

Bionatura

Latin American journal of Biotechnology and Life Sciences

**Estado de las Investigaciones en Productos Forestales no Maderables en Ecuador,
Universidad Técnica Particular de Loja (UTPL).**



Comparative study on Biochar salt absorption capacity in different saline concentrated solutions

Non-Timber Forest Products of the El Tundo community: a forested remnant of biodiversity and ancestral knowledge of southern Ecuador

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ÍNDICE / INDEX

EDITORIAL

- Uso sustentable de productos forestales no maderables en la Amazonía Ecuatoriana: Una oportunidad para el desarrollo armónico

2141

Omar Malagón

2137

LETTER TO EDITOR / CARTA AL EDITOR

- Chinese experience in the management and control of COVID-19 epidemic

2144

Ricardo Silva Rodríguez and Lissett Hermida Cruz

RESEARCH / INVESTIGACIÓN

- Comparative study on Biochar salt absorption capacity in different saline concentrated solution

2150

Vanessa Mishell Rocha Cabuyale

- Efecto de la aplicación de *Azospirillum* sp. y *Azotobacter* sp. sobre el crecimiento y productividad de kikuyo (*Pennisetum clandestinum*)

2156

Effect of the application of Azospirillum sp. and Azotobacter sp. on the growth and productivity of kikuyo (Pennisetum clandestinum)

Oscar Vivanco-Galván, Danny Carrión, Daniel Capa-Mora

- Productos Forestales No Maderables de la comunidad El Tundo:

2161

un remanente boscoso de biodiversidad y conocimiento ancestral del sur del Ecuador

Non-Timber Forest Products of the El Tundo community: a forested remnant of biodiversity and ancestral knowledge of southern Ecuador

Diana Maza Vivanco, Sergio Abad, Omar Malagón, Chabaco Armijos

- Economía agroecológica en una comuna rural del sur del Ecuador

2175

Agroecological economy in a rural commune in Southern Ecuador

Guido Prado, Janeth Sarango-Salazar, Zoila Herrera, Daniel Capa-Mora, Alex Padilla, Ángel Benítez

- Expression of the ANS, CHS and DFR genes involved in the biosynthesis

2180

of anthocyanins in *Vaccinium floribundum* Kunth from Ecuador, using RT-qPCR.

Viviana Chiluisa-Utreras, Doris Vela, Ivonne Vaca, Ramiro Acurio, Javier Chicaiza y Sofía Peñaherrera

- Optimization of cultural conditions affecting improved bioactive metabolite production by endophytic fungus *Trichoderma harzianum*

2187

Rashid Rahim Hateet, Zainab Alag Hassan, Abdulameer Abdullah Al-Mussawi and Shaima Rabeea Banoon

Potential effect of Imatinib on some sex hormones for male patients of Chronic Myelogenous Leukemia in Baghdad province <i>Abeer Anwer Ahmed, Khaleed J Khaleel, Alaa Abbas Fadhel</i>	2193
Isolation and molecular identification of microorganisms isolated from soils contaminated with heavy metals in Mosul city <i>Sana R. Alkazaz, Mohammad I. Khalil , Mazin N. Fadhel</i>	2196
Molasses as a new nutrition medium for <i>Scenedsmus quadricauda</i> growth and production of some bio compounds <i>Yousef J.I. AL -Shahery, Israa N. AL- Asady</i>	2202
Characteristics and quality of gray water and the possibility of reuse for irrigation purposes from the houses of some areas of the left side of the city of Mosul <i>Safa Arshed Saadoon , Ayad Fadeel Qasim</i>	2209
Micropropagación <i>in vitro</i> de naranja agria (<i>Citrus aurantium L.</i>) a partir de segmentos nodales In vitro micropropagation of sour orange (<i>Citrus aurantium L.</i>) from nodal segments <i>Angel David Hernández-Amasifuen, Alexandra Jherina Pineda-Lázaro , Hermila Belba Díaz-Pillasca</i>	2216
Determining conditions for best pollen quality of red-purple tree tomato (<i>Solanum betaceum</i> Cav.) germplasm <i>Andrea Sotomayor, Jorge Merino, William Viera</i>	2222
Comparative Study for Carrot Juice and Selenium Supplement in Many Physiological and Biochemical Parameters in Patients with Rheumatoid Arthritis in Kirkuk City <i>Wedad Mahmood Lahmood Al-obaidi, Mohanad Hassan Mahmood Al-Izzi, Aya Saad yaseen</i>	2228
Perfil bacteriano del shock séptico en una unidad de cuidados intensivos de la altitud del seguro social del Perú. Bacterial profile of septic shock in an intensive care unit of the Peruvian social security altitude <i>Amilcar Tinoco-Solázano, Jorge Chumbes Perez , Daniel Molano Franco , Jorge Luis Vélez-Páez, Antonio Viruez Soto</i>	2233
Análisis estadístico de las enfermedades asociadas a la mortalidad por COVID-19 en un Hospital de Ecuador durante el año 2020 Statistical analysis of diseases associated with mortality due to COVID-19 in a Hospital in Ecuador during the year 2020 <i>Sandra García-Bustos, Omar Ruiz-Barzola, Diana Cáceres , Kelly Márquez, Luis Valencia, Christian Vergara, Mariela González-Narváez</i>	2242
Phylogenetic and molecular evolutionary analysis of SENV DNA isolated from Iraqi Hepatitis Patients <i>Arwa Mujahid Al-Shuwaikh, Ealaf Abbas khudair, Dalya Basil Hanna.</i>	2251

Traditional practices in post-partum care among Indonesian and Filipino mothers: a comparative study <i>Marni Siregar, Sri Marasi Aritonang, Juana Linda Simbolon, Hetty WA Panggabean, Robert H Silalahi</i> Clinical Characteristics and Outcome of SARS-CoV-2 Patients. An Experience from Anbar province - West of Iraq <i>Hazim Ghazzay, Hamdi Saleh Al-Mutoriy, Mazin Saleh Al- Rudaini , Hamed Al Reesi</i> The inhibitory effect of aqueous and alcoholic extract of red pepper on some isolated pathogenic bacteria from different areas of human body <i>Lina Qays Yaseen , Sura Hameed Nayyef &Nadia Ibraheem Salih</i> Role of Leptin with hypothyroidism in Iraqi diabetic type 2 patients <i>Sulaiman M. Hasan</i> Prevalence of antibodies in Iraqi Urinary Tract Infection patients using radial immunodiffusion (RID) assay <i>Saja Mohammed Mohsen, Anas Wisam malik</i> Effect of cinnamon on blood sugar and anthropometric measurement in type two diabetes patients <i>Alaa Moyasser Sadiq , Najah Salman Abid, Ola Hussein Jasim , Besmeh Mohammed Ali</i> Determination of the prevalence of <i>blaOxa-like</i> gene and <i>ISAbal</i> elements among extensive-drug resistant (XDR) <i>Acinetobacter boumannii</i> isolates <i>Salah Sabah Muhsin, Wasan Abdul-Elah Bakir, Majeed Rasheed Sabah</i> Estimation of Vascular Cell Adhesion Molecule 1 (VCAM-1) Levels In Type 1 Diabetic Mellitus Patients <i>Ousamha Akram Saterr, Abeer J. Hassan, Qahtan Adnan Rasheed</i> Evaluación temporal de sistemas agroforestales de cacao en el trópico húmedo ecuatoriano <i>Temporal assay of cocoa agroforestry systems in the Ecuadorian humid tropic</i> <i>Carlos A. Tapia-Vera, Fernando D. Sanchez-Mora, Gregorio H. Vazcones-Montufar, Alexandra E. Barrera-Alvarez, Raúl V. Mora-Yela, Gorki T. Diaz-Coronel, Felipe R. Garcés-Fiallos</i>	2257 2265 2270 2274 2277 2280 2284 2292 2295 2301 2305
CASE REPORTS / REPORTE DE CASO	
Schizophrenia and refractory status epilepticus in a male patient with anti-NMDA auto-immune encephalitis: A case report <i>Pablo Andrés Llerena-Rengel, Luis Felipe Villamarín-Granja, Jorge Luis Vélez-Páez</i>	
REVIEW / ARTÍCULO DE REVISIÓN	
A review on emerging micropollutants: sources, environmental concentration and toxicity <i>Vadiraj K.T., Raghu Ram Achar and Sindhuja Sirigeri</i>	

Composition, hydrology, and health benefits of Zamzam water

2326

Amira Y. Boshra, Abdalbasit A. Mariod, Fatima A. Ali Massad, Eshraga M. Abdalrhman, Sabah M. Abbas, Amel A. Hassan, Manal M. Mahamedan, Nora M.A. Elatta, Huda Kh.A. Masaad, Amal M. Hamid, and Hammad A. Fadlalmola

Study review of camelid and shark antibodies for biomedical and biotechnological applications

2331

Esther Ivanova Matamoros Alcivar, Maily Selena González Avilés

Pruebas moleculares para el diagnóstico de COVID-19:

2341

La respuesta de Sudamérica

Molecular tests for the diagnosis of COVID-19: South America's response

Pool Marcos-Carbajal, Christian Allca-Muñoz, Macarena Ganoza-Farro, André Valdez-Olivera, Allison Gomez-Martel, Maribel Huaringa-Nuñez, Alberto Salazar-Granara

NEWS AND VIEWS / NOTICIAS Y OPINIONES

A scientific note of pest-insects associated with stingless bee hive

2348

Melipona eburnea in the Ecuadorian Amazon Region

Una nota científica de insectos-plaga asociados con las colmenas

de abejas sin aguijón Melipona eburnea en la región amazónica ecuatoriana

Fernando Valdivieso-Rivera, Michelle Pazmiño-Viteri, Alejandro Pinos-Tamayo, Marlon Estupiñan, Jonathan Liria, Cecilia Rodriguez-Haro

Molecular Photoacoustic Imaging

2351

Eduardo Cepeda and Katherine Narváez

EDITORIAL

Uso sustentable de productos forestales no maderables en la Amazonía Ecuatoriana: Una oportunidad para el desarrollo armónico

Omar Malagón

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En el mundo actual, aún en medio de una pandemia que asoló a la humanidad, y a los graves problemas ambientales que se avecinan, es urgente la búsqueda de opciones para el desarrollo sustentable de todo el complejo humano. Las respuestas pasan por revalorizar la funcionalidad ecosistémica de los bosques y por buscar alternativas para generar un desarrollo basado en una bioeconomía sustentable.

Hace pocos meses se firmó el Pacto Nacional para la Bioeconomía sustentable en Ecuador, que en su introducción establece: La Bioeconomía para el Ecuador representa una estrategia alternativa de desarrollo basado en la orientación de las actividades económicas enfocadas en la generación de conocimiento, uso y aprovechamiento sostenible de los recursos naturales de la biodiversidad, agro- biodiversidad y sus derivados, a través de un conjunto de políticas, procesos productivos y prácticas fundamentadas en la creación y transferencia de conocimiento, innovación y nuevas tecnologías, que provean productos y servicios, contribuyendo así en la transición hacia un sistema económico sostenible, socialmente inclusivo, competitivo y resiliente¹.

Los productos forestales no maderables (PFNM's) son aquellos derivados de ecosistemas forestales, diferentes a la madera, que prestan otros servicios y productos importantes a las comunidades que habitan en éstos. Existe una gran diversidad de productos que incluyen semillas y frutos alimenticios, fibra para construcción de estructuras, esencias, aromas y resinas; colorantes y especies medicinales. Gran parte de la riqueza originaria de nuestros bosques pasa por la existencia de una cantidad importante de estos productos, los cuales fueron adoptados por las culturas originarias; quienes encontraron en estos parte de su sustento.

Sin embargo, la paulatina deforestación con fines de extracción maderera; o para generar cultivos extensivos, en el caso de Ecuador, palma aceitera, café y cacao, entre otros; y la ganadería que ocupa cada vez más espacios boscosos ha hecho que los PFNM's no ganen la relevancia suficiente y, que incluso, pasen a estar en categoría de peligro por causa

de estas formas de explotación ecológica. En este entorno, es importante desde la investigación generar programas que permitan la búsqueda de aplicaciones de estos productos para que puedan ingresar al mercado actual, o generar usos sustentables de esta enorme diversidad de bienes que nos ofrecen los bosques.

Es por esto que en el contexto de Ecuador, a través de los Ministerios de Ambiente, Agua y Transición Ecológica y Ministerio de Agricultura y Ganadería se estableció el programa PROAmazonía, cuyo uno de sus objetivos es el desarrollo de cadenas de valor libres de deforestación, a partir de cultivos ya existentes y también de productos forestales no maderables.

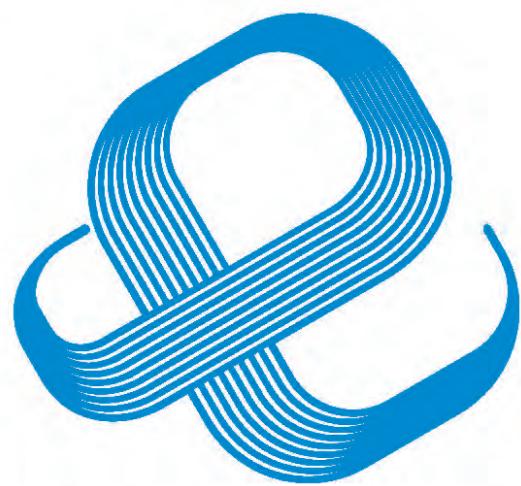
Bajo esta situación, la Universidad Técnica Particular de Loja busca a través del proyecto *"Asistencia técnica para la identificación de oportunidades de negocio a partir de PFNM's, productos de la biodiversidad, MFS/MST y factibilidad de planta para aprovechamiento sustentable en la Amazonía Centro y Sur, bajo el marco del programa PROAmazonía"*, la generación de alternativas viables para comunidades de la Amazonía Ecuatoriana en torno al uso sustentable de Productos Forestales No Maderables.

Es así que se ha dedicado este número especial de la revista Bionatura a formular un marco de colaboración nacional para la identificación de investigaciones alrededor de PFNM en todo el Ecuador, para así dejar patentes las oportunidades de negocios sustentables con las que cuentan nuestros bosques, las cuales podemos transferir a las comunidades locales, en búsqueda de llegar a un desarrollo sustentable; y la importancia de que la investigación genere lazos con el contexto y ganen en relevancia y pertinencia.

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Desde la investigación, **apoyamos**
a 7 comunidades de la Amazonía
centro y sur del Ecuador con personal
técnico, para el estudio y **desarrollo**
de Productos forestales no
maderables (PFNM). |



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LETTER TO EDITOR / CARTA AL EDITOR

Chinese experience in the management and control of COVID-19 epidemic

Ricardo Silva Rodríguez and Lisset Hermida Cruz

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In December 2019, a new epidemic of coronavirus disease appeared (COVID-19), caused by SARS-CoV-2 (formerly 2019-nCoV). The first reported disease cases occurred in Wuhan, Hubei province, resulting in the third zoonotic event related to lethal human coronavirus. Initially, the incubation period is 1-14 days (mean 5-6 days) in most cases but can be as long as 24 days^{1,2}. The most commonly seen characteristics of COVID-19 are fever, cough, tiredness, and abnormal chest computed tomography^{3,4}. So far, bat is thought to be the origin of SARS-CoV-2, based on sequence homology of 96% between SARS-CoV-2 and Bat-CoV-RaTG13⁵⁻⁷, but more impartial scientific investigations on the origin-tracing of the virus are required to elucidate the issue. Human-to-human transmission of SARS-CoV-2 occurs mainly via respiratory droplets¹, direct contact¹, asymptomatic transmission^{8,9}, and intrafamilial transmission^{3,4}.

At present, there are over 206 million cases of COVID-19 worldwide, with a 4.35 million death toll¹⁰. As of 12 August 2021, China had confirmed 94,260 cases with 4636 deaths (mortality rate 5%), and 87,740 recovered cases (93%)¹¹. Other countries, even though they had much more time to prepare for the arrival of the virus, delayed their response and that meant lost control¹². While the world is struggling to control COVID-19, China has been a good example of how to control the epidemic, and has shared information with other countries on the management and prevention of the disease. How was that possible?

Non-pharmaceutical and pharmaceutical intervention

COVID-19 began in China just before The Chinese New Year, the country's most important traditional holiday, which is associated with the world's most significant population flow. The New Year's holiday usually lasts about a week, and during that time, the population flow increases by more than 300 million. This population movement increased the transmission of COVID-19; as a result, the situation worsened significantly. The speed of China's response was the crucial factor in stopping transmission of the virus and saving lives. To this aim, China released the genomic sequence of the virus in early January 2020 and adopted a strategy that included a nationwide directive from the central government with governmental oversight, combining non-pharmaceutical and pharmaceutical intervention¹²⁻¹⁴. A series of rigorous measures were applied: a) early detection with an active screening at all levels; b) early diagnostic testing through the creation of new diagnostic tools (nucleic acid RT-PCR assay, gene sequencing, and IgM-IgG serology) aiming at performing as many tests as possible; c) early isolation of positive individuals, suspects, and close contacts, d) early treatment of all patients, those with mild symptoms and those with severe symptoms. Significantly, according to the Chinese experience, all patients should receive treatment¹⁵; e) protection of personnel: effectively protecting the most vulnerable groups and social distancing; f) local lockdowns along with systematic measures to control mobility

and reallocate resources effectively (e.g., medical resources to the epicenter of the infection in Wuhan). The government implemented policies to support different provinces and patients, classified into different groups according to the severity of the infection. In turn, other measures such as setting up of hospitals, diagnostic algorithms, and specific treatments based on the combination of Chinese and Western medicine were implemented to cope with the virus^{12,13}. Following the Diagnosis and Treatment Protocol for Novel Coronavirus Pneumonia (Trial Version 7) released by the National Health Commission and State Administration of Traditional Chinese Medicine (TCM) on 3 March 2020, different treatments were applied according to patient's conditions and careful evaluation of their adverse reactions and contraindications. Among these treatments were antiviral therapy [alpha-interferon, lopinavir/ritonavir, ribavirin (also suggested to be used jointly with interferon or lopinavir/ritonavir), chloroquine phosphate, and arbidol]. Antibiotic drug treatment was also used. In severe and critical-ill patients, effective oxygen therapy, non-invasive or invasive mechanical ventilation, rescue therapy, circulatory support, convalescent plasma treatment, glucocorticoids and immunotherapy were used. As for TCM, over 20 different products were applied according to physical conditions and clinical manifestations of patients¹⁶. Finally, the development of effective vaccines, capable of protecting against COVID-19 and its complications, was another tool incorporated into China's strategy to fight SARS-CoV2.

Vaccine development

As early as January 2020, Chinese researchers began to develop vaccines against SARS-CoV2. After one year and a half, five Chinese vaccines are already approved for Emergency use outside China. Of them three are inactivated formulations: 1) BBIBP-CorV (Sinopharm, Beijing), approved in 59 countries; 2) Coronavac (Sinovac), approved in 39 countries; 3) Inactivated Vero cells (Sinopharm, Wuhan), approved in 7 countries; 4) Replicative viral vector vaccine, Ad5-nCoV (Cansino), approved in 8 countries, and 5) Subunit vaccine, RBD-Dimer (Anhui Zhifei Longcom), approved in 2 countries. BBIBP-CorV (Sinopharm Beijing) and Coronavac (Sinovac) have been included in the Emergency Use List of WHO¹⁷.

Due to the high effectiveness of the non-pharmaceutical intervention in China, the Chinese vaccine developers had to move abroad to test the efficacy of their vaccines after finishing local phase I and II clinical trials¹⁸⁻²¹. This significantly delayed the release of efficacy results compared to the Western vaccines, which have been developed in countries with a high incidence of COVID-19. In the past three months, important audited data have come out validating the Chinese vaccines. In this respect, the efficacy of Sinopharm vaccines was published based on the interim report, including data collected from 40,411 participants in the United Arab Emirates and Bahrain last year²². The efficacy rate vs. symptomatic disease was 78.1% and 72.8% for the Beijing and Wuhan vaccines. In turn, the effectiveness

study of Coronavac vaccine in Chile, conducted from 2 February to 1 May 2021, with a cohort of approximately 10.2 million persons, was published²³. As a result, the effectiveness was 65.9% for the prevention of COVID-19, 87.5% for the prevention of hospitalization, 90.3% for the prevention of ICU admission, and 86.3% for the prevention of COVID-19-related death. In turn, according to the WHO, Sinovac's vaccine efficacy stands at 51% against symptomatic disease and 100% against severe disease, while Sinopharm's efficacy seems to fare slightly better at 79% against mild and hospitalized disease²⁴.

Although the level of immunogenicity induced by the Chinese inactivated vaccines is lower than that induced by other Western vaccines such as mRNA vaccines^{25,26}, in line with the difference of the effectiveness rates reported by the Chilean health authorities on 3 August 2021, among the vaccines currently underuse in Chile²⁷, this does not mean that inactivated Chinese vaccines are useless. First, the effectiveness rate is still over 50%, and second, the Chinese vaccines can be stored between 2-8° degrees Celsius, making them more feasible for a better cold chain distribution to developing countries, which may not have the required facilities to store large amounts of vaccine at very low temperatures, as is the case of the mRNA vaccines.

Since the beginning of July, COVID-19 cases in Chile started to go down. Based on the last Chilean report about Coronavac's effectiveness, the Chinese vaccine undoubtedly is contributing to this important result. Chile has now fully vaccinated more than 60% of its population, from which 70% are with Coronavac²⁸.

To date, China has exported vaccines to more than 60 countries, with the total amount exceeding 770 million doses²⁹. China has been the first country to work with other countries in the development and production of vaccines against COVID-19. Among these countries, Indonesia, Brazil, the United Arab Emirates (UAE) and Egypt are the first to establish production capacity for the Chinese vaccines²⁹.

Up to 14 August, 2021, China had delivered more than 1.83 billion doses to its population, including 60 million administered to those between the ages of 12 and 17, according to the National Health Commission, being fully vaccinated about 55% of the total population³⁰. Adding together doses administered in China and overseas, these may be the most widely used COVID-19 vaccines globally.

Of course, the development of updated schedules and potentially more effective vaccines is also of upmost priority for China and overseas. Early last month, the National Medical Products Administration had approved 22 COVID-19 vaccines for clinical trials using five different technologies. In summary, four vaccines have received conditional approval and three have been approved for emergency use³¹.

Concerning the administration of booster doses, one recent report described the potent boosting effect of one additional dose of Coronavac at 6-8 months upon administration of the second dose. According to recent news, in China and other countries using inactivated vaccines, this could be an appealing alternative to increase immunity for those working in risk areas, as well as for elderly³². On the other hand, in China, an alternative route of administration for the Cansino's vaccine was developed: The aerosolized Ad5-nCoV. As a result, two doses of aerosolized Ad5-nCoV elicited neutralizing antibody responses similar to one dose of intramuscular injection. In addition, an aerosolized booster vaccination 28 days after the first intramuscular injection also induced strong IgG and neutralizing antibody responses³³.

New Delta variant of SARS-CoV2. How to face it?

Since first appearing in India in late 2020, the Delta variant of SARS-CoV-2 has become the predominant strain in much of the world. Its fast-spreading nature (people infected with it produce far more virus than do those infected with the original version of SARS-CoV-2) and the combination of this high number of viruses (1000-fold) and a short incubation period (4 days) makes this Delta variant highly transmissible³⁴. Based on the published data about the high viral load associated to infection³⁵, the Delta variant is a real challenge for the current Chinese vaccines. The lack of yet effectiveness data of Chinese vaccines available against this important variant and its spreading around the world, make its control in China a real defiance. The latest COVID-19 surge with the Delta variant started in Nanjing on 20 July upon Nanjing Lukou International Airport's cabin cleaners tested positive for COVID-19 after cleaning an international flight. The recent virus cluster in Nanjing led to the outbreak spreading to other cities and other parts of China, but current epidemic control measures are still effective to prevent the spread of the Delta variant of the COVID-19 virus and the risk of seeing widespread outbreaks across the country is low, according to experts from the National Health Commission's Disease Prevention and Control Bureau^{30,36}. Big data and information technology are important tools to avoid a rebound³⁷. China has used big data to track people who have been in risk areas and are close contacts of new cases. These new, innovative and more specific anti-virus methods have led the Chinese authorities to control the emergence of the Delta variant quickly and effectively (Fig. 1)³⁸. Therefore, to accelerate the pace of vaccination today becomes a must to build the herd immunity in the population and achieve a balance between economic development and efforts to combat the Delta variant of COVID-19. The Chinese strategy is addressed to combine its highly effective non-pharmaceutical intervention with the high vaccination coverage with the current vaccines. According to the Chinese top epidemiologist Zhong Nanshan, from a preliminary analysis of more than 100 patients in Guangdong province, it was shown that Chinese vaccines are effective in preventing the development of pneumonia and severe infection produced by Delta variant. The epidemiologist urged more people to get vaccinated, meaning 80% or more of the population vaccinated in the country to build-up the immune barrier^{39,40}. The priority of vaccination in China is to prevent people from getting ill, but not from getting infected. That's why a proper combination of both interventions constitutes the unique way, in the short-term, to bring the Delta variant spread under control, whereas alternative vaccines and schedules of immunization are coming. The progress of the ongoing rebound of Delta variant in China will tell the last word, although no country is safe until all countries are safe, a statement very clear for China.

Conclusions

China offers an appealing solution to other countries and teaches the rest of the world that even the most difficult situations can be overcome. The high level of collaboration between government officials and health experts, has proven to be effective in containing and controlling COVID-19, which is greatly admired by the entire world. Today, normal life has returned in all regions in an orderly manner, including in Wuhan, the epicenter of the epidemic in China. Countries should take experience of China's response to COVID-19 and implement their own prevention and control strategies immediately, as

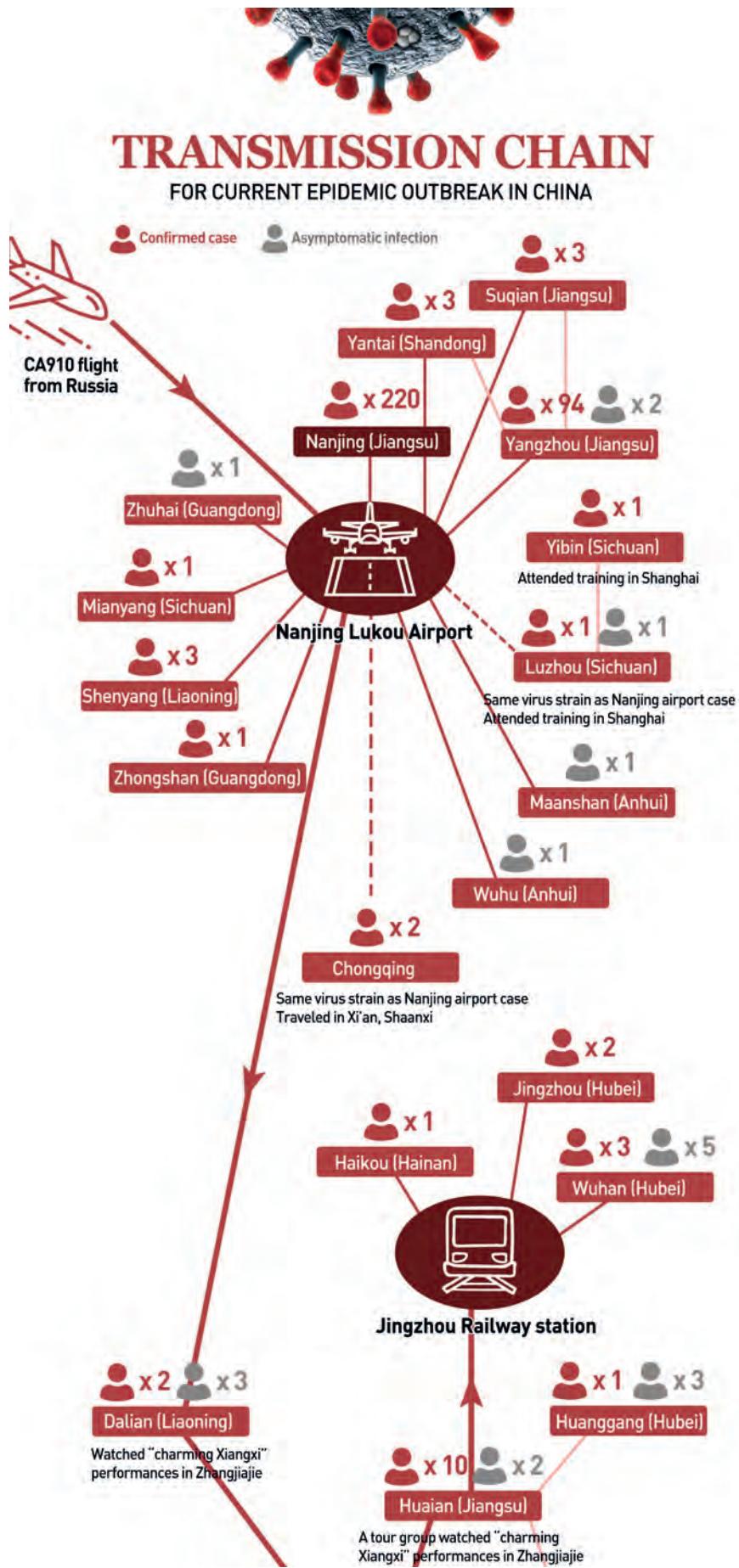


Figure 1. Transmission chain for current epidemic outbreak in China. Global Times. 3 August, 2021.

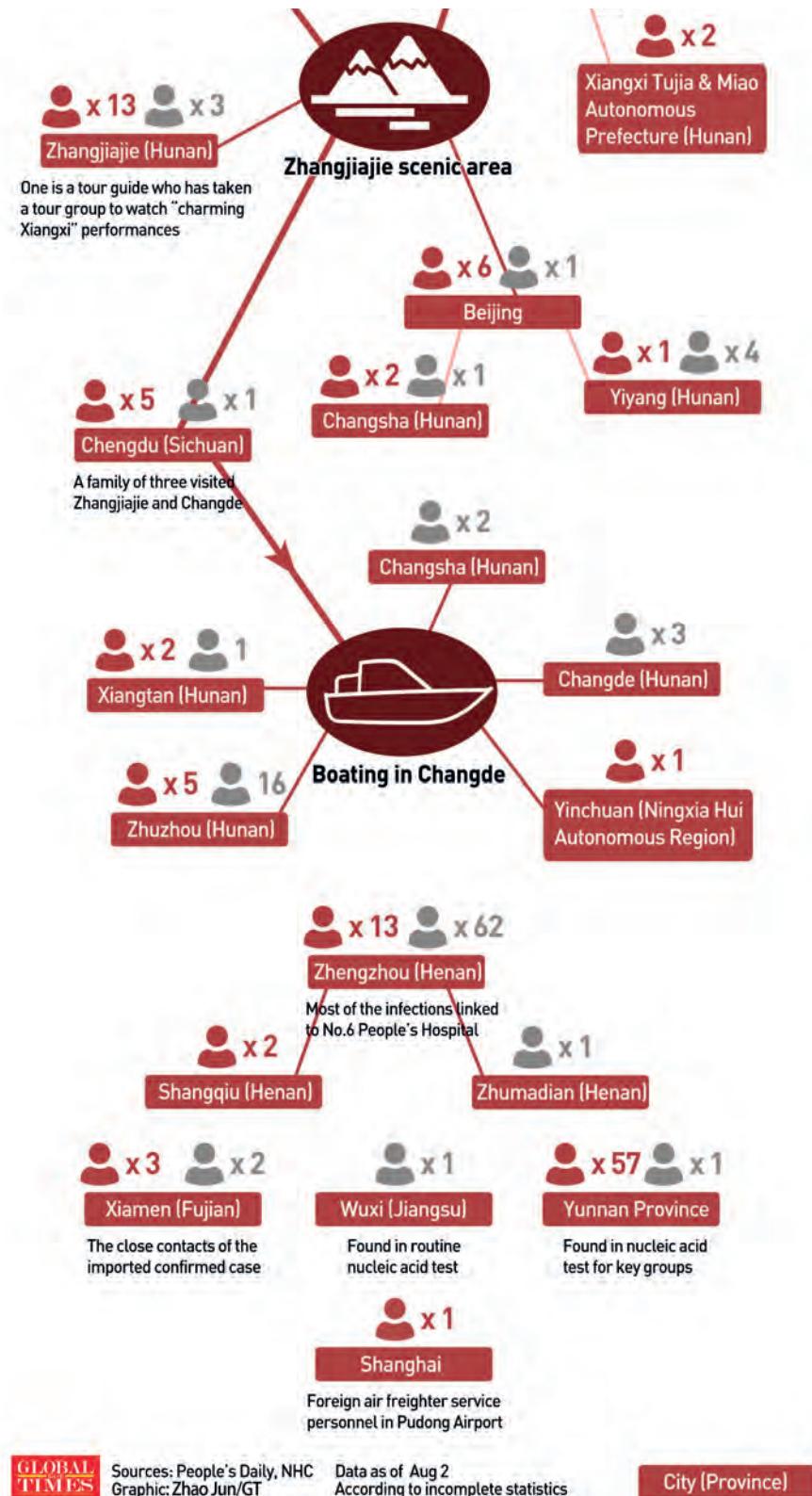


Figure 1. Transmission chain for current epidemic outbreak in China. Global Times. 3 August, 2021.

none is exempt from becoming the new epicenter of the virus. In addition, countries must do more to improve their health systems and personnel so as to be more prepared for future outbreaks and reduce their consequences. We recognize the huge contribution of China in the fight against the pandemic and call the international community to join to this fight and strengthen cooperation and solidarity⁴¹.

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RESEARCH / INVESTIGACIÓN

Comparative study on Biochar salt absorption capacity in different saline concentrated solutions

Vanessa Mishell Rocha Cabuyales

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2150

Abstract: Salt-affected soils are caused by excess accumulation of salts. As soil salinity increases, salt effects can result in the degradation of soils. Previous studies have determined that biochar has the potential to reduce salt stress in soils. In this study, the electroconductive properties of biochar to adsorb salts were investigated in different saline-concentrated solutions. Pelletized, fragmented and powdered biochar were placed in solutions with concentrations of 0, 50, 500, 1000, and 2000 parts per million sodium chloride, respectively. Control treatments consisted of deionized water mixed with salt and no biochar addition. A week after setting the experiment, the electroconductivity measurements were significantly higher relative to the first day. Significant differences were observed among treatments for pelletized, fragmented, and powdered biochar treatments. Increases in electroconductivity values are attributed to ambient temperature changes and differences in particle size. However, pelletized biochar declined in electroconductive values, which is attributed to ions being retained inside the pores of bigger particles. Our study concludes that biochar can adsorb salts at lower sodium chloride concentrations; therefore, it may help mitigate soil salt stress.

Key words: Biochar, salinity, electroconductivity, soil, salt.

Introduction

The accumulation of salts may cause soil degradation due to agricultural, industrial, or urban activities¹. Soil salinization is a widespread limitation where over 397 million hectares of the world's soils are unproductive saline patches of land². One of the most severe problems related to salinization is the progressive degradation of soil fertility¹. The excessive concentration of soluble salts in soils inhibits beneficial microbiological activity, causes accumulation of toxic ions, limits plant growth, and accelerates organic matter loss due to wind erosion or leaching^{1,3}. Because soil fertility is essential for nutrient cycling, structure, stability, and cation exchange processes, most management strategies aim to improve this property¹. There is currently much research regarding the amelioration of saline soils⁴. For this reason, biochar has been widely proposed to improve soil quality and crop productivity⁴. Biochar can enhance soil organic matter quality by dealing with soil salinity problems because of its salt sorption capacity⁵.

Biochar is obtained from a process known as pyrolysis, which is the thermo-chemical decomposition of organic biomass at temperatures generally ranging from 300 to 700 °C in the absence of oxygen^{6,7}. Pyrolysis processes stabilize the existing carbon in organic matter to be more resistant to chemical and biological decomposition⁸. When biochar is incorporated into soils, it degrades very slowly so that carbon is not emitted into the atmosphere, in contrast to non-pyrolyzed organic matter decomposition⁹. Moreover, biochar has the potential to decrease Na+ uptake by adsorbing salts¹⁰. These characteristics give biochar the capacity to improve the physical-chemical properties of soils, increase soil productivity, contribute to carbon sequestration, and alleviate salt stress in vegetation⁸.

To enhance the understanding of the salt adsorption properties of biochar, it is necessary to measure its ion electroconductivity (EC). The analysis of biochar's electroconductive properties allows measuring the concentration of salts present in a solution based on a conductivity meter, and this can be used to determine whether biochar might help mitigate salt stress in plants and soils^{11,4}. In addition, the effects of salinity amelioration on soils may vary depending on the biochar's phys-

ical characteristics¹². Biochars in this study range from pelletized, powdered, and fragmented particle types and at a range of sizes. It is essential to consider the use of different biochar morphologies because of their varied porosity. Porosity determines the conductivity and ability of biochar to retain ions on its surface¹³. It is predicted that biochar can reduce the electroconductivity of saline treatments because of its salt sorption capacity. Since biochar's salt adsorption potential has no tests, this study conducted electroconductivity measurements to understand biochar's salt adsorption capacity and identify the physical-chemical properties of biochar's particle morphology. Biochar salt adsorption analysis can be used to enhance the limited studies on biochar's amendment of salt stress on soils and vegetation. The objective of this study is to understand which variables affect biochar's salt adsorption capacity. This research paper focuses on the following specific objectives:

1. Measure the salt adsorption capacity of biochar through electroconductivity.
2. Evaluate the salt adsorption function of different biochar morphologies under different salinity conditions.

Methods

Biochar and salt stock solutions: Different types of biochar were analyzed based on particle morphology, including pelletized, fragmented, and powdered biochar. We used biochar pellets in the range of 2.8-2.00mm. These pellets were processed from highly compacted wood and manufactured by BioForest. We also used biochar fragments processed from sugar maple (*Acer saccharum Marshall*) and conifer wood. The size of the biochar fragments ranged from 0.5mm to 4mm. These sugar maple biochar fragments were manufactured at Haliburton Forest and Wildlife Reserve Ltd, whereas the conifer fragments and biochar powder were processed at Titan Carbon Smart Technologies. The titan fragments and titan powdered biochar were dried at 100°C for 24 hours.

In this study, five different salinity stock solutions were prepared with 99% pure reagent-grade sodium chloride (NaCl) and deionized water (DI) on a weight-in-volume basis. The choice of deionized water as solvent was motivated by its neutral electron conductance potential ($5.5\mu\text{S}/\text{m}$ at 23°C). Deionized water is characterized by a lack of minerals and contaminants usually present in tap water, which could otherwise affect the experiment. The addition of sodium chloride was motivated by the specific salt concentration concerning seawater, which known measure acted as our reference (35ppt or 35g/L)¹⁴. Thus, 0.5g of sodium chloride was weighted in a mechanical balance and dissolved in a 250mL bulb flask to obtain a stock solution with 500ppm Na⁺ solution per every 15mL of deionized water. We assumed that a more concentrated solution would require double the number of salts, so we prepared a series of stock solutions with 1000ppm and 2000ppm and a negligible salt concentration stock solution with 50ppm Na⁺. The saline solutions consisted of low (50mmol NaCl), medium (500mmol NaCl), high (1000mmol NaCl), and saturated (2000mmol NaCl) sodium chloride concentrations in a fixed amount of 15 milliliters of deionized water. All the stock solutions were prepared 24 hours to let the sodium chloride dissolve and come to equilibrium before mixing them with biochar.

Experimental procedure: Preliminary tests were conducted on biochar and salt solutions at room temperature ($23 - 25^\circ\text{C}$) to learn when equilibrium could be reached. The preparation of the preliminary samples took at least five days, so it was decided to conduct preliminary samples by adding solutions to biochar at 1:20 biochar: solution ratio. This ratio reflects the usual mix of 1g of solid biochar per every 20mL of liquid. Because of flask size, we adjusted the ratio to 15mL; thus, the fixed amount of biochar added was 0.75g (w:v). Biochar was weighted on a mechanical balance, and 0.75 grams were immersed in 25mL glass containers. Then, a plastic pipette was used to transfer 15 mL of each stock solution into lidded glass containers with moderate stirring. All of the treatments were

performed in triplicate as was the control group. For the control samples, we mixed sodium chloride with deionized water without the addition of biochar. All the prepared samples were shaken at a speed of 80 rpm on a WWR DS-500E Orbital Shaker for an hour at room temperature. Initial electron conductivity measurements were taken after one hour of shaking the samples. Electrical conductivity was measured at room temperature (23°C) using a bench-top conductivity meter (Orion Star A112, Thermo Electron Co., Mass., U.S.A.) coupled to a conductivity cell (Orion 013005MD). The conductivity cell was calibrated to two standard solutions at $1413\text{m}\mu\text{s}$ and 12.0mS . After every measurement, the conductivity cell was washed with deionized water to remove any adhering sample and was blotted before using it in the subsequent measurement. Once the results of the initial measurements were recorded, all the samples were placed in the shaker for seven days, and electroconductivity values were taken at room temperature ($25^\circ\text{C} \pm 0.5^\circ\text{C}$). At the end of the experiment, we dried all biochar samples in small aluminum sample holders so they could be used to measure salt content later on. After drying, the samples were transferred back to the identical washed vials, where the measurements were taken initially and stored with lids.

A detailed experimental design chart is depicted in Table 1. Statistical data and graphs were analyzed with R studio version 1.1.463 (R Core Team 2018). Data for each dependent variable were first analyzed for normal distribution by the Shapiro-Wilks normality test and equality of variances among biochar and salinity treatments by the Levene test. Data meeting these assumptions were further tested on the influence of biochar type and salinity level on the variability of electroconductivity by a two-way analysis of variance test or ANOVA. Statistics reported for each test are F-values, P-values, and standard errors. Microsoft Excel was used to show tables corresponding to the means and standard errors (Table 2). A line graph of electroconductivity grouped by salinity treatment is presented in Figure 1.

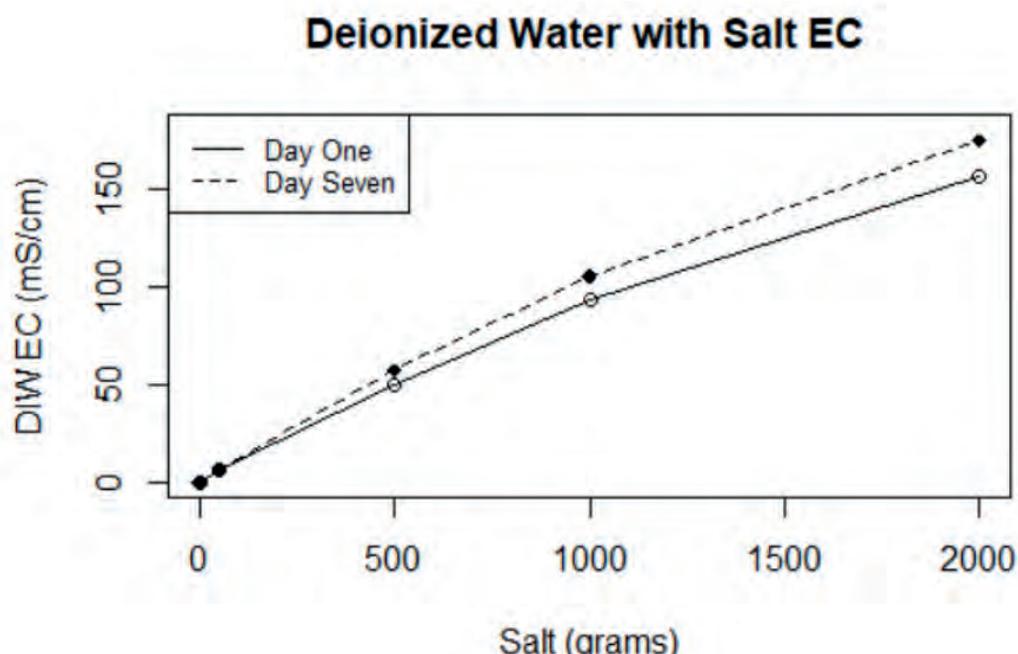


Figure 1. The graph shows the EC values obtained for the control group (deionized water with NaCl without biochar). The x-axis represents salinity levels in grams, whereas the y axis represents the EC of deionized water.

Control (DW) Deionized Water plus Salt (no biochar) & Deionized water plus biochar (no salt)	50 ppm NaCl solution in DW x 3 x 15ml mixed in 250 ml bulb flask	500 ppm NaCl solution in DW x 3 x 15ml mixed in 250 ml bulb flask	1000 ppm NaCl solution in DW x 3 x 15ml mixed in 250 ml bulb flask	2000 ppm NaCl solution in DW x 3 x 15ml mixed in 250 ml bulb flask
Biochar Pellets (BioForest) mixed wood biochar with binder in DW water 1:20 ratio biochar to water (0.75mg to 15ml water) x 3	Biochar Pellets in 50 ppm x 3 x 15ml	Biochar Pellets in 500 ppm x 3 x 15ml	Biochar Pellets in 1000 ppm x 3 x 15ml	Biochar Pellets in 2000 ppm x 3 x 15ml
Conifer Biochar Fragments (Titan) in DIW water at 1:20 ratio biochar to water (0.75mg to 15ml water) x 3	Titan Biochar in 50 ppm x 3 x 15ml	Titan Biochar in 500 ppm x 3 x 15ml	Titan Biochar in 1000 ppm x 3 x 15ml	Titan Biochar in 2000 ppm x 3 x 15ml
Sugar Maple Biochar Fragments (Haliburton) in DIW water 1:20 ratio biochar to water (0.75mg to 15ml water) x 3	Haliburton Biochar in 50 ppm x 3 x 15ml	Haliburton Biochar in 500 ppm x 3 x 15ml	Haliburton Biochar in 1000 ppm x 3 x 15ml	Haliburton Biochar in 2000 ppm x 3 x 15ml
Biochar Powder in DIW water 1:20 ratio biochar to water (0.75mg to 15ml water) x 3	Powder Biochar in 50 ppm x 3 x 15ml	Powder Biochar in 500 ppm x 3 x 15ml	Powder Biochar in 1000 ppm x 3 x 15ml	Powder Biochar in 2000 ppm x 3 x 15ml
Totals	225 ml (min.) total needed of 50ppm stock solution (mixed 0.75 g salt in 225 ml of DI water total)	225 ml (min.) total needed of 500 ppm stock solution (mixed 7.5g salt in 225ml DI water total)	225 ml (min.) total needed of 1000 ppm stock solution (mixed 15g salt in 225 ml DI water total)	225 ml (min.) total needed of 2000 ppm stock solution (mixed 30g salt in 225 ml DI water total)

Salt (g)	EC Day One				EC Day Seven			
	Biochar Pellets	Sugar Maple Fragments	Conifer Fragments	Biochar Powder	Biochar Pellets	Sugar Maple Fragments	Conifer Fragments	Biochar Powder
0	19.3 ± 1.05	14.95 ± 0.5	15.51 ± 1.44	0.275 ± 0.02	19.34 ± 1.05	14.95 ± 0.50	15.51 ± 1.44	0.275 ± 0.02
50	12.4 ± 0.02	8.53 ± 0.03	8.99 ± 0.03	6.07 ± 0.003	12.16 ± 0.17	7.77 ± 0.03	8.19 ± 0.07	6.71 ± 6.28
500	36.5 ± 0.28	34.92 ± 0.09	34.89 ± 0.12	50.93 ± 0.78	34.56 ± 0.26	41.12 ± 0.20	40.19 ± 0.06	56.63 ± 0.12
1000	85.5 ± 0.53	77.25 ± 0.2	77.46 ± 0.09	100.36 ± 0.32	80.33 ± 0.07	86.82 ± 0.15	86.99 ± 0.23	109.46 ± 1.98
2000	135.6 ± 0.56	138.32 ± 0.18	139.89 ± 0.25	159.89 ± 0.24	146.26 ± 0.46	153.82 ± 0.52	154.12 ± 0.12	175.79 ± 0.34

Table 2. Average (mean ± standard error N=3) for EC salinity treatments by biochar type (1:20 w/v ratio).

Results

Contrary to our initial hypotheses, biochar particles did not consistently result in decreased salinity. Electroconductivity values showed a considerable increase in response to biochar added to saline treatments except for the low concentration solutions, while powder biochar did not show any decrease in EC values at any saline level. A two-way analysis of variance was conducted on the influence of biochar type and salinity level on the variability of electroconductivity values. For day one, the ANOVA test yields an F-value of 4.528 and a P-value of 2.2×10^{-16} , indicating that the ANOVA is statistically significant according to the standard significance level of $p < 0.05$. This result demonstrates that EC does differ from one sali-

nity treatment to another. For day seven, where biochar and salinity are the independent variables, and EC values are the dependent variable, a significant relationship is found, supported by a p-value of 0.00191 (no log transformation). In other words, biochar type and salt concentration are the factors that influence EC values. Results of the variability in the EC measurements between saline solutions of the biochar samples are listed in Table 2. The results are discussed as the interaction of biochar on the different saline concentration treatments over time, and a comparison is made between the four different biochar types.

Table 1. Detailed experimental procedure and design.

Discussion

Electrical conductivity is a way to determine the concentration of ions present in an aqueous solution¹⁶. In an ionic solution, the specific conductivity measurement depends on the concentration and, therefore, on the number of ions (cations and anions)¹⁵. The more ions that are present, the higher the electroconductivity of the aqueous solutions¹⁶. These conductive ions include dissolved salts (NaCl) and organic and inorganic matter that composes biochar¹⁶. The duration of biochar's exposure to saline solutions had a pronounced effect on electroconductivity. As shown in Table 2, day-seven EC values were higher than day-one saline treatments and across the four types of biochar.

Salinity was not measured directly but is instead derived from electrical conductivity measurements¹⁵. For biochar pellets, the relationship between EC and salt is statistically significant ($p < 0.0026$). As shown in Figure 1a-b, low (50 ppm), medium (500ppm), and high (1000ppm) saline concentrations of Na⁺ presented a slight decrease in electroconductivity, with values ranging from 1% to 5% variation over a week. In contrast to other biochar types, pellets have a low surface area to volume ratio. For this reason, we believe that biochar pellets might be able to retain and hold more ions on their surface. However, the saturated saline concentration (2000ppm) presented an increment of 8% on the EC measurements. We attribute the observed EC variation to the fact that once biochar reaches equilibrium, it adsorbs salts, but these salts could also be released in the presence of saturated saline solutions because the particle might no longer be able to hold more ions.

EC values increased at the 500, 1000, and 2000 salinity levels (Table 2). The relationship between salinity level and EC

measurements is supported by a significant p-value of 0.00025 for sugar maple fragments and a significant p-value of 0.00026 for biochar conifer fragments (Figure 1c-d). The electroconductivity measurements show that the values increased significantly across all five salinity treatments ($p < 0.0029$). In contrast to pellets, values for powdered and fragmented biochar increased at each salinity level. Biochar powder particles have a higher surface area to volume ratio; such characteristics might allow the biochar to adsorb and de-adsorb Na⁺ salts at a different rate^{16,17}. Therefore, salt ions are prevented from holding onto the powder's particle surface and are subsequently leached into the salinity solution, and since these ions are leached into the solution, electroconductivity values tend to increase.

Biochar particle size, shape, and internal structure likely play essential roles in controlling sodium-ion storage because they might alter pore characteristics. For instance, biochar has pores inside of its particles. These pores are called intrapores and might provide additional space for ion storage beyond the pore space between particles or interpores¹⁶. As a result, electroconductivity values tend to decrease. A larger particle size, which is the case of pelletized and fragmented biochar, may increase sodium adsorption capacity, whereas a smaller particle size might not. Bigger biochar particles can store more ions inside their internal structure (Figure 3a).

In contrast, smaller biochar particles, such as powdered and fragmented biochar, might have limited ion storage capacity to hold ions on their internal structures (Figure 3b-c-d). Therefore, finely powdered biochar particles will not adsorb many ions, unlike bigger biochar particles like pellets. This addition of biochar grains with different shapes and sizes will eventually change the adsorption of minerals in the salinity solutions.

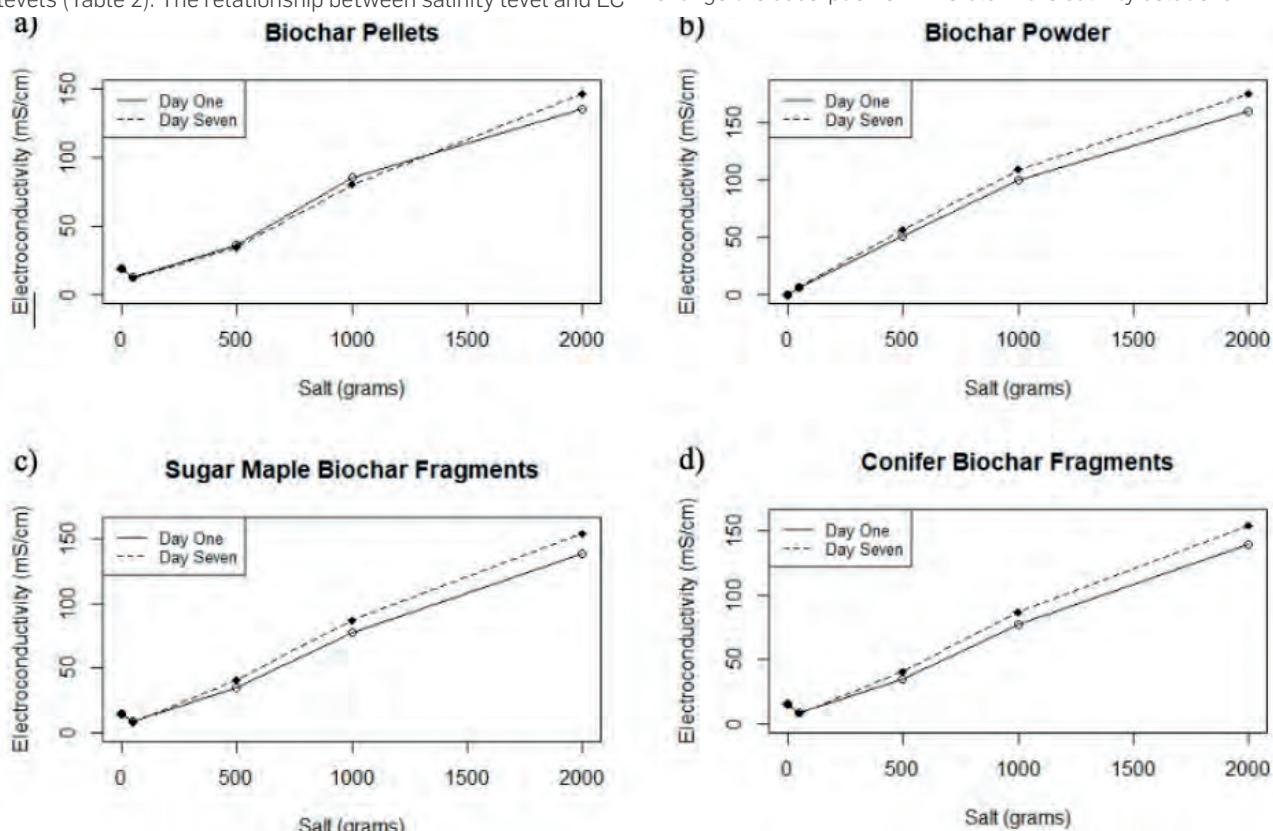


Figure 2. The figure illustrates the electroconductivity values for the four types of biochar tested: pellets, fragments (sugar maple and conifer) and powder. Graph a) shows biochar pellets, b) biochar powder, c) sugar maple fragments and d) conifer biochar fragments. For all four graphs, the x axis shows the salinity level concentration in grams whereas the y axis shows the electroconductivity values for the respective biochar in saline treatments.



Figure 3. The four different biochar types were used in the study. From left to right: Biochar pellets, Sugar Maple fragments, Conifer fragments, and Biochar Powder.

According to the Food and Agriculture Organization¹⁹, the higher EC variability of the salinity treatments may be attributed to increments in temperature. As shown in Table 2, the temperature of the saline solutions increased from $23^{\circ}\text{C} \pm 0.3^{\circ}\text{C}$ in day one to $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ on day seven. Unlike metallic conductivity, electrical conductivity increases at approximately 1.9% per Celsius degree increase in temperature¹⁹. When sodium electrolytes dissolve in water, they produce positive cation and harmful anion particles; this means that the electron conductance of the solutions increases with added ions¹⁰. An increase in EC may also be attributed to including substantial amounts of biochar ions and minerals into salinity solutions¹⁹. The presence of phenolic, carboxylic, pyrones, and ketone groups in biochar samples due to pyritization may in part explain the higher variability with the EC values between day one and seven¹⁰. An additional finding supporting this assumption is the increased electroconductivity presented by the control group, consisting of pure deionized water and different saline concentrations without biochar addition (Figure 2). Electroconductivity increased by approximately 0.5 to 12% from day one to day seven. It is important to note that temperature for day one was recorded to be $23^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ whereas temperature for day seven was $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ instead. Therefore, we believe that temperature played a significant role in rising EC values as higher temperatures might evaporate water, leaving saline ions behind.

Even though most of the biochar treatments showed increased EC values, low salt concentration treatments (50ppm) tend to progress toward a decrease in electroconductivity. All biochar types, except for powder, showed a decrease of 0.2-1.0 mS/cm in EC values at the 50ppm salinity concentration level. This finding suggests that biochar can adsorb salts in solutions with a small number of dissolved ions in contrast to solutions with a saturated number of ions^{20,21}.

Conclusions

This study concludes that biochar can adsorb salts at lower sodium chloride concentrations; therefore, it may help mitigate salt stress in soils. Under a chemical-biological approach, the findings of this research will help select the best Canadian

wood biochar options for different soil saline conditions in the environment. The presented information can serve as a guide to develop conservation strategies that aim to mitigate the adverse impacts of salt on vegetation and soils. However, further research still needs to be conducted to analyse a more precise interaction between biochar type and sodium sorption capacity. These analyses should implement different techniques to measure salinity values to get the exact sodium sorption levels.

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RESEARCH / INVESTIGACIÓN

Efecto de la aplicación de *Azospirillum* sp. y *Azotobacter* sp. sobre el crecimiento y productividad de kikuyo (*Pennisetum clandestinum*)

Effect of the application of *Azospirillum* sp. and *Azotobacter* sp. on the growth and productivity of kikuyo (*Pennisetum clandestinum*)

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2156

Resumen: El uso de bacterias promotoras de crecimiento vegetal (BPCV), es considerada como una alternativa para sustituir los fertilizantes químicos, ya que favorece la productividad de las especies vegetales. El presente estudio evalúo el efecto de BPCV de los géneros *Azospirillum* sp. y *Azotobacter* sp. en el cultivo del *Pennisetum clandestinum* (kikuyo), sobre altura de la planta, largo máximo de la hoja, largo de raíces, biomasa fresca y proteína total. La aplicación de las bacterias se realizó periódicamente sobre el cultivo, la primera inoculación fue luego del arado del terreno y posterior a ello cada 15 días, hasta el día 45. Los resultados muestran que *Azospirillum* sp. y *Azotobacter* sp. influyeron sobre el crecimiento y rendimiento de kikuyo. La aplicación de *Azospirillum* sp. mostró un incremento significativo en kikuyo sobre los parámetros altura de la planta y el largo de raíz, mientras *Azotobacter* sp. en la producción de biomasa fresca, largo de raíz y proteína total, por lo cual el uso de estos microorganismos benéficos podría ser de gran importancia en las actividades de producción de pasto para la ganadería, además de ser una alternativa para reducir el uso de productos químicos, con lo que se contribuiría a un mejor manejo de cultivos y al cuidado del medio ambiente.

Palabras clave: Bacterias promotoras de crecimiento vegetal, inoculación bacteriana, crecimiento y productividad de pasto.

Abstract: The use of plant growth-promoting bacteria (PGPR) is considered an alternative to substitute chemical fertilizers since it favors the productivity of plant species. The present study evaluated the effect of PGPR of the genera *Azospirillum* sp. and *Azotobacter* sp. on *Pennisetum clandestinum* (kikuyo) on plant height, maximum leaf length, root length, fresh biomass, and total protein. The bacteria were applied periodically on the crop, the first inoculation was applied after plowing the soil and every 15 days thereafter, until day 45. The results show that *Azospirillum* sp. and *Azotobacter* sp. influenced the growth and yield of kikuyo. The application of *Azospirillum* sp. showed a significant increase in kikuyo on the parameters plant height and root length, while *Azotobacter* sp. in the production of fresh biomass, root length, and total protein, so the use of these beneficial organisms could be of great importance in the activities of pasture production for livestock, besides being an aid to reduce the use of chemicals, which would contribute to better crop management and environmental care.

Key words: Plant growth promotes bacteria, bacterial inoculation, grass growth, and yield.

Introducción

La agricultura intensiva depende de una aplicación importante de fertilizantes nitrogenados, junto con otros nutrientes esenciales para maximizar la productividad de los cultivos¹. Como una alternativa al uso de fertilizantes químicos en la agricultura se ha incrementado el uso de bacterias promotoras del crecimiento vegetal (BPCV) como una efectiva herramienta para mejorar la salud de las plantas^{2,3}. Dentro de este grupo importante de BPCV encontramos a especies de *Azotobacter* y *Azospirillum* que son ampliamente asociadas con la fijación de nitrógeno^{4,5}. Por tal razón estas bacterias también estimulan la producción de hormonas de crecimiento como auxinas, citoquininas y giberelinas, mejorando la absorción de otros nutrientes como el fósforo. Por lo tanto, la inoculación con BPCV es una tecnología promisoria para el aumento de la producción agrícola, mientras se reduce los impactos ambientales por el uso inadecuado de fertilizantes⁶.

El *Pennisetum clandestinum* (kikuyo) es un hierba de zonas tropicales capaz de crecer mucho en verano y es usado en la alimentación de ganado vacuno. Sin embargo la producción de leche puede verse limitada por la calidad relativamente baja del kikuyo⁷, por esta razón es importante pensar en mejo-

rar la calidad del pasto a través de la inoculación de bacterias. Por ejemplo en el caso de *Azospirillum* la inoculación en hierba forrajera puede minimizar la degradación del suelo y mejorar la producción masiva de forraje. Esto es soportado por un reporte de Boddey *et al.*⁸ sobre bacterias fijadoras en la rizósfera de las hierbas forrajeras, lo que sugiere un futuro de menor uso de fertilizantes de N en los pastos forrajeros tropicales⁹. De hecho, la inoculación de la hierba *Brachiaria* spp. con cepas de *Azospirillum brasiliense* aumentó la producción media de masa de forraje en un 13%⁹. Asimismo, algunas investigaciones han mostrado que el crecimiento de diferentes plantas, incluidas hierbas anuales y perennes ha incrementado por la aplicación de *Azospirillum* y *Azotobacter*. Las bacterias son capaces de incrementar la germinación de las semillas, crecimiento y rendimiento de diferentes cultivos de plantas¹⁰⁻¹².

Con estos antecedentes y considerando que en Loja existen grandes extensiones de terreno ocupadas por kikuyo usado como alimento del ganado vacuno y como un cultivo importante, el presente trabajo tiene por objetivo evaluar el efecto del *Azospirillum* sp. y *Azotobacter* sp. sobre el crecimiento y la productividad del kikuyo.

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Materiales y métodos

Sitio experimental

El estudio se realizó en la Estación Agropecuaria-Universidad Técnica Particular de Loja (EA-UTPL) (Ecuador), situada en el sector de Cajanuma, al sur del cantón Loja, a 9 km desde la ciudad de Loja (X: -4,0886 y Y: -79,2066)¹³. El sitio de estudio está ubicado a una altura de 2.300 m s.n.m., además, presenta medias anuales de temperatura y precipitación de 15,4°C y 780 mm, respectivamente, según datos de la Estación Meteorológica "La Argelia"¹⁴.

Obtención cepas

Las dos cepas bacterianas fueron aisladas a partir de suelo de la rizósfera de kikuyo y preservadas a -80°C hasta su posterior uso. *Azospirillum* sp. y *Azotobacter* sp. fueron reactivadas y proveídas en cajas petri por la colección de microorganismos de la Universidad Técnica Particular de Loja (UTPL), para *Azospirillum* sp. en medio Agar Rojo Congo (27°C), mientras que, en agar Ashby la bacteria *Azotobacter* sp. (37°C), ambas permanecieron en incubación y en posición invertida en la obscuridad hasta su uso.

Incremento de biomasa del inoculo bacteriano

El ensayo se llevó a cabo en el Laboratorio de Cultivo y Conservación de Microorganismos de la UTPL. Para incrementar la biomasa de *Azospirillum* sp. una colonia de la caja petri fue colocada en medio JMV estéril (20 minutos, 120°C y 1.5 atm) sin nitrógeno, luego incubado (Yamato IC802) a 27°C entre 48 y 72 horas. En cuanto a *Azotobacter* sp. una colonia de la caja petri fue inoculada en medio JMV y mantenida a 37°C durante 48 y 72 horas. Se realizó una evaluación del número de unidades formadoras de colonias (UFC) de cada cepa bacteriana a través del conteo de bacterias por el método de Neubauer. Una concentración final de 1×10^8 g/mL fue ajustada en cada cepa bacteria en un volumen de 1L y mantenida a 4°C hasta su posterior aplicación en campo. Una hora aproximadamente transcurrió desde que las botellas de medio fueron transportadas hasta la EA-UTPL.

Diseño experimental

El experimento fue desarrollado en un área específica de 360 m², dividido en parcelas de 40 m² (9 parcelas experimentales), dejando entre parcela una distancia de 4 m, para minimizar efectos de borde. Los tratamientos evaluados fueron: i) *Azotobacter* sp., ii) *Azospirillum* sp. y iii) testigo (control, sin aplicación de bacterias). Cada tratamiento tuvo tres repeticiones bajo un diseño experimental de bloques al azar.

Aplicación bacteriana

Previo a la realización del ensayo un área geográfica con kikuyo y de características homogéneas del terreno fue preparada a través de arado y cruce por método tradicional a través de un tractor (Pascuali Sinea K5 AR) con el propósito de descompactar y airear el suelo para realizar el ensayo.

Para aplicar las bacterias sobre el kikuyo germinado se diluyó 1L del concentrado bacteriano en 20L de agua, obteniendo una concentración de 1×10^6 células por mL, se usó el proceso mecánico de pulverización¹⁵. Este procedimiento fue aplicado al kikuyo en varios días. La primera aplicación ocurrió al siguiente día (después de arado), luego a los 15 días (germi-

nación) 30 días (crecimiento bajo 10-15 cm), y 45 días (crecimiento medio 25-30 cm). El registro de datos se realizó luego de la primera aplicación bacteriana hasta los 60 días, fecha a partir de la cual el pasto está listo para el consumo animal.

Evaluación de variables

Se evaluaron algunas variables en el kikuyo para determinar el efecto de las cepas bacterianas: i) altura de planta: se colocó una regla graduada perpendicularmente al nivel del suelo y se tomó la altura (cm); ii) largo máximo de la hoja: fue medido una sola vez al final del ensayo (60 días), a partir del pecíolo, utilizando la vena central como referencia (cm); iii) largo de raíces: usando una cuadricula de 20x20 cm se hizo un corte y se obtuvo una muestra que contenía suelo y raíces, se separaron las raíces y se procedió a la obtención de los datos (cm) al final del ensayo; iv) biomasa vegetal fresca: se usó un cuadrante de 1m² para cortar y luego pesar en el momento de la cosecha, los datos se reportaron en kg/ha; v) proteína total a partir de la biomasa cruda: evaluada por el método de Kjeldahl validada por Harris¹⁶.

Análisis estadísticos

Para comparar el resultado del efecto de las bacterias sobre las variables evaluadas en *P. clandestinum* se realizó un análisis de varianza de una vía (ANOVA) ($p < 0.05$). Cuando se determinó significancia, se aplicó la prueba de Tukey subconjuntos homogéneos ($p < 0.05$). Previo al análisis de los datos y una vez calculados los residuos se verificó el cumplimiento de los supuestos de normalidad, independencia y homogeneidad de varianzas. El software estadístico SPSS v. 23.0.¹⁷, fue usado para los análisis. Para obtener datos precisos en todas las variables evaluadas se registró 5 mediciones de cada plántula de cada tratamiento al azar, con tres repeticiones.

Resultados y discusión

La aplicación de las cepas bacterias *Azospirillum* sp. y *Azotobacter* sp. en kikuyo, ha permitido evaluar a través de la medición de algunas variables los posibles efectos sobre la productividad y el crecimiento.

El uso de bacterias BPCV en la agricultura está incrementando continuamente, ya que ofrece una herramienta eficaz para sustituir el uso de fertilizantes químicos, pesticidas y otros suplementos dañinos^{2,3}. *Azospirillum* sp. y *Azotobacter* sp. son consideradas BPCV por la capacidad de mejorar la salud de las plantas¹⁸.

Los géneros *Azospirillum*, *Azotobacter* y *Pseudomonas* son ampliamente utilizados por sus características como fijadores de nitrógeno^{19,20}, la capacidad de producir índoles^{20, 21} y solubilizar fósforo^{22,23}, propiedades que hacen de estos microorganismos potenciales biofertilizantes.

Se registró la altura de la planta de pasto kikuyo y se determinó que el tratamiento con *Azospirillum* sp. presenta una mayor altura de la planta (Figura 1) en relación al tratamiento con *Azotobacter* sp. y al control (testigo). El análisis estadístico realizado evidencia las diferencias significativas entre el tratamiento *Azospirillum* sp. y el testigo.

Sobre la evaluación de la altura de kikuyo, el tratamiento con *Azospirillum* sp. mostró los registros de mayor altura, en relación al trabajo de Guimarães et al.²⁴ en un estudio con *Urochloa brizantha* (herba empalizada) inocularon nueve aislados de *Azospirillum* y determinaron plantas más altas usando la cepa *Azospirillum* (AZ02) cuando se comparó con el control. da

Silva Ramos *et al.*²⁵ evaluó el crecimiento de plantas de maíz inoculadas con *Azospirillum lipoferum* (BR cepa 11084) luego de 30 días de sembradas observaron un incremento en la altura de la planta.

Sobre el largo máximo de la hoja no se evidencia ningún efecto del uso de *Azospirillum* sp. y *Azotobacter* sp. ya que los valores registrados sobre el largo de la hoja son similares y no son estadísticamente diferentes entre tratamientos (Figura 2).

En cuanto al largo máximo de la hoja no se observó una diferencia de la aplicación de *Azospirillum* sp. y *Azotobacter* sp. sobre el control. En un estudio realizado por Reddy *et al.*²⁶ en la aplicación de *Azospirillum* sp. y *Azotobacter* sp. sobre tomate (*Lycopersicon esculentum*) donde se evaluó la variación del área de la hoja no se observaron diferencias con el control que no estaba inoculado por ningún microorganismo. Barassi *et al.*²⁷ reportó sobre el mejoramiento en los parámetros fotosintéticos de hojas, incluyendo el incremento en el contenido de clorofila y conductancia estomatal, además, de la producción de biomasa y altura de la planta.

El largo de la raíz que fue medido al día 60 permitió determinar que la presencia de *Azospirillum* sp. y *Azotobacter* sp. ejercen un efecto en el desarrollo de la raíz. Los resultados de la aplicación de bacterias muestran una clara diferencia estadística con el testigo (Figura 3).

El incremento en el desarrollo de las raíces se observó en los tratamientos con la aplicación de *Azospirillum* sp. y *Azotobacter* sp., debido al mejoramiento de la absorción de agua y nutrientes, lo que ha permitido un incremento de la capacidad

de la planta para tolerar estrés ambiental como sequía, salinidad, resultando en plantas más vigorosas y productivas²⁸⁻³⁰. En el 2017 Pérez y Sánchez³¹ determinaron que *Azospirillum* y *Azotobacter* asociadas al cultivo de *Ipomoea batata* tuvo incrementos significativos en la longitud de raíz. En *Azotobacter* se da la producción de la fitohormona auxina que ayuda a la producción de raíces largas e incrementa el número de pelos y raíces laterales que están involucradas en la absorción de nutrientes³².

La biomasa vegetal fresca de kikuyo se determinó por metro cuadrado, se pudo determinar que el tratamiento *Azospirillum* sp. presenta un incremento en la biomasa en relación al testigo (Figura 4).

La biomasa vegetal fresca del kikuyo resultó ser mayor en el tratamiento *Azotobacter* sp. con relación al testigo. En un estudio desarrollado por Mahato y Kafle en el 2018³³ en trigo donde se realizaron varios tratamientos se determinó que la biomasa del tratamiento *Azotobacter* fue mayor solo respecto al control, además, se observó un alto rendimiento de biomasa en aquellos tratamientos en los que *Azotobacter* acompañó al fertilizante químico.

La proteína total evaluada en el tratamiento *Azotobacter* sp. muestra un mayor porcentaje de proteína determinada en relación al tratamiento *Azospirillum* sp. y testigo (Figura 5).

El porcentaje de proteína determinado en el tratamiento *Azotobacter* sp. fue mayor en relación a los otros dos tratamientos. En el caso de la inoculación de *Azotobacter* dentro de condiciones controladas o *in vitro*, las plantas responden

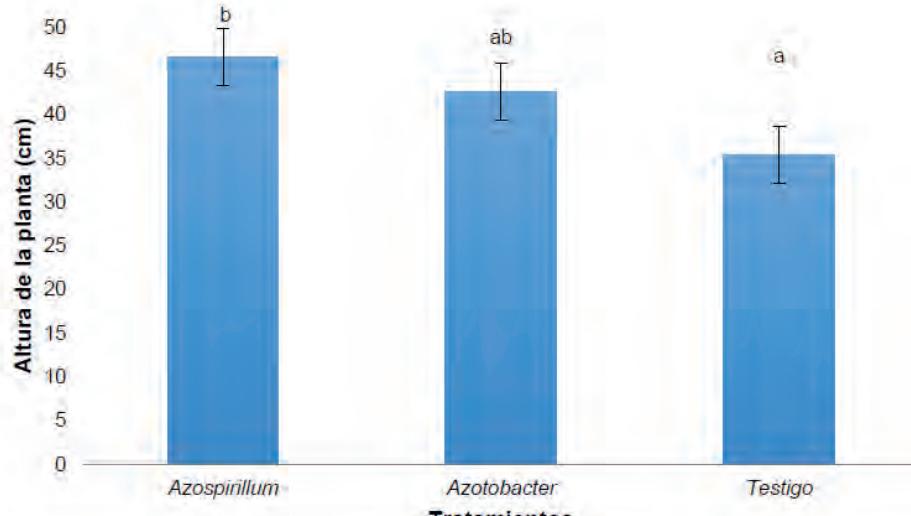


Figura 1. Altura de *Pennisetum clandestinum* inoculado con tratamiento 1 (*Azospirillum* sp.), tratamiento 2 (*Azotobacter* sp.) y tratamiento 3 (Testigo).

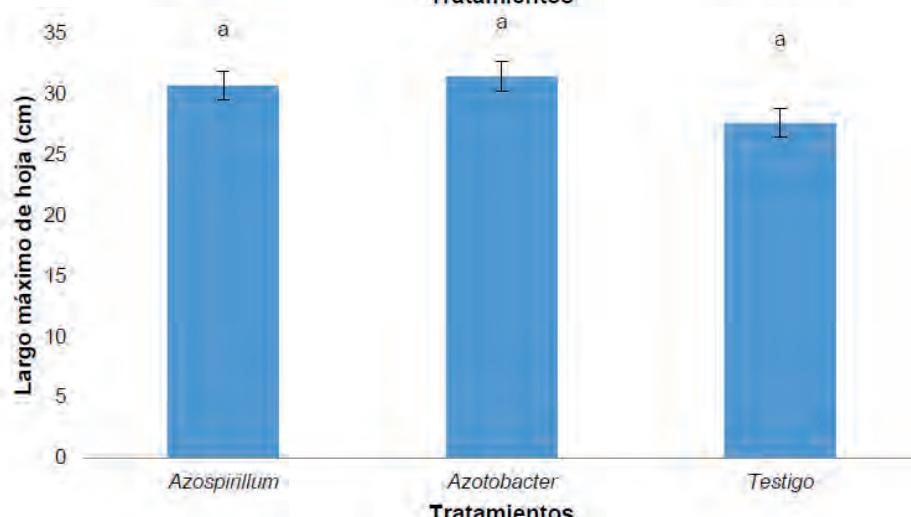


Figura 2. Largo máximo de hoja de *Pennisetum clandestinum* inoculado con tratamiento 1 (*Azospirillum* sp.), tratamiento 2 (*Azotobacter* sp.) y tratamiento 3 (Testigo).

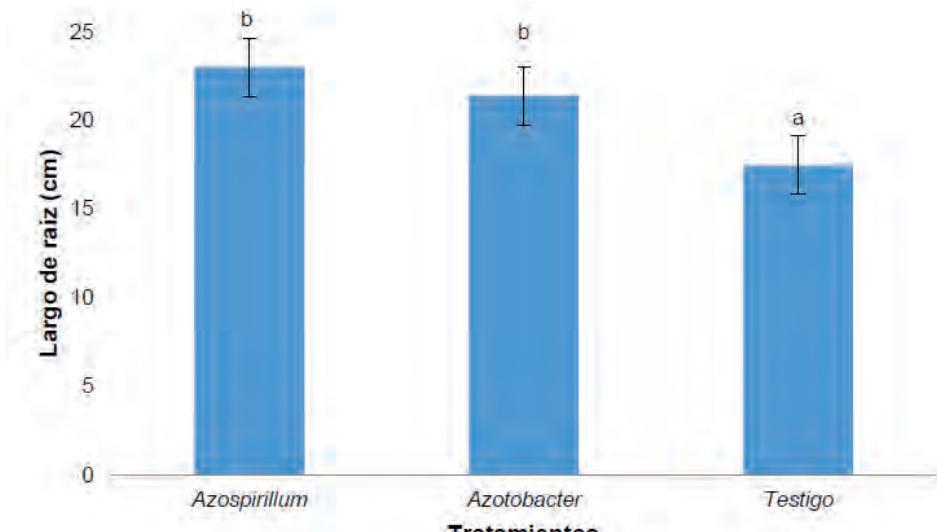


Figura 3. Largo de la raíz de *Pennisetum clandestinum* inoculado con tratamiento 1 (*Azospirillum* sp.), tratamiento 2 (*Azotobacter* sp.) y tratamiento 3 (Testigo).

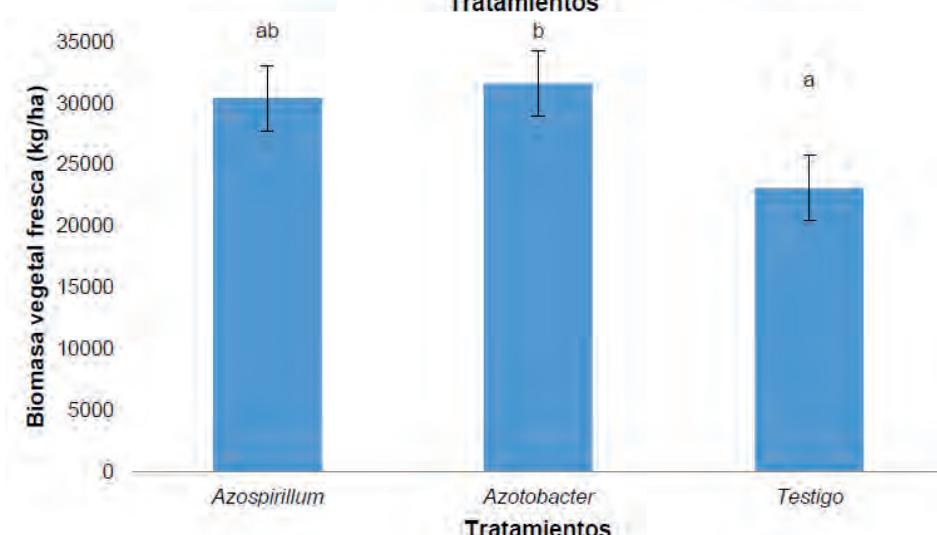


Figura 4. Biomasa vegetal fresca en Kg/ha de *Pennisetum clandestinum* inoculado con tratamiento 1 (*Azospirillum* sp.), tratamiento 2 (*Azotobacter* sp.) y tratamiento 3 (Testigo).

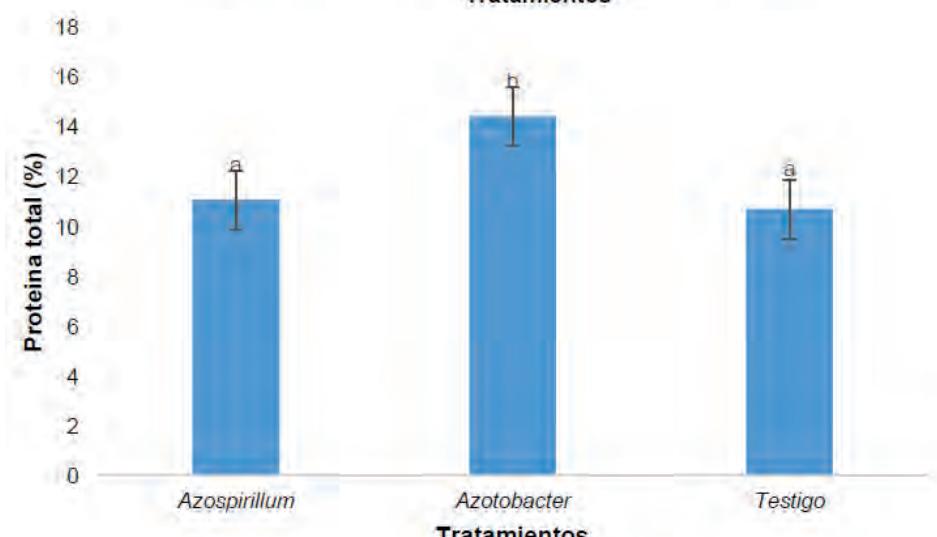


Figura 5. Proteína total en porcentaje de *Pennisetum clandestinum* inoculado con tratamiento 1 (*Azospirillum* sp.), tratamiento 2 (*Azotobacter* sp.) y tratamiento 3 (Testigo).

al estímulo bacteriano, entre ellos podemos destacar varios atributos como el contenido de proteína después de haber sido cosechado³⁴.

Como una perspectiva futura es importante evaluar el efecto de las dos bacterias fijadoras de nitrógeno como parte de un mismo tratamiento ya que se ha reportado en otros estudios que el uso de la especie *Azotobacter* como biofertilizador o en combinación con otras especies benéficas como

Azospirillum, mejora el rendimiento y la calidad de diferentes cultivos³⁴. Asimismo, las bacterias asociadas con la producción de gramíneas que se han estudiado más, son las de los géneros: *Azospirillum*, *Azotobacter*, *Klebsiella*, *Beijerinckia*, *Pseudomonas* y *Bacillus*. Algunas de ellas forman estructuras de resistencia para favorecer su supervivencia en condiciones de estrés, en especial sequía, la cual es común en los pastizales de zonas áridas³⁵.

Conclusiones

La aplicación de bacterias fijadoras del nitrógeno como *Azospirillum* sp. y *Azotobacter* sp. permiten mejorar los indicadores de crecimiento y rendimiento de kikuyo. La bacteria *Azotobacter* sp. presenta un efecto sobre la producción de biomasa fresca, largo de raíz y proteína total y la bacteria *Azospirillum* sp. sobre la altura de la planta y el largo de raíz favoreciendo los indicadores de crecimiento y calidad del kikuyo (*P. clandestinum*).

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RESEARCH / INVESTIGACIÓN

Productos Forestales No Maderables de la comunidad El Tundo: un remanente boscoso de biodiversidad y conocimiento ancestral del sur del Ecuador

Non-Timber Forest Products of the El Tundo community: a forested remnant of biodiversity and ancestral knowledge of southern Ecuador

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Resumen: Los Productos Forestales No Maderables (PFNM) constituyen una fuente importante de subsistencia para las comunidades rurales del Ecuador. El presente estudio buscó identificar los PFNM más relevantes, así como su uso tradicional y actual en la comunidad El Tundo del cantón Sozoranga, Provincia de Loja. La información etnobotánica y etnozoológica se obtuvo a través 30 entrevistas orales y observaciones de campo. Se determinó el consenso entre los informantes en cada categoría de uso mediante (FIC), así como otros índices cuantitativos que permitieron calcular la importancia o valor cultural (IVU), validación científica (UST) y uso común (IF) de las especies. Se registraron 123 PFNM, de los cuales 116 fueron vegetales y 7 animales distribuidos en 12 categorías de uso, de las cuales destacaron la medicina humana y materiales. Las especies más versátiles localmente son *Juglans neotropica* Diels, *Myroxylon peruferum* L. f. y *Verbena litoralis* Kunth. De las especies registradas, 33 constituyen recursos potenciales por su elevada aceptación socio-cultural, mientras que 17 de ellas presentaron los más altos niveles de fidelidad en toda la comunidad. Este trabajo contribuye a valorizar el conocimiento local para generar alternativas de aprovechamiento y uso sostenible de la biodiversidad.

Palabras clave: Biodiversidad, Productos Forestales No Maderables, El Tundo, Sur del Ecuador.

Abstract: Non-Timber Forest Products (NTFP) constitute an essential source of subsistence for rural communities of Ecuador. The present study identified NTFP and its traditional and current use in El Tundo community of Sozoranga canton, province of Loja. The ethnobotanical and ethnozoological information was obtained through 30 oral interviews and field observations. The consensus among informants in each category of use was determined using (FIC), as well as other quantitative indices that allowed calculating the importance or cultural value (IVU), scientific validation (UST), and everyday use (IF) of the species. 123 NTFP were registered, of which 116 were vegetables and 7 animals distributed in 12 categories of use, of which human medicine and materials stood out. The most locally versatile species are *Juglans neotropica* Diels, *Myroxylon peruferum* L. f. and *Verbena litoralis* Kunth. Of the registered species, 33 constitute potential resources due to their high socio-cultural acceptance, while 17 present the highest levels of fidelity in the entire community. This work contributes to value local knowledge to generate alternatives for the sustainable use and harvesting of biodiversity.

Key words: Biodiversity, Non-Timber Forest Products, El Tundo, Southern Ecuador.

Introducción

En la actualidad existen varias definiciones para el término Producto Forestal No Maderable (PFNM), una de las más aceptadas es aquella propuesta por la Organización de las Naciones Unidas para la Agricultura y la Alimentación¹ (1999) (FAO), quien los define como "bienes de origen biológico, distintos de la madera, derivados del bosque, de otras áreas forestales y de los árboles fuera de los bosques" (p.63). Existen muchas definiciones para este término que difieren unas de otras, existen dos aspectos fundamentales a la hora de considerar un PFNM, primero que sea dependiente de ecosistemas boscosos o terrenos análogos y segundo que sea excluyente de la madera².

En el Ecuador los PFNM han desempeñado un rol fundamental en la vida y el bienestar de los habitantes de diferentes sectores en cada una de las regiones naturales³, principalmente en la amazonía y la sierra⁴. En consecuencia, ha sido posible la instauración y el desarrollo de comunidades rurales campesinas e indígenas a lo largo del territorio ecuatoriano en particular en la región andina, en dónde la estrecha y longeva relación entre las diversas etnias que componen estas comunidades y el ecosiste-

ma andino ha generado un amplio conocimiento sobre el uso de especies forestales en diversos ámbitos⁵⁻⁷ como alimentación⁸⁻¹⁰, medicina¹¹, tintes¹², fibras y materiales de construcción^{13,14}.

A pesar de la gran riqueza vegetal (3039 spp.) y animal (568 spp.) existente en la provincia de Loja¹⁵, la información sobre PFNM aún es escasa. Por lo tanto resulta imprescindible conocer la interacción que mantienen las comunidades con la biota animal y vegetal, con la finalidad de lograr un manejo sostenible de estos recursos para promover la conservación de los ecosistemas forestales, y en especial de los bosques montanos andinos que a pesar de ser considerados como uno de los puntos calientes de biodiversidad o "hotspots" a nivel global, han sido muy poco estudiados y los más amenazados en los trópicos¹⁶⁻¹⁸.

Es así que tomando en cuenta estas premisas, se decide dar inicio con un estudio etnobiológico en la comunidad rural El Tundo en la provincia de Loja, la cual por sus condiciones bióticas y climáticas forma parte de los bosques de neblina montanos del Ecuador de acuerdo a la clasificación de Sierra¹⁹, mientras que correspondiendo con el sistema de clasificación

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más reciente del MAE²⁰, forma parte del Bosque siempreverde montano del Catamayo-Alamor (BsMn04). De todas maneras este ecosistema o formación vegetal constituye un sitio biodiverso con pocos remanentes naturales y en estado crítico en los Andes sur occidentales del país, por lo cual constituye un área natural prioritaria para la conservación²⁰⁻²².

El objetivo del presente estudio fue valorizar el uso tradicional de los PFNM de la comunidad El Tundo del cantón Sozoranga en la provincia de Loja. A través de los resultados se podrán identificar potenciales PFNM y establecer acciones correctivas o generar alternativas que promuevan un uso sustentable de las especies forestales.

Materiales y métodos

Localización

El estudio se realizó en la comunidad rural El Tundo, situada dentro del Bosque Protector Jatumpamba-Jorupe en el cantón Sozoranga de la provincia de Loja, sur de Ecuador. Geográficamente se ubica entre las coordenadas geográficas X: 632767 y Y: 9523280, a una altitud de 1.800 – 2.400 m s.n.m. (Figura 1).

Recopilación de información

Para esta fase se realizó previamente un Acta de Permiso de Consentimiento Previo Fundamentado, la cual permitió acceder al conocimiento ancestral ligado al uso tradicional de especies útiles. Se realizaron varios recorridos por todas las 18

viviendas que forman parte de esta comunidad. Se aplicaron 30 encuestas semiestructuradas correspondientes al 54% de la población total, considerando personas de ambos sexos con rangos de edad entre 28–94 años.

Algunos de los datos considerados en la encuesta para especies vegetales y animales fueron los siguientes: nombre común, categoría de uso (alimentación, bebida, combustión, cuidado personal, forraje, material, medicina animal, medicina tradicional, medicina humana, miel de insectos, ornamental y tintura), parte utilizada, forma de recolección, modo de preparación y administración, manejo, y abundancia; mientras que para los informantes se consideró la edad, sexo, nivel de educación formal, actividad económica, tiempo de estancia en la comunidad, y tipo de vivienda.

Recolección de muestras botánicas

Durante varios recorridos de campo por zonas boscosas y de cultivo, se colectaron las muestras botánicas de las especies mencionadas por los informantes. Esta actividad se realizó con la participación y acompañamiento de un grupo de personas de la comunidad. Para la colecta, procesamiento y preparación del material vegetal se utilizó la guía propuesta por Cascante²³.

Identificación de especies de flora y fauna

La determinación taxonómica de las muestras vegetales recolectadas se realizó en el ex Herbario de Plantas de Productos Naturales (HPPN), de la sección de química básica y aplicada del Departamento de Química de la Universidad Técnica Particular de Loja (UTPL), mediante la verificación con

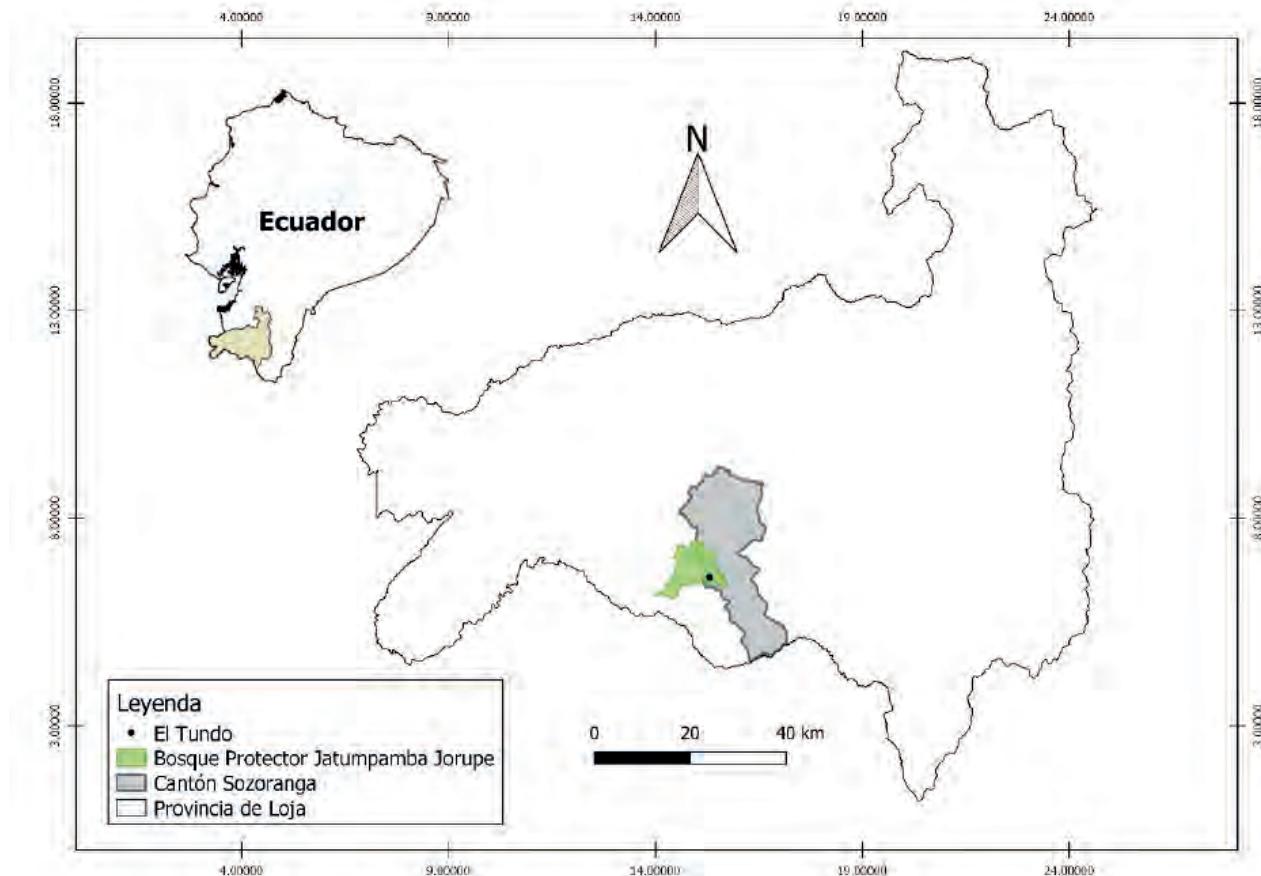


Figura 1. Posición geográfica del cantón Sozoranga y el área de estudio.

pliegos de muestras botánicas ya existentes en dicho herbario. A su vez, se utilizó literatura complementaria de estudios etnobotánicos como la de Andrade *et al.*²⁴; Tinitana Imaicela²⁵; y Tene *et al.*²⁶.

Para corroborar la información en cuanto a su taxonomía y origen biogeográfico de las especies vegetales, se consultó la base de datos on-line Tropicos del Missouri Botanical Garden²⁷ (<https://www.tropicos.org>), el Catálogo de plantas vasculares del Ecuador²⁸, y el Catálogo de la Vida²⁹ (<https://www.catalogueoflife.org>); mientras que para las especies animales se consultó el portal de FaunaWebEcuador³⁰ (<https://bioweb.bio/faunaweb.html>) y la base de datos de Global Biodiversity Information Facility (GBIF)³¹ (<https://www.gbif.org>).

Para conocer el estado de conservación de las especies se revisó la Lista Roja de la Unión Internacional para la Conservación de Naturaleza³², el Libro Rojo de las plantas endémicas del Ecuador³³, y el Libro Rojo de los mamíferos del Ecuador³⁴. La nomenclatura taxonómica utilizada fue acorde a la propuesta por Jørgensen y León-Yáñez²⁸; Tirira³⁵; y Tirira *et al.*³⁶.

Índices etnobotánicos cuantitativos calculados

La información registrada a partir de las encuestas realizadas fue ingresada y organizada en una matriz de datos para su análisis cuantitativo. Para determinar los acuerdos (coincidencias) de los informantes sobre los PFNM utilizados en cada categoría de uso, se calculó el Factor de Consenso del Informante (FIC)^{37,38}, usando la siguiente fórmula:

$$FIC = \frac{Nur - Nt}{Nur - 1}$$

Dónde:

Nur = número total de citaciones reportadas para una categoría de uso.

Nt = número total de especies utilizadas para esa categoría de uso.

De la misma forma se calculó el Índice de Valor de Uso (IVU), para determinar la importancia o valor cultural de una determinada especie para todos los informantes de la comunidad³⁹, empleando la siguiente fórmula:

$$IVU = \frac{\sum UV}{ns}$$

Dónde:

UV = número de usos mencionados por cada uno de los informantes para cada especie.

ns = número de informantes encuestados.

Asimismo se calculó el Índice de Nivel de Uso Significativo TRAMIL (UST) para estimar en base a la frecuencia de usos ($\geq 20\%$), si una especie merece su evaluación y validación científica⁴⁰, para ello se aplicó la siguiente fórmula:

$$UST = \frac{\text{Uso Especie(s)}}{ns} * 100$$

Dónde:

Uso especie(s) = número de citaciones para cada especie.
 ns = número de informantes encuestados.

Finalmente para conocer la existencia de una posible especie con un mismo uso común en cada categoría de uso por toda la comunidad, se aplicó el Índice de Nivel de Fidelidad (FL)⁴¹ utilizando la siguiente fórmula:

$$FL = \frac{Ip}{It} * 100$$

Dónde:

Ip = número de informantes que mencionan el uso de una especie para una categoría en particular.

It = número total de informantes que reportan esa especie para cualquier categoría.

Resultados y discusión

Especies y familias reportadas

En este estudio se reportaron 226 usos diferentes para 116 especies vegetales y 7 especies animales proveedoras de PFNM (Tabla 1). Estas especies en su mayoría crecen en zonas boscosas, mientras que otras son cultivadas en huertos agroforestales y jardines. Estos resultados guardan relación a los de Añazco⁴², quien inventarió en cuatro formaciones vegetales de la región andina ecuatoriana 100 PFNM, de los cuales fueron 90 especies vegetales y 10 especies animales. Asimismo, en otros estudios realizados anteriormente dentro de la provincia de Loja, se muestran cifras similares en número de especies vegetales reportadas como PFNM. En el cantón Zapotillo se han registrado 87 especies vegetales⁴³, en el cantón Saraguro se reportan 89 spp.⁴⁴, en el cantón Macará se enlistan 111 spp.⁴⁵, y en el Parque Nacional Yacuri en el cantón Espíndola se registran 209 spp.⁴.

Las especies registradas se agruparon sistemáticamente en 54 familias y 95 géneros. Para las especies vegetales, las familias botánicas más representativas fueron Asteraceae (8 spp.), Poaceae (8 spp.), y Solanaceae (7 spp.). En cuanto a las especies animales, las familias registradas fueron Apidae, Dasypodidae, Didelphidae, Felidae, Mephitidae, Procyonidae, y Tayassuidae cada una con 1 especie. En diversos estudios etnomedicinales la familia Asteraceae se ha registrado como la más numerosa en especies⁴⁶⁻⁴⁸ y consecuentemente la más relevante en la comercialización de sus especies con fines terapéuticos entre la población ecuatoriana⁴⁹. La gran cantidad de metabolitos secundarios⁵⁰, y compuestos aromáticos con propiedades carminativas y desinfectantes⁵¹ que esta familia botánica produce puede estar relacionado con el valor medicinal que se le atribuye a la misma.

Categorías de uso registradas

Entre las diferentes categorías de uso reportadas, el mayor número de registros se obtuvo para (i) medicina humana (60 spp.), seguido del uso para (ii) elaboración de bebidas (39 spp.) y (iii) alimento (33 spp.). Es importante resaltar que una misma especie en algunos casos estuvo presente en más de una categoría de uso, lo cual demuestra la importancia de los usos o aplicaciones que tienen los recursos forestales para los pobladores de la comunidad El Tundo (Figura 2).

En Ecuador y particularmente en la provincia de Loja también se destaca y prevalece el uso medicinal como el más predominante y a su vez importante al igual que el uso de alimentación humana^{43-45,52-54}. Estos reportes corroboran el uso habitual y constante de la medicina herbolaria en la población como una

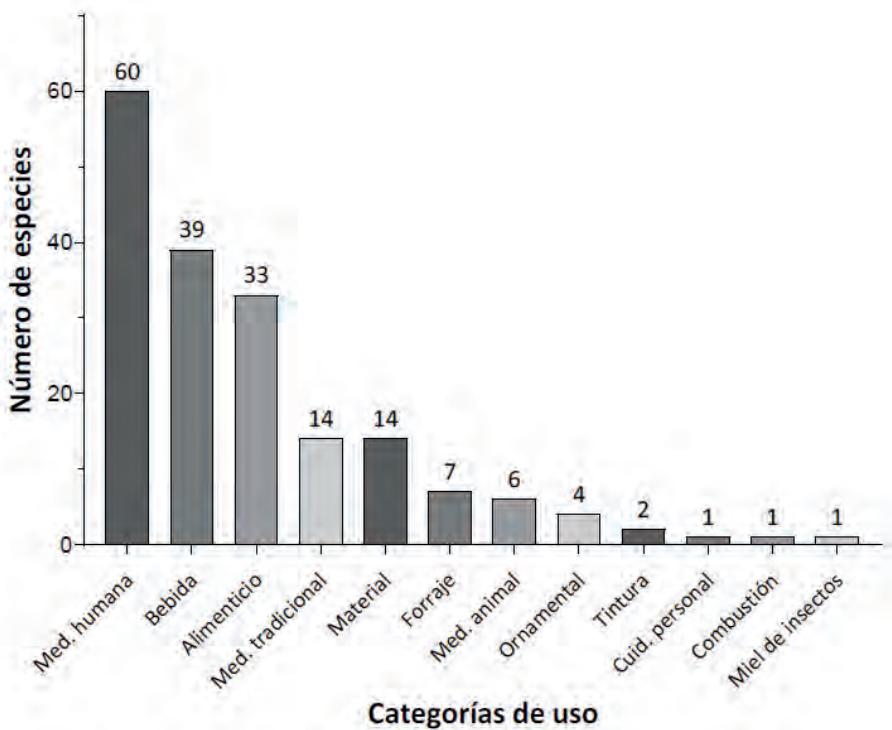


Figura 2. Número de especies reportadas como PFNM para cada categoría de uso en la comunidad El Tundo.

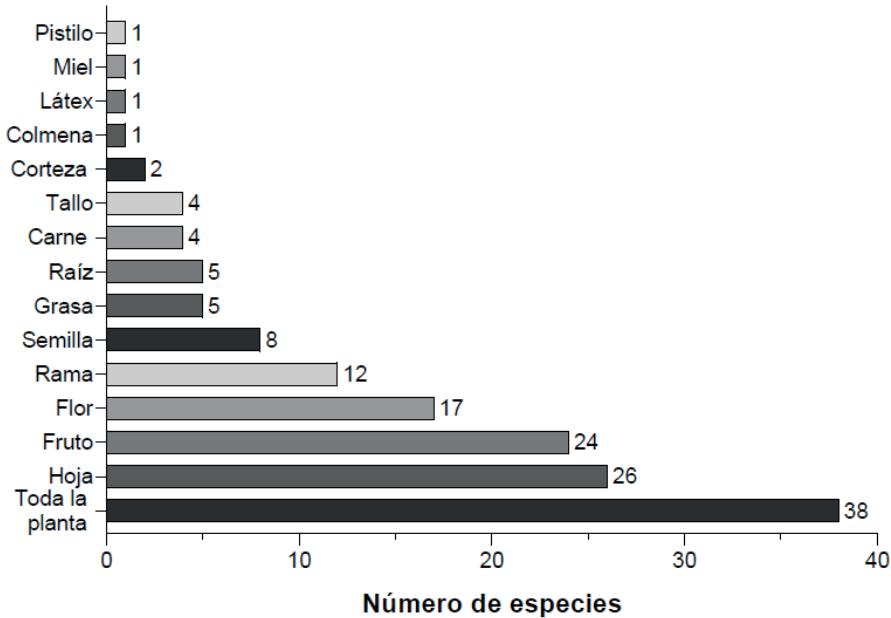


Figura 3. Estructuras útiles de las especies proveedoras de PFNM en la comunidad El Tundo.

alternativa natural de sanación. Por consiguiente se demuestra que el gran número de las especies que forman parte de estas categorías de uso ejercen un papel fundamental en la vida de los habitantes de la comunidad El Tundo, en especial aquellas relacionadas para atenuar problemas de salud puntuales.

Por otro lado la relación ser humano-fauna constituye también una importante interacción que las personas de esta comunidad han mantenido. Si bien, las especies de este grupo taxonómico reportadas en este estudio (7 spp.) no son tan numerosas en comparación a las especies vegetales (116 spp.), se han encontrado importantes usos para las mismas como son: (i) medicina humana (6 spp.), (ii) alimenticio (4 spp.), (iii) medicina animal (1 spp.), (iv) medicina tradicional (1 spp.), y (v) miel de insectos (1 spp.).

A nivel nacional se han realizado estudios etnozoológicos con fines de conservación, y registro de usos de PFNM en donde se incluyen las categorías de medicina humana y alimentos como las más habituales⁴², además de otras utilidades complementarias como ritual, animal de compañía⁵⁵, y melífera⁵⁶.

Formas de vida, rango biogeográfico, y estado de conservación de las especies

Las 123 especies animales y vegetales se encuentran organizadas morfológicamente en ocho formas de vida. Las hierbas representan la mayor asignación con un total de (60 spp.), seguido de los arbustos (27 spp.), árboles (23 spp.), mamíferos (6 spp.), trepadoras (2 spp.), e insectos, lianas, y subarbustos (1 spp.) cada uno. Del total de plantas vasculares documentadas

en el Ecuador hasta el momento, las hierbas representan el hábito más numeroso en especies²⁸ y en especies útiles para alguna categoría ocupa el segundo lugar después de los árboles⁵³.

La relevancia del hábito herbáceo en términos de uso medicinal en la región sur del Ecuador también se ha documentado en diversos estudios previos^{6,24,46,57,58}. Por otro lado el predominante uso de las hierbas, puede estar relacionado con el ecosistema boscoso montano del que forma parte el área de estudio, en dónde el estrato herbáceo es abundante^{20,22}, y por la gran concentración de metabolitos secundarios que este grupo posee⁵⁹. Esto refuerza la idea de la alta frecuencia del uso del hábito herbáceo por parte de la comunidad.

Del total de especies reportadas (123 spp.), se lograron identificar taxonómicamente hasta nivel de especie 109 spp., de las cuales 38 son nativas, 35 son introducidas y cultivadas, 13 son nativas y cultivadas, 10 son introducidas, 8 son cultivadas y 3 son endémicas; mientras que de las 2 restantes no se dispone de información con respecto a su origen en publicaciones especializadas.

De las 38 especies nativas, 22 de ellas tiene usos en medicina humana. En estudios realizados en las provincias de Loja y Zamora Chinchipe se muestran cifras superiores de especies nativas medicinales^{24,26,57,58}. Estos datos posiblemente sean el resultado de la gran diversidad de especies vegetales útiles nativas identificadas en el Ecuador (4.591 spp.), cifra que representan el 89% de la totalidad de especies útiles registradas en el país⁵³.

Sin embargo esta biodiversidad nativa se puede ver comprometida por la presencia de una gran cantidad de especies introducidas e introducidas y cultivadas en la zona, pues según Nuñez y Simberloff⁶⁰ las especies exóticas o invasoras constituyen una seria amenaza para la persistencia de la diversidad. El extenso uso de plantas introducidas posiblemente sea consecuencia del gran valor alimenticio y medicinal que estas poseen.

Es así que en Ecuador especies introducidas como *Coffea arabica* L. (café), *Saccharum officinarum* L. (caña de azúcar), y *Musa paradisiaca* (banano) constituyen especies emblemáticas con fines alimenticios⁶⁰ (Nuñez y Simberloff, 2005). Mientras que en el ámbito de la medicina, las especies introducidas destacan por su versatilidad para el tratamiento de diversas patologías, y por su amplia disponibilidad en todo el territorio nacional⁶¹. Lo cual reafirma su elevado grado de utilidad por parte de la población local.

Para la clasificación de acuerdo al estado de conservación, se consideraron las 109 especies animales y vegetales identificadas taxonómicamente hasta nivel de especie. Estas se encuentran distribuidas en seis categorías en función al sistema de clasificación de la UICN. La mayor parte de las especies (68 spp.) se encuentran en el primer nivel de clasificación, No Evaluado (NE), le sigue la categoría de Preocupación Menor (LC) con (28 spp.). Con menor número de especies están aquellas que presentan Datos Insuficientes (DD) (7 spp.), En Peligro (EN) se encuentran (3 spp.), Casi Amenazadas (NT) están (2 spp.), y en estado Vulnerable (VU) se encuentra (1 spp.).

De las ocho categorías, tres de ellas [En Peligro Crítico (CR), En Peligro (EN), y Vulnerable (VU)] implican amenaza⁶². En este contexto, cuatro especies de las identificadas se encuentran bajo algún grado de amenaza. En el caso de las especies vegetales, aquellas que se encuentran En Peligro (EN) son el café (*Coffea arabica* L.), el nogal (*Juglans neotropica* Diels.) y el monte del oso (*Siparuna eggersii* Hieron.) tanto en la Lista Roja de la UICN³² como en el Libro Rojo de las plantas endémicas del Ecuador³³ para el caso de las dos últimas espe-

cies. En cuanto a las especies animales, únicamente el león o puma (*Puma concolor*) se encuentra catalogado como Vulnerable (VU) a nivel global en la Lista Roja de la UICN³² y a nivel nacional en el Libro Rojo de los mamíferos del Ecuador³⁴.

En términos de uso, los que más destacan para estas especies amenazadas son el uso medicinal y alimenticio. Ambas utilidades conllevan para el caso de las especies vegetales la recolección de partes aéreas de la planta, lo cual en modo constante genera la destrucción total de los individuos, mientras que para los animales implican la muerte inmediata del individuo. Probablemente estas circunstancias han inducido a nivel global a que buena parte de las especies medicinales principalmente (alrededor de 15.000), formen parte del listado de especies amenazadas registradas por la UICN⁶³. A su vez factores de presión adicionales como la deforestación, fragmentación de hábitats, prácticas agroforestales inadecuadas, sobreexplotación de la flora y fauna silvestre, entre otros ponen en riesgo la perdurabilidad de los PFNM y por ende los medios de subsistencia especialmente de las familias de escasos recursos⁶⁴⁻⁶⁹.

El análisis de las cifras indica a su vez que cerca del 68% de especies útiles para la comunidad, no poseen una evaluación en cuanto a su riesgo de extinción, lo cual las posiciona en las categorías de No Evaluado (NE) o Datos Insuficientes (DD); sin embargo esto no quiere decir que no se encuentren amenazadas⁶². A nivel de la región, los estudios sobre el estado de conservación son escasos para los grupos taxonómicos de plantas medicinales⁴⁶ e insectos tales como las abejas⁷⁰. Es por ello crucial dar inicio con investigaciones que aporten información suficiente para esclarecer las probabilidades de extinción de estas importantes especies y poder establecer prioridades de conservación.

Estructuras morfológicas y derivados utilizados de las especies

Con respecto a las partes vegetales más aprovechadas, la planta entera presenta el mayor porcentaje de uso que reúne 38 spp. (31%), seguido de las hojas con 26 spp. (21%), y frutos con 24 spp. (20%); menores proporciones tienen las flores que registran 17 spp. (14%), ramas con 12 spp. (10%), semillas con 8 spp. (6%), raíces con 5 spp. (4%), tallos con 4 spp. (3%), cortezas con 2 spp. (2%), por su parte el látex y los pistilos con 1 sola spp., representan el (1%) cada una. Por otro lado en cuanto a los derivados de origen animal, la grasa (manteca) con 5 spp., y carne con 4 spp. se utilizan en mayores proporciones (4 y 3% respectivamente), mientras que la miel y colmenas con 1 spp. se emplean muy poco (1%) (Figura 3).

Si bien, se ha identificado a las hojas y frutos como las formas vegetativas más utilizadas como PFNM en los bosques andinos ecuatorianos⁴²; toda la estructura vegetal representa también una importante estructura con diferentes utilidades. En tal sentido en el presente estudio se incluyen 6 categorías de uso diferentes para las 38 especies de las cuales se aprovecha toda su estructura como materia prima. Entre las diferentes utilidades más frecuentes descritas para esta parte morfológica están el uso con fines medicinales, para elaboración de bebidas y para fabricación de herramientas domésticas.

Estos resultados guardan relación con lo reportado al sur del país, en la provincia de Loja por Bussmann y Sharon⁴⁷; y Andrade *et al.*²⁴, quienes identifican a toda la planta como la estructura morfológica mayormente utilizada con fines medicinales, y Ríos *et al.*⁴⁶ a las hojas y flores como los órganos vegetales principalmente utilizados en la elaboración de bebidas

De todas maneras, las hojas al igual que las flores tam-

bién forman parte de esta estructura útil (toda la planta) de las herbáceas frecuentemente reportada; por ende su uso habitual puede estar relacionado con las sustancias activas y compuestos químicos almacenados en forma de metabolitos secundarios que contienen las hojas principalmente de las hierbas medicinales^{71,72}.

Para el caso de las partes y derivados de origen animal, se aprovecha la grasa, carne, y miel principalmente para la medicina humana y alimento. Es así que los animales silvestres constituyen para las comunidades rurales una fuente de calorías, proteínas y grasas esenciales⁷³. Entre las especies mencionadas para el consumo de carne están: armadillo (*Dasypus novemcinctus*); león o puma (*Puma concolor*); sajino (*Pecari tajacu*); y shushano o coatí (*Nasua narica*). Aunque se esperaba que por la posición geográfica del área de estudio el impacto hacia la fauna silvestre sea aún más significativo, esto no fue así debido a que los pobladores de la comunidad emplean con mayor frecuencia fuentes de proteína alternativas procedentes de la crianza de animales domésticos.

Sin embargo el valor medicinal que se le atribuye a la grasa de los mamíferos es de gran importancia local, pues se registran beneficios curativos y preventivos que no lo poseen las plantas; por ejemplo para tratar manchas de la piel se emplea la raposa (*Didelphis marsupialis*), fracturas de huesos el shushano o coatí (*Nasua narica*), y combatir enfermedades reumáticas el añango (*Conepatus semistriatus*); shushano o coatí (*Nasua narica*), y respiratorias el armadillo (*Dasypus novemcinctus*). Todo esto se corrobora con los registros de usos similares para la grasa y carne de estos y otros mamíferos en Ecuador^{55,74}, Colombia^{75,76} y México⁷⁷⁻⁸⁰. Cabe resaltar que para todas estas especies animales mencionadas existen criterios de selección por parte de los consumidores, es así que son capturados y aprovechados aquellos individuos más desarrollados que presenten mayor masa corporal. Esta particularidad puede conllevar a un desequilibrio en la estructura poblacional de las especies, afectando así las posibilidades de desarrollo y reproducción de las mismas⁷⁵.

Por otro lado el uso medicinal de la miel de abeja como PFNM se ha reportado también en la comunidad de Quimis en la provincia de Manabí, debido a sus propiedades curativas⁵⁶. En este estudio se reporta el uso de la miel de abeja (*Apis mellifera Linnaeus*) para el restablecimiento de cortes y heridas, uso que está relacionado con la acción anti-bacteriana, anti-oxidante y anti-inflamatoria de los componentes de la miel para la reparación y cicatrización de tejidos^{81,82}. Sin embargo, actualmente esta práctica de extracción de miel a partir de apíarios ya no se realiza entre los campesinos de la comunidad, por ende, se ha generado una pérdida del conocimiento tradicional en torno a esta práctica.

Estado de uso o consumo de las especies

El uso o consumo de los PFNM se distribuye en cinco estados, de los cuales algunas especies se emplean en más de un estado. En la mayor parte de los casos se aprovecha el material animal y/o vegetal de las especies en estado fresco (82 spp.), sin embargo, también se emplea bien sea fresco o seco (17 spp.), o por su parte únicamente en seco (13 spp.). Para el caso de frutos y algunas semillas comestibles, se consumen con más frecuencia en estadio maduro (26 spp.), y muy poco inmaduro (2 spp.).

En otros estudios realizados en la provincia de Loja, el uso de material vegetal fresco para preparar remedios y bebidas también es más común que en estado seco^{46,47,58}. Asimismo el consumo de frutos y semillas es mayor en estado maduro⁸³⁻⁸⁵.

De acuerdo con Jima y Megersa⁸⁶; Mata Pinzón *et al.*⁸⁷, el uso de plantas frescas o recién cosechadas se debe a que las sustancias activas de las mismas se conservan en su mayoría en estado fresco, que por el contrario se perderían durante el transcurso de secado.

Por su parte el aprovechamiento frecuente de una gran parte de frutos en estado maduro puede atribuirse a las características organolépticas (textura, color, aroma, sabor, nivel de dulzura) que presentan los frutos en este estado de desarrollo, lo cual genera un aspecto atractivo para su aceptación y consumo^{88,89}. Sin embargo por su estado de madurez, los frutos con esta característica se clasifican como productos perecibles, lo cual supone una tendencia a sufrir deterioro fisiológico y por ende de sus propiedades físico-químicas desde el momento de su cosecha⁹⁰. Es así que por este motivo los informantes mencionan que el consumo o preparación (para algunos casos) de los frutos es inmediato. Sigue lo contrario con las semillas y granos ya que al ser consumidas en estado seco estas contienen bajos niveles de humedad, es decir menos del 50% de agua en su composición, a esto se suman sus propiedades antioxidantes lo cual a su vez favorece una larga preservación y más aún si se almacenan en lugares libres de humedad^{91,92}. En la comunidad El Tundo las cosechas de gramíneas y leguminosas se conservan por largos períodos de tiempo en cajones de madera, ya que al ser una fuente rica de proteínas y carbohidratos⁹³, estas forman parte de la dieta diaria de las familias de esta comunidad.

Modo de consumo, empleo y preparación de las especies

La forma de preparación o consumo de las especies animales y vegetales varía según la categoría de uso y parte utilizada. En este contexto, la infusión es la forma más usual de preparación de bebidas y remedios (53 spp.), seguida de la decocción (14 spp.), y con menor frecuencia están los atados, zumos, manojos, macerados, triturado, estrujado, licuado, frito, picado, molido, tostado, entero, lijado, partido, caliente y tejido.

En la comunidad El Tundo, el consumo de bebidas mediante infusión es una tradición. Dentro de estas bebidas se encuentra una de carácter medicinal, la cual es conocida localmente como "tisana". Dos de las formas más comunes para preparar una tisana son la infusión y la decocción⁹⁴. En este caso, se trata de una infusión "bajeada" de 27 especies vegetales herbáceas y arbustivas frescas y/o secas, de las cuales se emplean partes suaves (hojas, tallos y ramas tiernas, y flores) para su preparación.

La preparación de estas partes tiernas de plantas medicinales mediante infusión, es el procedimiento más idóneo para la obtención de una buena tisana; debido a que se logra de una manera efectiva la extracción de los principios activos de las mismas, reduciendo la alteración de su estructura por acción del calor⁹⁵. El consumo de esta infusión es habitual debido a sus efectos antiinflamatorios, además se le atribuye propiedades terapéuticas que tratan el "calor interno", y contrarrestan dolores renales.

En la provincia de Loja se ha registrado una bebida tradicional muy similar en aspecto y forma de preparación, comúnmente conocida como "horchata" que posee también propiedades antiinflamatorias⁴⁶. El efecto antiinflamatorio en este tipo de bebidas puede estar relacionado con el proceso de preparación y con los compuestos químicos que poseen las plantas que forman parte de la fórmula de preparación. De acuerdo con Villegas-Novoa *et al.*⁹⁶, las infusiones herbales presentan diversos compuestos químicos que actúan en forma conjunta produciendo una respuesta antiinflamatoria. Entre

los compuestos químicos de las plantas medicinales García Bacallao *et al.*⁹⁷ mencionan a los flavonoides y taninos, los cuales contribuyen a generar una respuesta antiinflamatoria.

A su vez dentro de esta categoría de infusiones se han mencionado otras propiedades terapéuticas por parte de los informantes como: analgésico, anticancerígeno (cáncer de la próstata), antigripal, infecciones del tracto urinario, sedante, hidratante, entre otras. Esta última es aprovechada mediante el consumo de "aguas aromáticas". Cabe mencionar que en algunos casos las infusiones se preparan mediante combinaciones, es decir se emplean dos o más especies; mientras que en otros casos se adicionan compuestos químicos como el bicarbonato de sodio, e incluso bebidas alcohólicas destiladas como el aguardiente de caña de azúcar. En un único caso en particular se registró el empleo de derivados de origen animal para preparación de infusiones medicinales; tal es el caso del nogal (*Juglans neotropica* Diels.), cuyas hojas se consumen en infusión con leche de vaca para producir un "aumento de la cantidad de sangre". Esta forma de preparación se ha documentado también en comunidades del Azuay y Cañar para tratar inflamaciones, afecciones bronquiales y anemia⁹⁸.

Forma de uso o administración de las especies

Se han identificado 17 formas de uso o administración diferentes. Cabe mencionar que las formas de administración están relacionadas con aspectos referentes a la medicina, alimentación y bebidas; mientras que las formas de uso hacen énfasis a aquellas utilidades vinculadas de la obtención de un material o producto en especial. La administración vía oral fue significativamente la más frecuente (93 spp.). Otras formas de administración fueron baños y aplicaciones tópicas, inhalación, lavados y limpias, frotación, pomada, paños y emplasto, y soplos. Por otro lado, la forma de uso más frecuente fue como herramienta (11 spp.), y en menor proporción ornamento, construcción, tinte, calefacción, cocción, y cordelería. Una misma especie en algunos casos tiene más de una forma de uso o administración.

La forma oral, ha sido descrita también en varios estudios dentro de la provincia de Loja como la vía más frecuente de administración de remedios y bebidas^{47,57,58}. Esto corrobora el empleo frecuente de la vía oral como principal mecanismo de administración de uso interno por parte de los usuarios de la comunidad, además de ser un medio de fácil y rápida asimilación de las propiedades medicinales^{71,99}.

Por su parte entre las formas de administración para uso externo están las limpias y los baños, las cuales se han registrado por parte de los informantes como aplicaciones (i) terapéuticas para el "resfrío de la sangre y de los huesos", "calor interno", "contrarrestar dolores de cabeza", "combatir resfriados", "endurar los bebés" y (ii) tradicionales para el "mal de aire" y "espanto de los niños". Estas vías de administración al igual que sus aplicaciones (terapéuticas y tradicionales) son de uso común en Loja y el resto de provincias que forman parte de los Andes ecuatorianos^{11,26,98,100,101}.

A su vez las pomadas también forman parte de estas dos aplicaciones, principalmente para "aliviar hinchazones", "contrarrestar problemas reumáticos", "contrarrestar problemas de asma", "contrarrestar dolores de huesos y fracturas" y "mal de aire". Estos beneficios pueden estar relacionados con los efectos emolientes que posee esta forma de administración semisólida¹⁰².

Asimismo, las inhalaciones, frotaciones y soplos son formas adicionales de administración externa para tratar diferentes afecciones. En Ecuador, estas prácticas forman parte de la

medicina ancestral andina⁷. Dentro de la comunidad El Tundo, se emplean principalmente en el campo de las aplicaciones tradicionales para tratar el "mal de aire" y el "espanto de los niños". En la comunidad de Saraguro de la provincia de Loja, los soplos por la boca corresponden a una práctica habitual para tratar enfermedades sobrenaturales tales como el "mal aire" o "susto"¹⁰³. Mientras que en las comunidades rurales de México, las inhalaciones y frotaciones son métodos de administración externa frecuentes para atender el "mal de ojo" y el "susto"⁸⁷.

Esto demuestra que estas formas de administración son sinérgicas para el tratamiento de este tipo de afecciones relacionadas con la medicina tradicional. La importancia que denotan las inhalaciones a la hora de contrarrestar el "mal de aire" puede atribuirse a los efectos que producen los aromas fuertes de las plantas utilizadas en el cuerpo de la persona afectada. Esta noción concuerda con lo reportado por (104) en su estudio etnobotánico con curanderos de la sierra ecuatoriana, quienes afirman que los olores fuertes de los órganos de las plantas generan una atracción intrínseca de "el mal" presente en el cuerpo humano. Tal es el caso de *Ruta graveolens* L, especie muy aromática¹⁰⁰ que genera un efecto relajante en las personas posterior a su inhalación¹⁰¹. Sin embargo al tratarse de una especie con potencial tóxico, su uso debe ser controlado para evitar cuadros letales¹⁰⁵.

Por otro lado, desde el punto de vista de los recursos materiales, el uso de los PFNM como herramientas ha desempeñado un rol importante para cubrir ciertas necesidades de uso doméstico en las comunidades rurales del Ecuador¹⁴. Los objetos manufacturados que se han empleado por los informantes como herramientas son escobas, recipientes para transporte de agua, y productos tejidos denominados "shikras"; de los cuales estos dos últimos ya no se utilizan actualmente debido a que han sido sustituidos por utensilios plásticos o de otro material. Es así que el acceso al mercado es uno de los factores que promueve el desuso de las plantas como materia prima para confeccionar sus propios utensilios por parte de las comunidades¹⁰⁶.

De igual manera en el pasado se extraían tintes de color marrón de las hojas del nogal (*Juglans neotropica* Diels.) y se realizaban sogas a partir de las fibras foliares de cabuya (*Furcraea andina* Trel.), prácticas que actualmente han desaparecido entre los pobladores pero que son conocidas por muy pocos adultos mayores, quienes han estado durante toda su vida relacionados a este medio natural. Esto demuestra que la comunidad El Tundo ha sufrido un proceso de transculturización promovido por la no valorización de los conocimientos ancestrales, lo cual ha generado un abandono de estas prácticas que implican el desuso de especies clave en las culturas andinas. A nivel de los Andes ecuatorianos *Juglans neotropica* Diels., ha formado parte de las especies vegetales útiles para teñir lana¹⁰⁷; mientras que *Furcraea andina* Trel. figuraba como uno de los principales PFNM más relevantes productores de fibra para la fabricación y comercialización de diversos utensilios¹⁰⁸. Sin embargo, en los últimos años el uso tradicional de ambas especies ha sufrido un declive a causa de la globalización y disponibilidad de artículos industriales de menor precio^{44,109,110}.

Sistemas de manejo de las especies

En cuanto al método de obtención o manejo de las especies que proporcionan PFNM, los cultivos representan la principal fuente de sustento para la comunidad (65 spp.), seguido del bosque (19 spp.), cultivos o pastizales (12 spp.), bosque o cultivos (10 spp.); un menor número de especies provienen de

los jardines (8 spp), la caza (6 spp.), apicultura (1 spp.), compra (1 spp.), y de la compra o cultivos (1 spp.).

En la comunidad El Tundo es común el cultivo de plantas en huertos agroforestales y jardines por parte de hombres y mujeres, los cuales proveen alimentos y medicinas naturales que cubren sus requerimientos diarios. Estos sistemas productivos están compuestos por una diversidad de especies entre las que destacan especies base de su alimentación (hortalizas, tubérculos, cereales), árboles y arbustos frutales, hierbas medicinales, y especies avícolas y pecuarias. A nivel de la provincia de Loja, el cultivo en huertos agroforestales es una práctica habitual de las comunidades rurales¹¹¹. La instauración de estos sistemas diversificados de producción especialmente en los sectores rurales tiene como finalidad garantizar la subsistencia de las familias campesinas¹¹², y a su vez contribuir a la conservación *in situ* de la biodiversidad¹¹³. Esto ratifica la importancia que representan los cultivos agroforestales para esta comunidad.

Por otro lado, el bosque corresponde a una fuente silvestre de cosecha de PFNM, de donde los pobladores mencionan extraer inusualmente pocos productos únicamente para consumo propio. Esta tendencia es similar principalmente para especies medicinales, las cuales de acuerdo a los estudios de Ansaldi *et al.*⁶ y Solano Rivera⁵⁷, se extraen muy poco del cerro o bosque debido a que son cultivadas en huertos y jardines. El hecho de que los pobladores de la comunidad no obtengan beneficios económicos de la venta de productos forestales medicinales, reduce en parte el impacto hacia este ecosistema. Pues se ha determinado que las cosechas y capturas masivas en los bosques se ven influenciadas por la demanda y el acceso a los mercados¹¹⁴. Cabe resaltar que el bosque en la comunidad El Tundo se encuentra en buen estado de conservación en las partes altas con pendientes fuertes que hacen el acceso limitado, mientras que en las zonas bajas y en parte planas la cobertura forestal se encuentra fragmentada o intervenida a causa del establecimiento de cultivos, pastizales y la deforestación, generando así un mosaico paisajístico en el lugar.

En cuanto a los PFNM de origen animal, estos se obtienen de la caza de especies silvestres. La cacería es una actividad bastante conocida en las comunidades rurales para la obtención de PFNM tales como carne y pieles¹¹⁵. Esta práctica es exclusivamente masculina, y a su vez poco frecuente entre los pobladores de esta comunidad, pues mencionan que los pocos ejemplares cazados son el resultado de una mera casualidad, y que además últimamente no se han observado animales silvestres por el sector y cuando aparecen se presentan muy pocos individuos, mientras que en otros casos algunas especies como el armadillo (*Dasypus novemcinctus*) han desaparecido por completo de la zona. Esto puede ser el resultado de la influencia de esta práctica sobre las estructuras poblaciones de las especies silvestres.

Al respecto Bennett y Robinson¹¹⁶, afirman que algunos de los efectos significativos de la caza en las poblaciones silvestres son la reducción de la densidad de las poblaciones y la extinción local de especies vulnerables. Lo cual se corrobora con el registro de una reducción poblacional de mamíferos silvestres a causa de la cacería como medio de subsistencia en las comunidades indígenas de los Sionas y Secoyas en el Ecuador¹¹⁷. Otra causal pude atribuirse a la intervención antrópica que ha sufrido el bosque en la comunidad, lo cual ha generado la migración de las especies dependientes del bosque hacia otros remanentes boscosos¹¹⁸. De todas maneras la comprensión del manejo de los recursos de vida silvestre, sean plantas o animales por parte de los miembros de un pueblo o

comunidad, resulta un hecho determinante para fomentar la conservación del bosque¹¹⁹.

Factor de Consenso del Informante (FIC)

En función de la utilidad dada por parte de los informantes, los PFNM han sido clasificados dentro de 12 categorías de uso. Los factores de consenso del informante se han calculado para cada categoría de uso (Tabla 2). El valor más alto de FIC (0.78) se obtuvo para la categoría de medicina humana, mientras que, para las categorías de cuidado personal, combustión y miel de insectos, los valores fueron nulos (0.00), debido al reducido número de citaciones para una sola especie en estas categorías.

Los valores de FIC obtenidos muestran que existe un consenso entre los pobladores de la comunidad El Tundo respecto al uso de los PFNM en todas las categorías de uso a excepción de las tres últimas. Para el caso de las nueve primeras categorías de uso se evidencia que existe un intercambio de conocimientos y criterio de selección bien definido entre la comunidad. Estos resultados guardan relación a lo registrado por Briceño Fonseca *et al.*¹²⁰, quienes señalaron a las categorías alimenticia y medicinal como las de mayor valor de FIC (0.67 y 0.66 respectivamente) en cuanto al uso tradicional de PFNM.

Para el caso de las categorías de cuidado personal, combustión y miel de insectos, no se registró un consenso entre los informantes con respecto al uso específico de las especies registradas en estas categorías. Este escaso consenso podría ser el resultado de un proceso de transculturación o deterioro de la transmisión del conocimiento de generación en generación, lo cual ha generado una erosión de conocimiento tradicional sobre el uso de las especies en dichas categorías en esta comunidad^{121,122}. Esta idea se ve reforzada por el hecho de que especies como el nogal (*Juglans neotropica* Diels) y la abeja (*Apis mellifera* Linnaeus), actualmente ya no se emplean por los pobladores para el oscurecimiento del cabello y extracción de miel de abeja respectivamente, puesto que el acceso a productos comerciales ha provocado la pérdida de dependencia de estos PFNM.

Dentro de la categoría de medicina humana se encuentran subcategorías de uso, entre las más comunes y responsables del alto consenso (24 especies y 152 citaciones) para esta categoría están la influenza (gripe) y los resfriados. Este elevado acuerdo entre los informantes demuestra que ambas afecciones son comunes dentro de la comunidad, las cuales posiblemente aparecen como consecuencia de las condiciones climáticas (bajas temperaturas) propias de la zona; por lo cual a su vez se refleja la importancia que tienen las plantas medicinales en la localidad. Esto se corrobora con lo reportado por Tinitana *et al.*⁵⁸ y Ríos *et al.*⁴⁶, quienes registraron un elevado consenso (FIC= 0.91 y 0.83 respectivamente) entre mujeres expendedoras de plantas medicinales para tratar resfriados y la influenza en mercados tradicionales de la provincia de Loja. Dichas afecciones forma parte de las enfermedades del sistema respiratorio, y constituye una de las principales causas de mortalidad a nivel nacional¹²³. Por su parte Ullah y Muhammad¹²⁴, identificaron a los resfriados como la categoría de afecciones medicinales con mayor consenso (FIC= 0.98) entre los informantes del valle de Shamozaí, norte de Pakistán, donde el clima es frío en temporada invernal.

Índice de valor de uso (IVU)

De las 123 especies registradas, únicamente 9 de ellas presentaron los valores de uso más altos (IVU= 0.57 - 1.47).

Esto puede atribuirse a la pérdida progresiva del conocimiento ancestral o interés de la población local en cuanto al uso de PFNM. Las especies que denotan mayor importancia o valor cultural para los usuarios de la comunidad El Tundo son aquellas de origen vegetal. Entre las que destacan están el nogal (*Juglans neotropica* Diels; 1.47), chaquino (*Myroxylon perufiferum* L. f; 0.90), verbena (*Verbena litoralis* Kunth; 0.77), manzanilla (*Matricaria chamomilla* L; 0.70), guíneo (*Musa sapientum* L; 0.60), sauco (*Cestrum peruvianum* Willd. ex Roem. & Schult; 0.60), malva olorosa (*Pelargonium odoratissimum* (L.) L'Her; 0.57), tilo (*Sambucus nigra* L; 0.57), y toronjil (*Melissa officinalis* L; 0.57) (Tabla 3). El resto de especies presentaron valores de uso por debajo de 0.50.

Debido a la composición de aminoácidos presentes en *J. neotropica* Diels, esta especie constituye una buena fuente proteica para la nutrición humana¹²⁵. Así mismo por sus propiedades tónicas en el pasado varias mujeres de la comunidad elaboraban un "jarabe de alimento" a partir las hojas del nogal para tratar diferentes afecciones de salud, el cual era comercializado en plazas de la ciudad de Loja. Sin embargo en la actualidad esta práctica ya no se realiza, ya que su proceso de elaboración es extenso y además conocido por muy pocas personas dentro de la comunidad. Esta y otras prácticas ancestrales de la provincia de Loja, han sido documentadas para *J. neotropica* Diels en los estudios previos^{24,26,53,98,125}, lo cual demuestra el valor potencial que ha tenido principalmente en el ámbito medicinal y la importancia que aún posee dicha especie para los pobladores locales.

Dentro de la provincia de Loja el nogal (*J. neotropica* Diels.) también ha sido identificado como uno de los PFNM con mayor valor de uso. Tanto en las comunidades de Bellavista, Cequer, El Durazno, San Antonio de Manú, y Udushi de la parroquia Manú del cantón Saraguro⁴⁴, como en las comunidades de la parroquia Santa Rufina del cantón Chaguarpamba¹²⁶, esta especie registra un (IVU= 2) con utilidades en el ámbito de la medicina humana para el tratamiento de diversas afecciones medicinales, y para la obtención de tintes y colorantes. Estos registros ponen de manifiesto la importancia de dicha especie a nivel provincial.

Las múltiples utilidades que presentan las especies anteriormente mencionadas para los informantes¹²⁷, así como su abundancia en la zona (en el caso de *J. neotropica* Diels.)¹²⁸, han sido determinantes para su posicionamiento como las de mayor relevancia para la comunidad El Tundo. En contraste, las especies registradas con un menor IVU (≤ 0.50), tienen en la mayoría de los casos un solo uso específico, como aquellas de uso alimenticio, material, y ornamental. Esta situación puede deberse a que varias de estas especies están entrando en desuso o su conocimiento es restringido por muy pocas personas de la comunidad, por lo cual su importancia o valor cultural no es significativo.

Nivel de uso significativo TRAMIL (UST)

Mediante el nivel de uso significativo, se registraron 33 especies vegetales con valores de UST iguales o superiores al 20%. La especie con mayor UST el nogal (*Juglans neotropica* Diels) con 83.33%, y las otras 32 especies tienen valores de UST entre 20% y 63.33% (Tabla 3).

Los valores de UST obtenidos muestran una elevada aceptación social y cultural por parte de la comunidad El Tundo principalmente para las especies introducidas (15 spp.), en comparación a las nativas (14 spp.) y cultivadas (2 spp.); a pesar de que las especies con origen biogeográfico nativo han sido registradas como las más abundantes en este estudio.

Possiblemente esto puede estar relacionado con la categoría de uso designada para estas especies introducidas, las cuales en su mayoría han sido nombradas por constituir una fuente de alimento, y soluciones rápidas de curación de afecciones relacionadas con la medicina animal, tradicional y humana.

Esto se corrobora con lo mencionado por Bennett y Prance¹²⁹ y Begossi *et al.*¹³⁰, quienes afirman que el predominio de la flora introducida es muy común principalmente para el uso alimenticio, medicinal, y ornamental en países tropicales, de los cuales Ecuador no es la excepción. El nivel de uso significativo que poseen estas plantas introducidas, se debe en gran parte a su uso frecuente por parte de los pobladores locales, lo cual se ve reflejado en el número de citaciones obtenidas para cada especie. Las 15 especies introducidas registradas entre las 33 con los porcentajes de UST= $\geq 20\%$ son: *Musa sapientum* L. (56.67%; 15 citaciones), *Matricaria chamomilla* L. (56.67%; 17 citaciones), *Pelargonium odoratissimum* (L.) L'Her (43.33%; 12 citaciones), *Citrus x aurantium* L. (36.67%; 11 citaciones), *Phyla dulcis* (Trevir.) Moldenke (30%; 9 citaciones), *Cymbopogon citratus* (DC.) Stapf (30%; 9 citaciones), *Coffea arabica* L. (26.67%; 8 citaciones), *Rosa x alba* L. (26.67%; 8 citaciones), *Ruta graveolens* L. (26.67%; 8 citaciones), *Zea mays* L. (23.33%; 7 citaciones), *Saccharum officinarum* L. (20%; 6 citaciones), *Mentha piperita* L. (20%; 6 citaciones), *Eriobotrya japonica* (Thunb.) Lindl. (20%; 6 citaciones), *Phyla scaberrima* (A. Juss. ex Pers.) Moldenke (20%; 6 citaciones), y *Aloe vera* (L.) Burm.f. (20%; 6 citaciones). La importancia de algunas de estas plantas no nativas está bien definida en el país, pues *C. x aurantium* L., *C. citratus* (DC.) Stapf; y *S. officinarum* L. forman parte de la farmacopea indígena del Ecuador¹²⁹.

En diferentes estudios ethnomedicinales realizados a nivel nacional, algunos autores también han identificado varias de las especies anteriormente mencionadas con altos porcentajes de aceptabilidad social y cultural^{99,131-134}. Estos registros confirman la alta preponderancia y versatilidad de dichas especies introducidas a nivel regional y nacional, estableciéndose como recursos vegetales importantes con posibilidades de ser objetos de estudio en futuras investigaciones orientadas a producir medicamentos o productos herbolarios a partir de ellas y de esta manera conseguir un aprovechamiento y uso de los compuestos activos que estas poseen.

Índice de Nivel de Fidelidad (FL)

Mediante el índice de nivel de fidelidad, se identificaron para las categorías de uso un total de 17 PFNM potenciales en términos de uso para la población local de El Tundo (Tabla 6). Para este propósito se consideraron las especies con un total de al menos 9 citaciones entre categorías de uso (It= 9) y altos niveles de fidelidad. Cabe señalar que para las categorías de combustión, miel de insectos, y ornamental no se registraron especies, puesto que presentan citaciones inferiores a 9 (It= 1).

Un FL del 100% indica un elevado acuerdo entre la mayoría de los informantes de la comunidad El Tundo con respecto a su uso para una misma categoría. Un total de 8 especies vegetales presentaron un FL=100% (*Verbena litoralis* Kunth, *Gynoxys verrucosa* Wedd., *Phyla dulcis* (Trevir.) Moldenke, *Myroxylon perufiferum* L. f., *Peperomia congona* Sodiro, *Cestrum racemosum* Ruiz & Pav, y *Sambucus nigra* L.); sin embargo, una de ellas no se encuentra identificada taxonómicamente por lo cual no se considerará para su análisis con otros estudios.

El uso de recursos vegetales para la elaboración de productos manufacturados tales como las escobas está claramente documentado en el Ecuador¹³, en donde *V. litoralis* Kun-

th forma parte de las 10 especies más útiles tanto en el ámbito medicinal y como material¹⁰. En el presente estudio para la categoría de materiales, *V. litoralis* Kunth destaca por su uso para la fabricación de escobas de uso doméstico. El empleo de esta y otras especies para esta finalidad también ha sido registrado en las comunidades de Chaguarpamba, Puyango, Paltas, Catamayo y Sozoranga¹²⁵. Lo cual demuestra que, en las comunidades rurales y campesinas de la provincia de Loja, aún prevalece el uso de plantas como *V. litoralis* Kunth para ser utilizadas como materia prima para la elaboración de escobas artesanales que por el contrario deberían ser adquiridas con dinero efectivo en los mercados.

La medicina tradicional incluye varias enfermedades de carácter espiritual o sobrenatural. El espanto forma parte de una de estas afecciones, la cual afecta principalmente a niños por tratarse de un grupo susceptible¹⁰⁰, y su tratamiento requiere plantas medicinales como agentes terapéuticos⁸⁷. En la comunidad El Tundo *G. verrucosa* Wedd es utilizada constantemente mediante baños o limpias corporales para contrarrestar el "espanto" de los niños. *G. verrucosa* Wedd es una especie de la familia botánica Asteraceae ampliamente utilizada dentro de la medicina tradicional en la provincia de Loja, a la cual se le atribuyen múltiples propiedades curativas para tratar la alergia, dermatitis, y el "espanto"^{125,26,53}.

Las 5 especies con mayores valores de fidelidad registradas en la categoría de medicina humana se usan para contrarrestar dolores de cabeza (*M. peruferum* L. f., *P. congoana* Sodiro, y *C. racemosum* Ruiz & Pav), dolores estomacales (*P. dulcis* (Trevir.) Moldenke), cortes y heridas (*M. peruferum* L. f.), prevención de los nervios y dolores de oído (*P. congoana* Sodiro), como antígrital (*M. peruferum* L. f., *P. congoana* Sodiro, *C. racemosum* Ruiz & Pav, y *S. nigra* L.), anticancerígeno y energizante (*M. peruferum* L. f.). Lo anterior coincide con varios autores quienes mencionan a estas especies para tratar los mismos problemas de salud en la provincia de Loja^{24,25,47,56,134}.

Tanto la bibliografía científica como los elevados niveles de fidelidad obtenidos para *Verbena litoralis* Kunth, *Gynoxys verrucosa* Wedd., *Phyla dulcis* (Trevir.) Moldenke, *Myroxylon peruferum* L. f., *Peperomia congoana* Sodiro, *Cestrum racemosum* Ruiz & Pav, y *Sambucus nigra* L., confirman la efectividad gracias a sus componentes activos y sobre todo demuestran un acuerdo considerable y un íntimo conocimiento entre los informantes de las comunidades rurales de la provincia de Loja, entre ellas la comunidad de El Tundo para el uso medicinal de estas especies.

A su vez los elevados niveles de fidelidad sugieren que estas especies están incorporadas dentro de las actividades diarias de los usuarios y por lo tanto se consideran habituales en la zona de estudio. Entre estas especies *Myroxylon peruferum* L. f., debería ser considerado objeto prioritario en cuanto a conservación y manejo sostenible. Puesto que al ser una especie tan aceptada por sus múltiples propiedades medicinales, la extracción constante de su corteza conlleva a la destrucción definitiva de estos individuos arbóreos, y más aun teniendo en cuenta que es una especie especialista con distribución restringida en la provincia de Loja, lo cual aumenta sus posibilidades de extinción¹³⁶.

Conclusiones

El presente trabajo constituye uno de los primeros estudios etnobiológicos que incluye plantas y animales desarrollados en el sur del Ecuador. A su vez demuestra la estrecha relación que mantienen los pobladores de la comunidad El Tundo

con la flora y fauna forestal, así como el amplio conocimiento tradicional adquirido y que todavía prevalece entre las personas adultas y adultas mayores de la localidad. Todo esto queda reflejado con las 123 especies proveedoras de PFNM registradas, las cuales han sido y son una fuente natural e indispensable principalmente para las categorías de medicina humana, alimentos y bebidas en esta comunidad.

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RESEARCH / INVESTIGACIÓN

Economía agroecológica en una comuna rural del sur del Ecuador Agroecological economy in a rural commune in Southern Ecuador

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Resumen: La producción agrícola y pecuaria en las comunidades rurales contribuyen de manera relevante en el crecimiento económico y la seguridad alimentaria de un país. Los huertos son espacios clave para la producción como para el aporte a la recuperación de los recursos naturales, cuando se trabaja con un enfoque agroecológico. Se evaluó los cambios en la economía familiar de la Comuna Indivisa de Chinchanga, ubicada entre los cantones de Calvas y Sozoranga de la provincia de Loja, bajo la intervención de organizaciones públicas y privadas durante el período 2006-2016. Se realizaron 169 encuestas dirigidas a jefes/as de hogar relacionadas con el ingreso, egreso y rentabilidad mensual de las familias. Los resultados señalaron un incremento en el ingreso familiar en un 84.82% y la rentabilidad en 137.22% por la venta de sus nuevos productos agropecuarios y productos de ciclo corto. Además, se evidenció cambios significativos en los ingresos y rentabilidad entre los dos períodos relacionado con la diversificación de la producción agrícola y huertos familiares. La educación influyó en la economía familiar de esta comuna, dado que las personas con mayores ingresos, son los que han cursado un nivel secundario.

Palabras clave: Economía familiar, ingresos, prácticas agroecológicas, seguridad alimentaria.

Abstract: Agricultural and livestock production in rural communities contributes significantly to the country's economic growth and food security. The orchards are very important and for food production as for contribution to the recovery of natural resources. This is possible when we work with an agroecological approach. We measure the changes in the family economy of the Community "Indivisa" in Chinchanga, located between the towns Calvas and Sozoranga in the province of Loja, evaluated under the intervention of public and private organizations during the years 2006-2016. We conducted 169 surveys for heads of households related to income, expenses, and monthly profitability of families. The results indicated an increase in the family income by 84.82% and profitability by 137.22% due to the sale of their new agricultural products and short cycle products. In addition, significant changes in income and profitability were evidenced between the two periods related to the diversification of agricultural production and home gardens. Education influenced the family economy of this commune; therefore, people with the highest income attended a secondary level.

Key words: Agroecological practices, family economy, food security, income.

Introducción

La agroecología se fundamenta en el manejo ecológico y sostenible de los recursos naturales, vinculando a la sociedad para desarrollar un modelo alternativo frente al modelo convencional industrial, intentando establecer estrategias de producción y consumo, encaminados hacia el bienestar de los productores y conservación del ecosistema¹⁻⁴. Las prácticas agroecológicas, también se vinculan a procesos que procuran la recuperación del conocimiento tradicional, sumado al trabajo con redes sociales y economías comunitarias, acceso a los mercados y el manejo integrado de agricultura, ganadería y silvicultura, influyendo directamente en la seguridad alimentaria de los pueblos⁵.

La agroecología surge en la década de los años 70, como respuesta a los impactos negativos que la revolución verde generó a nivel ecológico, social y económico^{6,7}, buscando recuperar el protagonismo de la familia campesina y de la comunidad, revalorizando el conocimiento ancestral para el manejo de los recursos naturales. Por lo que, se ha homologado permanentemente con la agricultura orgánica y es una opción para producir sin utilizar paquetes tecnológicos de agroquímicos⁸. En este contexto, existen varios estudios en donde enfocan la agroecología como una alternativa sostenible para los campesinos, pueblos indígenas y sus comunidades⁹⁻¹².

El desarrollo de prácticas agroecológicas en el Ecuador no ha sido altamente notorio, de manera que permita ser compe-

titivo frente a la producción convencional, y posicionarse como alternativa productiva enfocada a la gestión y conservación de los recursos naturales. A pesar que, la constitución ecuatoriana considera el derecho a la alimentación, y constituye que la aplicación de estos derechos se explique por medio de leyes y políticas públicas¹³.

En la provincia de Loja los valles más productivos están dedicados a la producción de monocultivos de caña de azúcar, arroz y maíz amarillo principalmente, que se destinan para la agroindustria¹⁴. Detrás de esta propuesta tecnológica están los grandes monopolios productores de semillas, agroquímicos y maquinaria agrícola, que hacen dependientes de esta nueva tecnología a los productores agrícolas⁹.

En la provincia de Loja se iniciaron procesos formativos en agroecología con el Consorcio Latinoamericano de Agroecología (CLADES), que fueron impulsadas por la Coordinadora Ecuatoriana de Agroecología (CEA). En los eventos de formación participaron profesionales y líderes campesinos miembros de la Federación Unitaria Provincial de Organizaciones Campesinas y Populares de Sur (FUPOCS) creada en 1981, la Unión Popular de Mujeres de Loja (UPML), creada en 1984 y de la Red Agroecológica Loja establecida en el 2007, quienes han trabajado activamente en la producción agroecológica en sus organizaciones de base.

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Es por ello, que evaluamos la intervención de Fundación Ayuda en Acción, Ministerio de Agricultura y Ganadería (MAG) y Gobierno Autónomo Descentralizado de la parroquia Colaisaca (GAD – Colaisaca) durante el período 2006 -2016 en la economía familiar campesina (ingresos, egresos y rentabilidad) de la comuna Indivisa de Chinchanga ubicada en el Sur de Ecuador. La intervención de estos organismos se basó en un apoyo integral a las familias más vulnerables, fomentando la producción agropecuaria a través de la propuesta de prácticas agroecológicas.

Materiales y métodos

2176

Localización

El área se localizó en la comuna rural de Indivisa de Chinchanga ($4^{\circ}14'14.9''\text{S}$ $79^{\circ}41'41.3''\text{O}$), en los cantones de Calvas (parroquia de Colaisaca) y Sozoranga (parroquia Santa Fátima), cuenta con un área aproximadamente de 20 mil hectáreas, con 40 comunidades, denominado desde tiempos coloniales. La comunidad rural es una circunscripción territorial legítima, respetada por los diferentes órdenes administrativos locales y gobernada por un personero y los cabildos que representan a las comunidades.

La población del territorio en estudio tiene un total de 1854 habitantes con 941 hombres y 913 mujeres, la Población Económicamente Activa (PEA) representa el 32.47 % (602 habitantes); la población en edad de trabajar (PET) representa el 45.41 % (842 habitantes) del total de la población de la parroquia de Colaisaca, lo que representa que el 22.12 % de la población son desempleados¹⁵.

Las comunidades analizadas de la Comuna Indivisa Chinchanga fueron: Attilo (A), Belamine (BE), Bella María (BM), Carango (CA), Colaisaca (CL), El Batán (BA), El Limón (L), El Molle (MO), El Parco (PR), El Pongo (PO), Guamba (GU), Lagunas (LA), Moras (M), Piedras Negras (PN), Pichinamaca (P), Pitas (PI), Riodopamba (RI), Santa Anilla (SA), Shocopa (SH), Surunuma (SU), Tarume (TA), Tunas (TU) y Yurarrumi (Y); en donde intervinieron algunos organismos de desarrollo como la Fundación Ayuda en Acción, Ministerio de Agricultura y Ganadería (MAG) y Gobierno Autónomo Descentralizado de la parroquia Colaisaca, en la propuesta y desarrollo de prácticas de manejo agroecológico a la producción agropecuaria.

Diseño y recolección de datos

Se aplicó una encuesta a los jefes/as de hogar correspondientes a 169 familias, que representan el 56.33% del total de la población beneficiaria de los proyectos, por tratarse de una zona de influencia muy dispersa geográficamente, por la deficiente accesibilidad vial, es difícil llegar a toda la población con los apoyos necesarios para mejorar sus sistemas de producción agropecuaria; aclarando que, el empoderamiento de la propuesta agroecológica fue involucrando a todos los productores de los sectores en diferentes etapas. Se determinó una muestra representativa (encuestados), utilizándose la fórmula de cálculo para el efecto de muestreo aleatorio simple (n), con un nivel de significación del 95% y un error estadístico máximo del 5% como se detalla a continuación:

$$n = \frac{Z^2 p q N}{E^2 (N - 1) + Z^2 p q}$$

Donde:

n = Tamaño de la muestra a calcular

Z = Nivel de confianza para 95%, es 1.96

p = Variabilidad positiva es 50%

q = Variabilidad negativa es 50%

N = Tamaño de la población es 300

E = Precisión o el error es 5%

$$n = \frac{(1.96)^2 (0.5)(0.5)(300)}{(0.05)^2 (300 - 1) + (1.96)^2 (0.5)(0.5)} = 168.69$$

Por tanto, la muestra fue de 169 encuestas

En las encuestas se recolectó información relacionada con el estado civil, sexo, nivel de instrucción; variables cuantitativas discretas como edad, número de miembros de la familia; así como la evolución de variables cuantitativas continuas como, ingresos anuales por venta de producción agropecuaria; ingresos por concepto de venta de fuerza de trabajo, bono de desarrollo humano y remesas; total de ingresos anuales; egresos anuales por satisfacción de necesidades básicas, autoconsumo y rentabilidad (Anexo 1).

Análisis de datos

Se analizó la relación entre los niveles de ingresos, egresos, rentabilidad entre los dos períodos de las diferentes localidades, mediante un análisis no paramétrico pareado de Wilcoxon, debido a que los datos según la prueba de Shapiro-Wilk no presentaron una distribución normal ($p < 0.05$), y la percepción de las diferentes comunidades (nivel de formación académica) con los ingresos, egresos y rentabilidad se determinó con una prueba no paramétrica Kruskall-Wallis.

Resultados

Los resultados arrojan que en la comuna de Indivisa Chinchanga, sus familias están conformadas por un promedio de 5 miembros familiares, de la población, el 45.83 % corresponde al género femenino, y el 54.17 % al masculino. La edad media de las personas está 50.82 años, y que alrededor de un 86 % solo cuenta con educación primaria, 6.54 % educación secundaria, y el resto de la población no ha cursado ningún tipo de formación educativa. Los ingresos, egresos y rentabilidad en toda la comuna y en cada una de las localidades, son diferentes al inicio y al final de la investigación (Figura 1).

En cuanto a los ingresos totales al inicio del estudio se podía apreciar un ingreso medio total de 1348.46 ± 52.36 USD/año, mientras que para 2016 estos ingresos subieron a 2492.22 ± 89.26 USD/año. Así también, los egresos mostraron cambios estadísticamente significativos (2006: 908.78 ± 47.72 USD/año y 2016: 1444.16 ± 70.22 USD/año), con lo cual la rentabilidad también varía de manera significativa durante el período de evaluación, la cual de en 2006 fue de 439.67 ± 7.54 USD/año y al finalizar fue de 1043.05 ± 58.06 USD/año.

En cuanto al aspecto económico, se pudo evidenciar que la variación de ingresos ($W=10.01$, $p < 0.0001$), gasto ($W=7.01$, $p < 0.0001$) y la rentabilidad ($W=9.15$, $p < 0.0001$) obtenida al inicio y final del período de evaluación señaló cambios significativos, mostrando que, en todas las variables económicas, dado el tiempo transcurrido se presentan cambios.

En otro aspecto, se observó cambios en los niveles de ingresos, egresos y rentabilidad en función de la formación académica. La formación secundaria señaló los niveles de ingresos más altos (Figura 2), frente a las personas que solo tienen una formación primaria o ninguna formación, así también, el

egreso de este nivel de formación es más alto y, en cuanto a la rentabilidad este mismo grupo, obtiene la rentabilidad más alta, seguido del nivel de educación primaria (Figura 2). Corroborando estos hallazgos se aprecian diferencias significativas para ingresos ($KW=21.42$, $p<0.0001$), egresos ($KW=23.88$, $p<0.0001$) y rentabilidad ($KW=10.67$, $p=0.013$) en relación a la formación académica.

Discusión

Los resultados obtenidos para la Comuna Indivisa de Chinchanga evidenciaron que los niveles de ingresos de la comuna en general (en todas las localidades) son mayores a los egresos, tanto al inicio como final del periodo de evaluación; lo cual, a su vez está en relación directa con la rentabilidad positiva obtenida. En este contexto, Chiriboga¹⁶ hacen conocer que la producción agrícola y pecuaria contribuyen de manera relevante en el crecimiento económico como de la seguridad alimentaria en diferentes comunidades, lo que ha evidenciado en nuestro estudio ya que la intervención de estos organismos ha permitido mejorar la producción agropecuaria y por ende la sostenibilidad económica de las familias de esta comuna.

Por lo tanto, un manejo adecuado de las fincas productivas, garantizará la sostenibilidad social, productiva y económica de los agricultores, además, que contribuirá con la sostenibilidad ambiental del ecosistema¹⁷. Sin embargo, para lograr esto, es indispensable el uso de las buenas prácticas agroecológicas, que buscan alcanzar esta sostenibilidad y resiliencia en el funcionamiento eficiente de los sistemas de producción alimentarios¹⁸. Por lo cual, el apoyo de organismos públicos o privados a los pequeños agricultores, en la capacitación un

manejo agroecológico de sus predios, beneficiaría en la sostenibilidad en general de estos sectores.

A pesar que la implementación de las prácticas agroecológicas y apoyos de instituciones vinculadas a desarrollar proyectos agroecológicos en las huertas, ha tenido un efecto positivo en el aporte a la mejora de ingresos y rentabilidad, el componente de egresos sigue el mismo patrón, debido a que los sistemas productivos de estas localidades son pequeños y más de carácter familiar, sacando solo parte de sus producciones a la venta local. Así, Tahiru *et al.*¹⁹ mencionan que las participaciones de las ONGs mediante diferentes instrumentos de alguna manera no generan efectivamente un incremento del ingreso, debido a la dificultad de la comunidad local para generar y absorber efectivamente los ingresos en diferentes actividades. Además, cabe considerar que, debido a la cultura local y posiblemente a una falta de conocimiento sobre la conservación de medio ambiente, un producto agroecológico no tiene un valor añadido que un producto producido de manera convencional²⁰. Sin embargo, en nuestro caso este tipo de prácticas agroecológicas propuestas en estas unidades productivas agropecuarias, estaría contribuyendo con la recuperación de los recursos naturales²¹.

Las localidades de El Pongo, Altillo, Tunas y Yurarrumi obtuvieron una mayor rentabilidad, relacionado con un alto nivel organizativo y lograron ubicar sus productos en las ferias libres del cantón con el reconocimiento de su calidad agroecológica. Apoyando nuestros resultados estudios previos han documentado que comunidades con una adecuada estructura organizativa pueden acceder eficazmente a proyectos de financiamiento y desarrollo, siendo impulsadoras de oportunidades productivas, de cooperación y de gestión de recursos²².

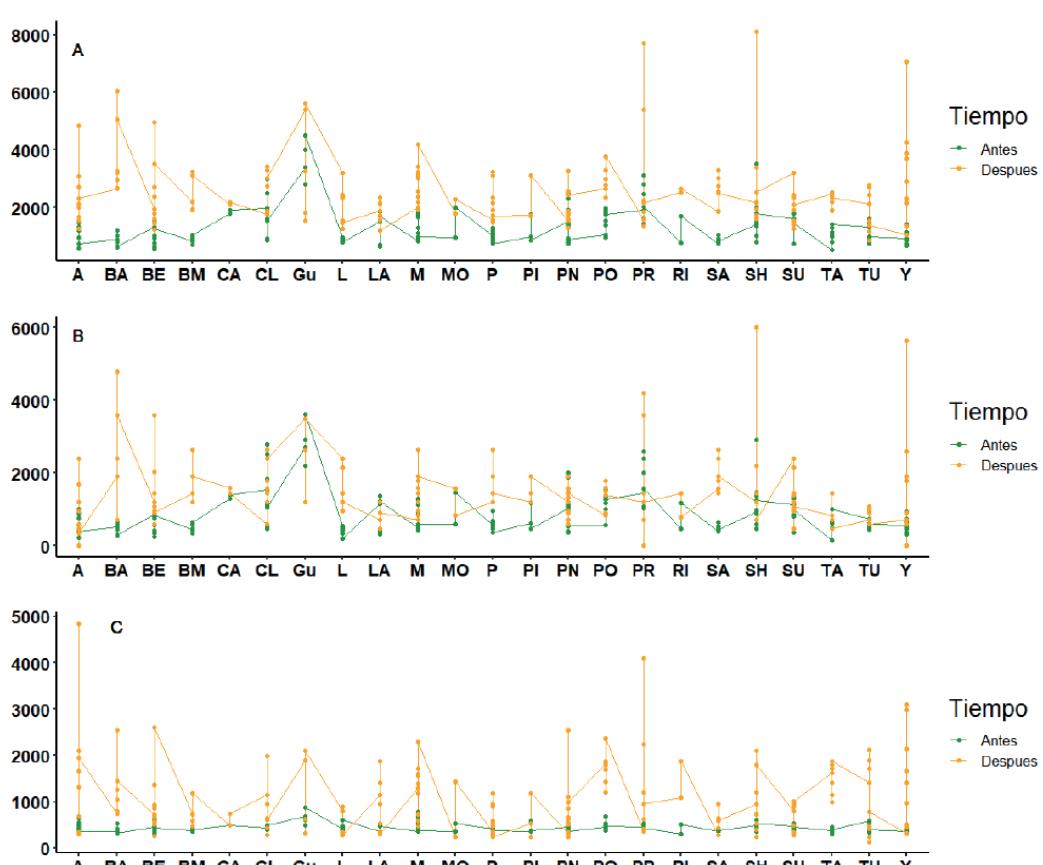


Figura 1. Variación de ingresos, egresos y rentabilidad en las diferentes localidades entre los años 2006 y 2016 en la comuna Indivisa de Chinchanga.

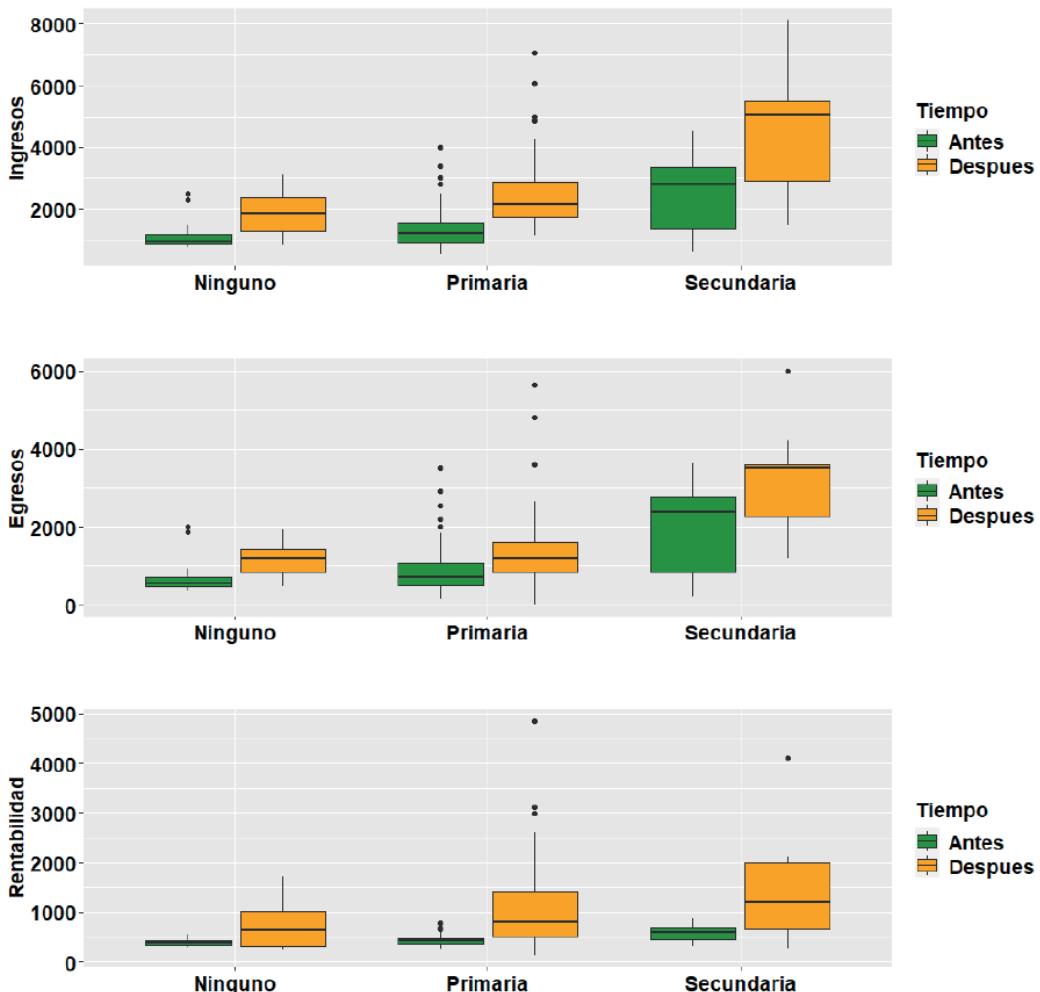


Figura 2. Nivel de formación educativa frente al nivel de ingreso, egreso y rentabilidad entre los años 2006 y 2016 en la comuna Indivila de Chinchanga.

Por otra parte, en la mayor parte de localidades el porcentaje de ingresos es por concepto de producción de la huerta campesina, la cual en un 70% es destinada al autoconsumo y el 30% restante corresponde a la producción que se destina a la venta. Una hipótesis explicativa podría ser que los ingresos provenientes de la venta de productos agropecuarios, no sean suficientes para cubrir las necesidades básicas de la familia, por lo que, los pobladores deben acudir también a actividades no agropecuarias²³. Otras actividades están relacionadas con la venta de la fuerza de trabajo, bono de desarrollo humano y remesas de inmigrantes. Sin embargo, se ha evidenciado un efecto positivo de las diferentes organizaciones ya que el autoconsumo tiene la tendencia al alza, basada en la implementación de huertos y la producción de animales menores garantizándoles consolidar la propuesta de seguridad alimentaria a través de la producción agroecológica. Por ejemplo, Acevedo-Osorio *et al.*²⁴ señala que las estrategias agroecológicas mejoraron la resiliencia socioecológica de los agroecosistemas manejados por agricultores, y que el incremento en los niveles productivos implica la aplicación de prácticas sostenibles²⁵. Por lo que, Sanchez y Chicaiza²⁶ señalan que a pesar que las comunidades ven la necesidad de invertir en cultivos más rentables, la agricultura familiar es esencial, debido a que cumple una función social y cultural de una comunidad.

Se evidenció una relación directa entre las variables nivel de ingresos y formación académica, es decir que, a un mayor nivel de formación académica, incrementan los ingresos y la

rentabilidad económica de las personas de esta comuna. Gallas y Andrade²⁷ muestran que la educación es considerada como uno de los principales factores que influyen en los ingresos económicos de las personas, aunque también dicen que este factor dependerá de aspectos demográficos. En este caso, la mayoría de las personas solo poseen educación primaria o ninguna educación (mayor al 90%), lo que lleva a una diferencia de ingresos económicos frente al 6.54 % que poseen la educación secundaria. Briceño²⁸ comenta que la educación debe ser considerada como una inversión por parte de las personas, ya que el capacitarse les permite aumentar los capitales económicos, todo esto se estaría evidenciando en este estudio.

Conclusiones

Concluimos que en la Comuna Indivila de Chinchanga se evidenció un efecto positivo de las diferentes organizaciones en la economía familiar, debido a que los niveles de ingresos y rentabilidad en todas las localidades fueron mayores al final del período de evaluación (ingresos 84.82% y rentabilidad 137.22%), relacionado con la diversificación de la producción agrícola y huertos familiares. Además, se evidencia que la educación está influyendo de manera directa en la economía familiar de esta comuna, dado que las personas con mayores ingresos, son las que han estudiado un nivel secundario (Bachillerato).

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RESEARCH / INVESTIGACIÓN

Expression of the ANS, CHS and DFR genes involved in the biosynthesis of anthocyanins in *Vaccinium floribundum* Kunth from Ecuador, using RT-qPCR

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Abstract: *Vaccinium floribundum* Kunth, a wild native species of berry in Ecuador, presents a lot of phenolic compounds, specifically anthocyanins; hence it is considered a natural nutraceutical due to all its nutritional properties. The comparison of the expression of genes involved in the biosynthesis pathway of anthocyanin of several populations. The aim of the research was to analyze the expression levels of three genes involved in the biosynthesis of anthocyanin in this species collected in two areas of the province of Pichincha: Machachi population of the Mejía canton, with geographic coordinates 0° 31'04.8 " S 78° 37'07.4 " W and altitude 3200 meters above sea level, and Pintag population of the Quito cantón, with geographic coordinates 0° 24'00.0 " S 78° 24'00.0 "

W and altitude 3000 meters above sea level. The gene expression analysis was performed using the quantitative polymerase chain reaction technique and reverse transcription (RT-qPCR). For the population of Machachi, the glyceraldehyde-3-phosphate dehydrogenase gene had an average concentration of 648.59 ng/µL, followed by the chalcone synthase gene with 143.71 ng/µL, then by the dihydroflavonol 4-reductase gene with 59.58 ng/µL and finally by the anthocyanin synthase gene with 39 ng/µL. For the population of Pintag, the glyceraldehyde-3-phosphate dehydrogenase gene has an average concentration of 667.32 ng/µL, followed by the chalcone synthase gene with 157.22 ng/µL, then by the dihydroflavonol 4-reductase gene with 60.42 ng/µL, and finally by the anthocyanin synthase gene with 44.40 ng/µL. Each gene has a similar expression level in both populations, but there are differences when comparing the expression level among genes. Many enzymes, structural genes, and regulatory elements have been observed as transcription factors involved in anthocyanin biosynthesis.

Key words: Anthocyanin synthase, chalcone synthase, dihydroflavonol 4-reductase, glyceraldehyde-3-phosphate dehydrogenase, Mortiño.

Introduction

The *Vaccinium floribundum* Kunth, known as Mortiño in Ecuador, is an endemic species from the north of South America between Colombia and Ecuador and native of the Ecuadorian Moors¹. There are no commercial crops of this species, but only parcels of land where the species grows wildly, and its fruits are sold in the last months of the year². In Ecuador, it is usually consumed as fresh fruit or it is processed in artisanal jams. The consumption of the fresh fruit is intended for preparing the traditional "colada Morada," a typical drink made in Ecuador at October and November.

Besides being a fruit of great importance for the conservation of the moorlands and culture of Ecuador, Mortiño is a wild shrub still in the process of domestication in the country but with slow development and requires more in-depth studies. It possesses excellent nutritional properties related to its high content of phenolic compounds: anthocyanin³.

Anthocyanin is a type of flavonoid mainly involved in fruit ripening. These phenolic compounds are found in leaves, flowers, and especially in fruits since they produce the bluish or reddish coloration typical of the ripe fruits⁴. It is considered that fruits with intense bluish colors possess greater nutritional, medicinal, and antioxidants properties⁵, which have been associated with reducing coronary, neurological, and cardiovascular diseases⁶, cancer, diabetes, and inflammatory processes; this has been observed in various *in vitro* and *in vivo*^{7,8} studies.

The metabolic synthesis of flavonoids is well described for some species; however, its synthesis and anthocyanin concentration in *Vaccinium floribundum* are still unknown concerning

the ripening process. Several genes involved with the synthesis of anthocyanin from phenylalanine have been identified; three of these are chalcone synthase (CHS), which participates at the beginning of the biosynthetic route, dihydroflavonol 4-reductase (DFR) and anthocyanin synthase (ANS) that are practically observed at the end of the synthesis^{4,9}.

Molecular analyses are tools for managing biodiversity for the conservation and monitoring of endemic species and natural populations⁹. One of the molecular techniques used to observe gene expression patterns is the RT-QPCR (reverse transcriptase quantitative PCR)¹⁰, through which the concentration of one or more specific transcripts can be quantified¹¹.

The objective of this research is to quantify the expression of the chalcone synthase, dihydroflavonol 4-reductase, and anthocyanin synthase genes that are directly involved in the biosynthesis of anthocyanin in two populations of Pichincha, namely Machachi and Pintag, since as mentioned by Li et al.¹²; there are insufficient specific genomic and transcriptomic profiles data to understand the molecular mechanisms associated with antioxidants, especially in *Vaccinium* species.

Materials and methods

Location and collection of samples

Ripe fruits of *V. floribundum* were collected in two wild populations of Pichincha, namely Machachi at Mejía with geographical coordinates 0°31'04.8"S 78°37'07.4"W and 3200 masl,

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and Pintag in Quito with geographical coordinates 0°24'00.0"S 78°24'00.0"W and 3000 masl.

According to the regulation for food collection of the INEN (Instituto Ecuatoriano de Normalización), 1kg of ripe fruits was collected from each native population and placed in a small tank with liquid nitrogen. The collected sample of *V. floribundum*, consisted of ripe fruits with the following characteristics: dark color in the peel, a diameter of 1 cm, and shrubs taller than 1.2 m. Although these moors are highly intervened, native populations of the species under study can be found.

RNA extraction

The extractions by columns were made using the Pure Link® RNA Mini Kit (Ambion, Life), according to the manufacturer's instructions. The final column was placed in a recovery tube, adding 50 µL of RNase free water to perform the elution and centrifuging it for 2 min at 12000 x g; afterward, 1 µL of RNase inhibition Protector (Roche) was added, and this preparation was retained at -20 °C to be processed on the same day.

Reverse transcription

A reverse transcription kit was used to obtain the cDNA, specifically a transcriber First Strand cDNA synthesis kit and Random Primer of Roche, used according to the manufacturer's directions. The final microtube was taken to the conventional ThermoCycler (Labnet) using a protocol of 10-min at 25 °C, 30-min at 55 °C, 5-min at 85 °C, and cooling at ∞ 4 °C; finally, the cDNA was obtained and quantified in the Fluorometer Qubit® 2.0.

Elaboration of the standard curve

Serial solutions of the cDNA were prepared, obtained from a ripe *V. floribundum* fruit collected in Machachi, and the glyceraldehyde-3-phosphate dehydrogenase gene was used as a control to establish a patterned curve since it is a house-keeping gene.

The samples were quantified using Qubit® 2.0 Fluorometer with the Qubit® DNA Assay Kit (Life), following the instructions of the manufacturer; this was carried out using the DsDNA High sensitivity program, obtaining specific concentrations that will be entered into the RT-qPCR equipment for expression analysis of the genes.

With the quantified cDNA, amplification was performed using the Light Cycler Fast Start DNA Master^{PLUS} SYBR Green I (Roche), with the primers of the glyceraldehyde-3-phosphate dehydrogenase gene to enter the data in the Light Cycler 2.0 software (Roche).

In this experiment, programming of absolute quantification was performed, where the data obtained from the concentrations are entered through the Qubit, which are the positive standard controls for the subsequent analysis of the interest samples¹³.

The amplification run is initiated, giving the standard curve of cDNA concentration of the control gene, comparing the subsequent samples for their quantification in ng/µL.

Finally, it should be considered that the error values and the curve efficiency must be from 0.0 to 0.3 points and from 1.8 to 2.2 points, respectively, to be further later used in the runs of the RT-qPCR for quantification of samples with unknown concentrations. The standard curves with the control gene are the basis for the quantification at RT-qPCR¹⁴.

Quantification in RT-qPCR

The Master Mix Light Cycler Fast Start DNA Master^{PLUS}

SYBR Green I (Roche) was previously prepared. For the master mix of each sample, the protocol of 20 µL of the final volume was standardized with 9.8 µL of Ultra-Pure H₂O, 0.6 µL of the first forward, 0.6 µL of the first reverse, in a 10 micromolar concentration, plus 4 µL of the Master Mix and 5 µL of the previously obtained CP *V. floribundum* cDNA. On the other hand, a negative control, H₂O PCR grade, and positive control, a sample of Mortiño, were required for each of the genes, and two more capillaries were prepared¹⁵. The sequences of the first GAPDH used were

F:5'CAAACTGTCTTCCCCACTT3',
R:5'CAGGCAACACCTTACCAACA3', for DFR,
F:5'GAAGTGATCAAGCCGACGAT3',
R:5'ATCCAAGTCGCTCCAGTTGT3', for CHS,
F:5'CCAAGGCCATCAAGGAATG 3',
R:5'TGATACATCATGAGTCGCTTC3', for ANS,
F:5'TCTTCTACGGAGGGCAAATGG3',
R:5'ACAGCCCAGAATCTGAC3'.

In the case of the negative control, 5 µL of the sample were replaced by 5 µL of Ultra-Pure H₂O, and for the positive control the capillaries were carefully centrifuged at 12000 x g for 15s to ensure that the specimen is at the bottom of the sample and have a correct reading.

The amplification protocol was programmed in the Light cycler 4.0 software (Roche): initial denaturation at 95 °C, for 1 cycle, then 60 denaturation cycles at 95 °C, hybridization at 60 °C and extension at 72 °C, to finally reach the last step of cooling for 1 cycle at a temperature of 40 °C. Finally, the previously saved external standard curve must be imported to obtain the quantitative data¹⁶.

4x2 Factorial statistical analysis

A factorial evaluation of the four genes was carried out in the INFOSTAT program 2018a, namely chalcone synthase, dihydroflavonol 4-reductase, anthocyanin synthase, and glyceraldehyde-3-phosphate dehydrogenase in the two populations from Pichincha; i.e., with this analysis, it can be reported the relationship of each of the independent variables: each of the genes and all the genes with each one population and with two populations.

Using the variance analysis (ANOVA) with $\alpha=0.01$, it is verified if two or more averages of two or more data groups are similar or if some differ significantly from the others¹⁷.

The Duncan test was also performed to compare the degree of similarity in the expression of the 4 genes involved in the biosynthesis of the anthocyanin and the relationship between the two populations since this statistical test is used to examine all the differences among means considering that the number of repetitions is constant.

Results

Standard curve using the glyceraldehyde-3phosphate dehydrogenase gene

The standard curve generated was effective, as it yielded an efficiency of 2.121 points and an error of 0.202 points, which are in the acceptable ranges required by the ThermoCycler LightCycler 2.0; hence, it is concluded that the data obtained are reliable.

Figure 1 shows a curve in which the X axis represents the logarithmic concentration of the serial dilutions data that were obtained in the Qubit, whereas the Y axis represents the CP or

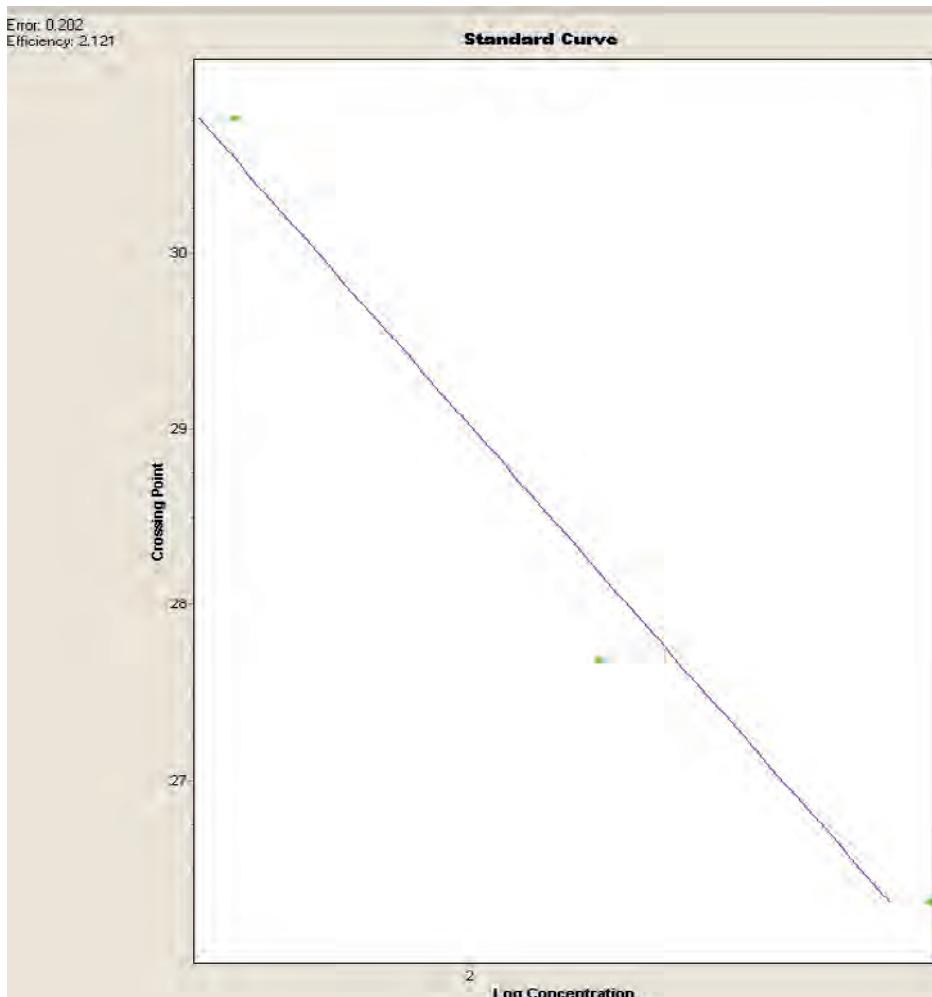


Figure 1. Standard curve for the *V. floribundum* with the glyceraldehyde-3-phosphate dehydrogenase gene. Error and efficiency are shown in the appropriate parameters.

crossing point in which the samples generated the amplification; the blue line is the trend line between the two axes, and it represents the normalization of the curve.

Determination of the expression levels of the four genes in the species *Vaccinium floribundum*

The cream formulated was evaluated for its organoleptic properties (color, state, and odor). The appearance of the cream was analyzed by its color and roughness visually and by touch. Results are listed in Table No. 2.

The glyceraldehyde-3-phosphate dehydrogenase gene has the highest expression levels in both locations, with an average of 648.59 ng/ µL for Machachi (Table 1) and 667.32 ng/µL for Pintag (Table 2). Ten replications of the exact measurements were performed for each gene.

The second gene with a higher expression level is the chalcone synthase with an average of 143.71 ng/µL in Machachi and 157.22 ng/µL in Pintag, then the dihydroflavonol 4-reductase gene with an average of 59.58 ng/µL in Machachi and 60.42 ng/µL for the population of Pintag; finally, the anthocyanidin synthase gene with an average of 39.29 ng/µL in Machachi and 44.40 in Pintag.

In Figure 2, one of the replications for the amplification curves of the four genes of the Machachi population is observed; this curve is inversely proportional to the concentration because while the curve rises in a minor cycle, the amount of the target will be higher. Four curves represent the four genes under study, plus the curve of negative control; the blue cur-

ve is almost linear until the end of the run without CP point. The green curve represents the glyceraldehyde-3-phosphate dehydrogenase gene, with a CP point of almost 27 cycles and a concentration of 727.10 ng/ µL; the red curve represents the chalcone synthase gene, with a CP of 38 cycles and a concentration of 120.20 ng/µL; the brown curve represents the dihydroflavonol 4-reductase gene, with a CP of 47 cycles and a concentration of 52.70 ng/µL; finally the black curve represents the anthocyanin synthase gene with a 60-cycle CP and a concentration of 39.40 ng/µL.

Regarding the Pintag population, Figure 3 shows one of the repetitions of the amplification curves of the four genes: the blue curve is almost linear until the end without CP point, and is the negative control. The green curve represents the glyceraldehyde-3-phosphate dehydrogenase gene with a 25-cycle CP point and a concentration of 780 ng/µL; the light blue curve represents the chalcone synthase gene with a 32-cycle CP and a concentration of 189.90 ng/µL; the brown curve represents the dihydroflavonol 4-reductase gene, with a 46-cycle CP and a concentration of 56 ng/µL; and finally, the black curve represents the anthocyanin synthase gene with a 59 cycle CP and a concentration of 44.30 ng/µL.

Comparison of the expression gene levels of *Vaccinium floribundum* in the two populations of Pichincha

When comparing the two populations under analysis, no significant differences were found in any of the four genes ($F = 0.47$, $p = 0.4965$). On the contrary, the expression averages

Replications	Machachi GENES			
	Chalcone synthase	Dihydroflavonol 4-reductase	Anthocyanins Synthase	Glyceraldehyde-3-phosphate dehydrogenase
1	120.20	52.70	39.40	727.10
2	163.20	54.27	36.50	720.80
3	160.40	57.06	33.60	627.00
4	210.88	58.20	38.30	401.00
5	120.00	54.26	57.70	639.00
6	145.50	56.35	48.30	633.00
7	124.00	52.77	20.00	459.90
8	130.00	51.30	35.80	712.60
9	143.60	73.20	48.80	739.70
10	119.30	85.72	34.50	825.80
Average	143.71	59.58	39.29	648.59

Table 1. cDNA concentrations (ng/μL) of the *V. floribundum* Machachi population for the four genes and their averages.

Replications	Pintag GENES			
	Chalcone synthase	Dihydroflavonol 4-reductase	Anthocyanins Synthase	Glyceraldehyde-3-phosphate dehydrogenase
1	189.90	56.00	44.30	780.00
2	130.00	59.00	42.30	823.00
3	140.00	55.50	44.20	596.00
4	160.00	41.60	39.90	560.00
5	199.54	51.30	40.80	573.00
6	155.50	58.35	50.30	653.00
7	144.00	72.77	40.00	479.90
8	160.40	55.70	38.80	732.60
9	163.60	78.20	58.80	749.90
10	129.30	75.82	44.60	725.80
Average	157.22	60.42	44.40	667.32

Table 2. cDNA concentrations (ng/μL) of the Pintag population of *V. floribundum* for the four genes and their averages.

of each gene are very similar in both populations. In contrast, highly significant differences were observed when comparing the expression levels among the four genes analyzed ($F = 433.24$; $p < 0.0001$).

This study showed an expression level related to the biosynthetic route, where CHS has the highest expression level and ANS the lowest one. The differential expression of each gene observed in ripe fruits would be related to the maturation stage of this species and the function of each gene in the synthesis of anthocyanin. It would be essential to observe the expression of these genes in *V. floribundum* under different maturation stages. The results of this study (Duncan test) Show (Figure 4) that there are no significant differences in the expression levels when comparing the Machachi and Pintag populations. However, three expression levels can be observed; the anthocyanin synthase gene presented the lowest level, the intermediate level by the dihydroflavonol 4-reductase gene, and the highest expression level was observed in the chalcone synthase and glyceraldehyde-3-phosphate dehydrogenase (control) genes.

The ANOVA analysis among populations of *V. floribundum* ($F = 0.08$ and $p = 0.9688$) indicates no significant differences between such populations. Instead, it is observed that each of the genes analyzed (chalcone synthase, dihydroflavonol 4-reductase, anthocyanin synthase and glyceraldehyde-3-phosphate dehydrogenase) has a different expression level; this expression level is similar in both populations of *V. floribundum*; Chen *et al.*¹⁸ and Wang *et al.*¹⁰ showed that internal factors such as the maturation degree and environmental factors such as humidity, temperature and amount of light can affect the synthesis levels of anthocyanin; therefore, knowing the metabolic pathways can allow the implementation of management plans and avoid the extinction of this species¹⁹.

Discussion and conclusions

The glyceraldehyde-3-phosphate dehydrogenase gene presents the highest concentrations concerning other genes, and therefore it enables the normalization of a standard curve and generates reliable data, as has been proven in other stu-

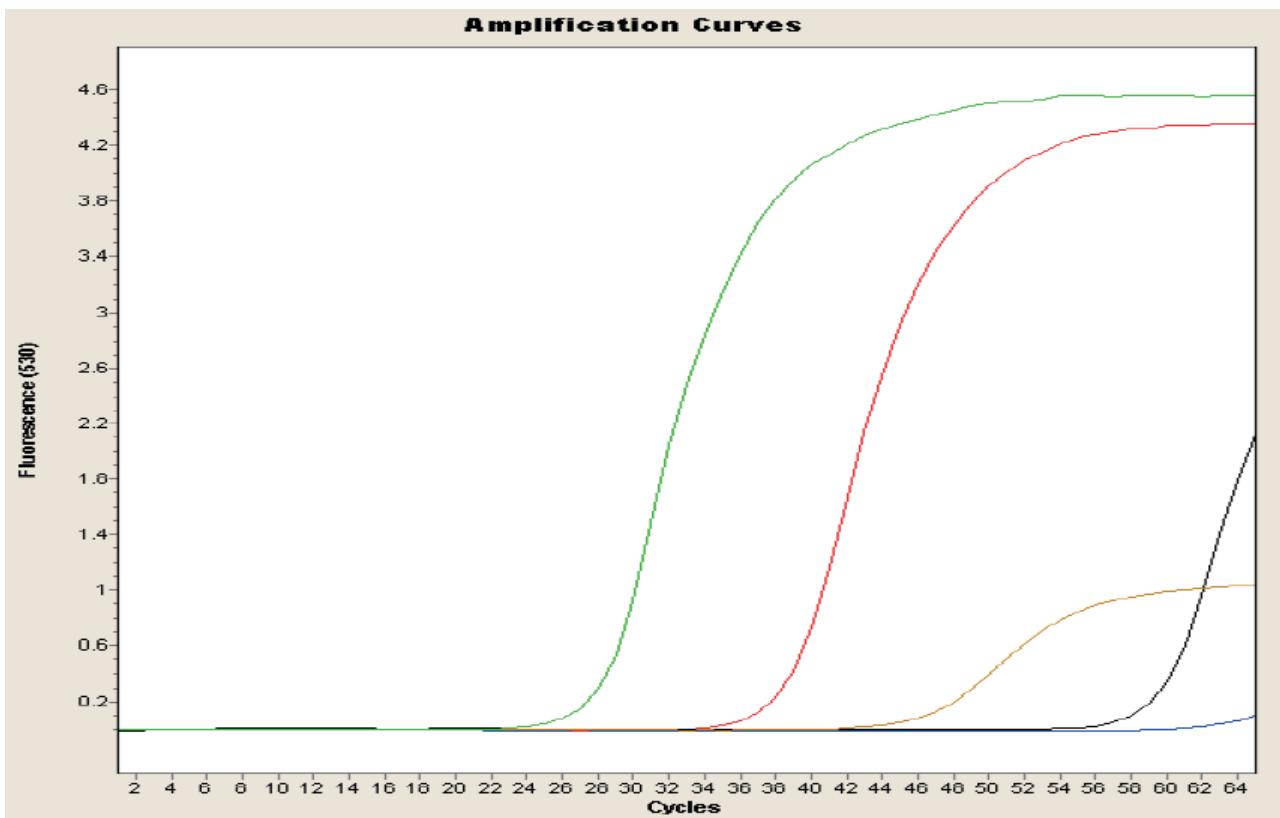


Figure 2. Amplification curve of the four population genes of Machachi population.

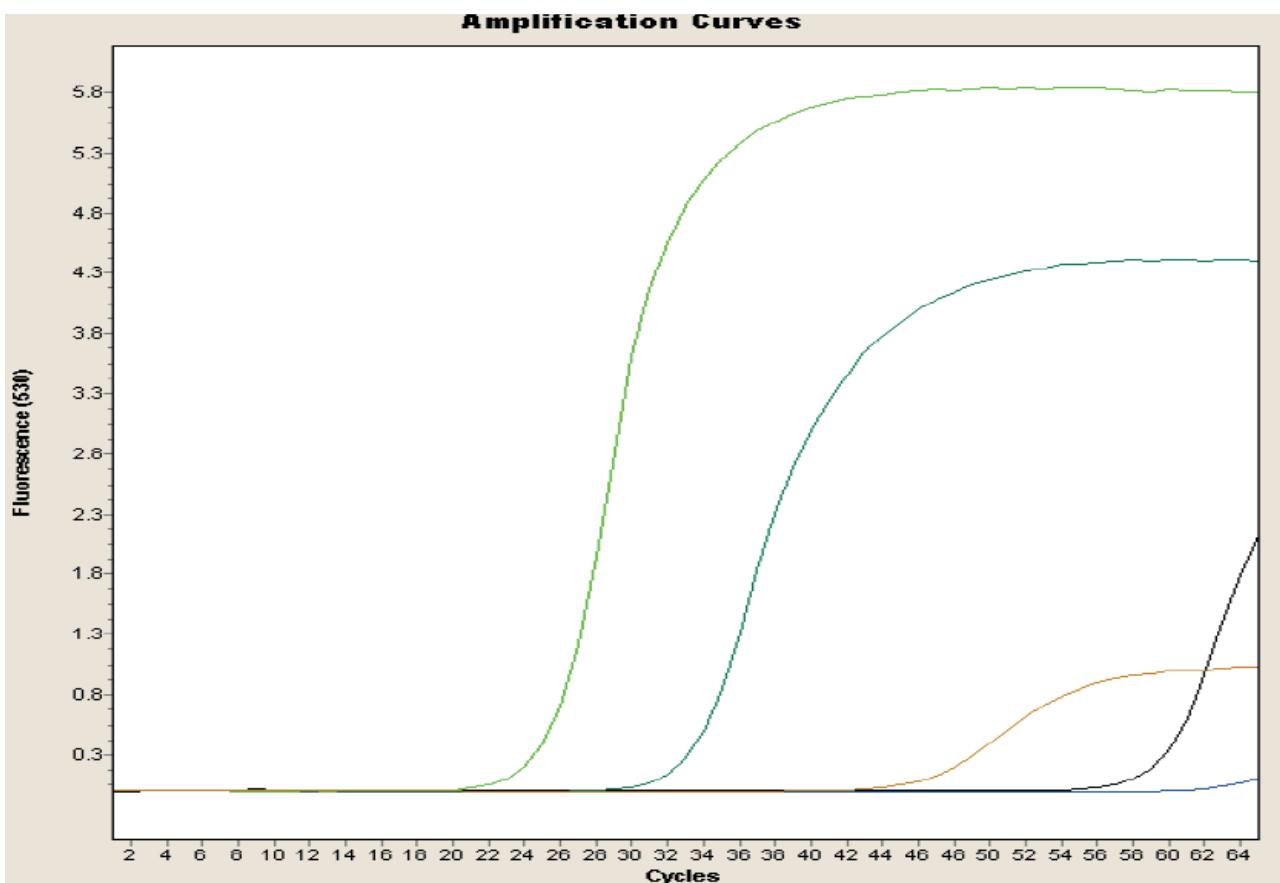


Figure 3. Amplification curves of the four genes of the Pintag population.

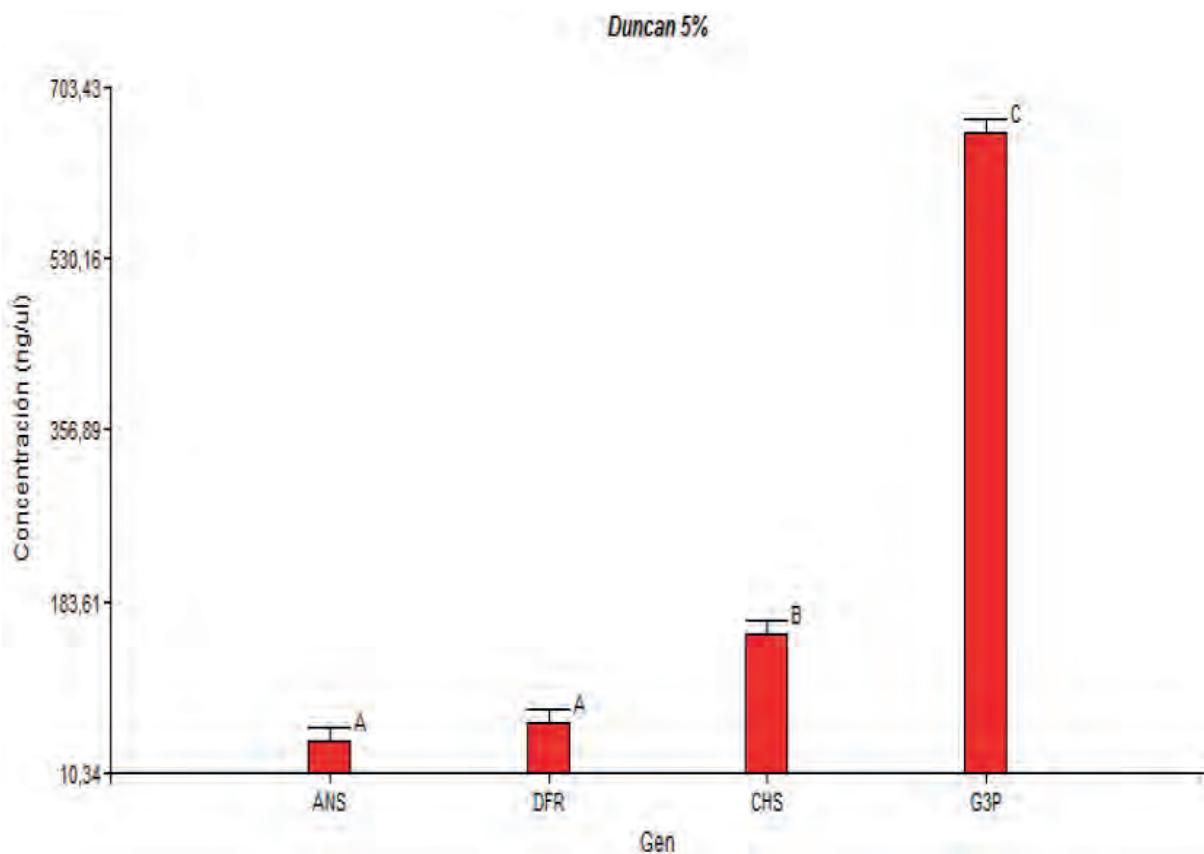


Figure 4. Duncan Test for comparing the averages according to the cDNA gene concentrations in ng/μL.

dies in Bilberry conducted by Jaakola *et al.*¹⁴ and in *V. Myrtillus* conducted by Martz *et al.*³. The results obtained indicate a high correlation in the expression of the chalcone synthase and dihydroflavonol 4-reductase found in *Clivia miniata*²⁰, suggesting that these genes are subjected to coordinate the regulation of anthocyanin in the species, i.e., the two genes have direct action in the translation process regulating the final products. According to the expression values obtained for these two genes, something similar could be happening in the *V. floribundum*.

In the two populations, the anthocyanin synthase gene has the lowest expression level. It is possible that, as mentioned by Aguilera *et al.*²¹, some fruits belonging to the family of the Rosaceas, Vitaceae, Solanaceae, Ericaceae, and Passifloraceae are rich in anthocyanin, but in the case of Mortiño, the delphinidin is codified by another gene, the F3'5'H (Flavonoid 3'5'-hydroxylase), which is a crucial precursor compound that could be found in higher proportion compared to the anthocyanin synthase²².

There are no significant differences when comparing the expression level of the three genes (CHS, DFR, and ANS) among the populations since each gene has a similar expression level in both; therefore, the altitudinal differences do not affect the metabolic processes, and in this case the biosynthesis route of the anthocyanin in *V. floribundum*. These results agree with the research conducted by Li *et al.*²³ and Wang *et al.*¹⁰, who observed the concentration of anthocyanin and flavonoids in *Vaccinium uliginosum* berries from seven locations in the Khingan Mountains only increased when there was a wide variation in altitude among populations.

The expression level of the CHS, DFR, and ANS genes is different and is related to the expression order in the biosynthesis path of anthocyanin, so the first to express and the one with the highest expression level is CHS, then DFR, and finally

ANS. These data agree with Jaakola *et al.*¹⁴ in Bilberry, where procyanidins and quercetin were the main flavonoids in the early development stages of the berry, but their levels decreased dramatically during the maturation progress and the last stages. The content of anthocyanin increased significantly, and these were the main flavonoids in the ripe berry. Therefore, there is a correlation between the accumulation of anthocyanin and the expression of flavonoids during the ripening of berries.

Each of the genes presents a determining function in each stage of growth and maturation of the species, and it can be concluded that in the ripening stage when the fruit has a dark violet coloration and a diameter of about 1 cm, the gene that is most expressed is the chalcone synthase.

Many enzymes, structural genes, and regulatory elements have been observed as transcription factors involved in the biosynthesis of anthocyanin²⁴. At the beginning of the biosynthesis, structural genes are expressed as chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3-hydroxylase (F3H), which are necessary for the synthesis of anthocyanin precursors and derivatives of flavone and flavonols; then biosynthetic genes such as the dihydroflavonol 4-reductase (DFR) and the anthocyanin synthase (ANS) are expressed, which are necessary for the synthesis of anthocyanin and proanthocyanin²².

V. floribundum Kunth can be considered as a source of anthocyanin; therefore, the expression analysis of the genes involved in the biosynthesis route of anthocyanin in *V. floribundum* offers essential information that will enable the implementation of conservation programs for the species.

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RESEARCH / INVESTIGACIÓN

Optimization of cultural conditions affecting improved bioactive metabolite production by endophytic fungus *Trichoderma harzianum*Rashid Rahim Hateet^{1*}, Zainab Alag Hassan², Abdulameer Abdullah Al-Mussawi² and Shaima Rabeea Banoon¹DOI: [10.21931/RB/2021.06.04.8](https://doi.org/10.21931/RB/2021.06.04.8)

2187

Abstract: The present study aimed to optimize cultural conditions for optimum bioactive metabolite production by endophytic fungus *Trichoderma harzianum*, isolated by surface sterilization method from the leaf of the eucalyptus plant. The fungus was identified based on morphological characterization. Fungal metabolites were carried out by ethyl acetate solvent. The antibacterial activity was tested against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (NCTC 6571). Various carbon, nitrogen sources, pH, temperature, incubation period, and NaCl on the antibacterial metabolite production were studied. Bioactive metabolite production of *T. harzianum* exhibits a broad spectrum of in vitro antibacterial activity against two strains of bacteria. For the optimum production of bioactive metabolites, Dextrose and Glucose were found to be the best sources of carbon and the best sources of Nitrogen Yeast extract (YE) and (NH4)2SO4. The maximum production of bioactive metabolites occurs at pH 7 and 25°C; the NaCl showed a positive influence on bioactive metabolites.

Key words: Endophytic fungus, *Trichoderma harzianum*, Optimum conditions, bioactive metabolite, eucalyptus.

Introducción

The genus *Trichoderma* Pers. consists of species with agricultural, biotechnological, and industrial benefits¹. The first complete genus description date back to 1969² and comprised nine species complexes grouping; species morphologically inseparable but genetically diverse. Recent advances in *Trichoderma* taxonomy have brought the present system of more than two hundred biologic species, supported by the molecular analysis of the variable genomic DNA regions with phylogenetic and taxonomic significance³. *Trichoderma* inhabits the root, soil, and foliar environments are highly interactive and free-living fungi and successfully used to control many crop pathogens in field trials⁴. Endophytes are microbes, which colonize plants' internal tissues without causing harmful, apparent adverse effects⁵. Complex interactions with host fungi and numerous endophytic fungi are beneficial for their hosts in many ways, including promoting host growth and nutrient gain and enhancing host resistance to phytopathogens, pests or abiotic stress⁶. *Trichoderma* spp. produce secondary metabolites with various biological activities affecting plant metabolism⁷. Endophytic fungi are now regarded as an outstanding source of bioactive metabolites because so many of them are cultivated in unusual environments, occupied by millions of unique biological niches (higher plants)^{8,9}. Of the 300,000 higher plant species on the earth, one or more endophytes are present in each plant, of the millions which exist here¹⁰. Fungi are an excellent natural source of bioactive secondary metabolites production containing several bioactive agents, including antibiotics, anti-tumor, antidiabetic, and antioxidants¹¹⁻¹⁶. The exploration of natural resources to new antimicrobial compounds has become more and more relevant.

Nevertheless, natural products provide important drug sources used in various fields of therapy¹⁷. Auto-regulators administer secondary metabolism by carbon, nitrogen, phosphate sources, trace elements, precursors, secondary metabolism induction enzymes, catabolism suppression and inhibition, and feedback suppression¹⁸. Numerous microbes live in extreme environments, such as high temperatures, high salt concentrations, low pH, and high radiation. Fungal growth and

metabolite production also influence some physical factors. The biotechnological production of microorganisms usually depends on their specific environmental adaptations. The production of bioactive and antimicrobial agents, many new and exciting bioactive metabolites such as antibiotics, antivirals, and antioxidants have pharmaceutical, industrial and agricultural importance, isolated and characterized from soil fungi which affected by the physical and chemical parameters such as pH, temperature, incubation period, carbon and nitrogen and amino acid sources⁸. The majority of the study indicated a different ecological and cultural factor influencing the biosynthesis of secondary fungal metabolites¹⁹. This study aimed to assess the optimal cultural conditions for producing bioactive metabolites by *Trichoderma harzianum* endophytic fungus.

Materials and methods**Sample collection**

The *Euclyptus camaldulensis* leaf from the southern province of Maysan in Iraq. 48 hours after collection, The surface sterilization of the leaf was based on (20).

Isolation and characterization of fungi

In Petri dishes containing medium potato dextrose agar (PDA), the segments of surface-sterilized leaf were evenly separated. In the light chamber with 12 hours of light and 12 dark hours, the dishes of Petri were sealed by Parafilm and incubated at 26°C. The Petri dishes were monitored every day to monitor the growth of endophytic fungal colonies from the leaf segments. Identification was made using Rifai's identification keys² and Bissett²¹⁻²³.

Microbial target organisms

Staphylococcus aureus (NCTC 6571) and *Escherichia coli* (ATCC 25922) standard test bacteria used in the current study.

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Cultivation was sub-cultured on a nutrient agar medium before the antibacterial test (Oxoid, England).

Fungal metabolite extraction

The filtrate for fungal culture was extracted three times using a separating funnel with 1:1 (Vol) ethyl acetate. The organic layer has been collected and dehydrated with Na_2SO_4 .

Antibacterial activity

A filter paper disc diffusion technique²⁴ was used for determining the antibacterial bioactivity of the fungal extract. Petri dishes were prepared, and the bacterial suspension containing 1×10^6 cells per ml of Muller Hinton Agar²⁵.

Minimal inhibitory concentration.

The standard serial dilution analysis determined the minimum inhibitory concentration (MIC) values²⁶. The inhibitory test was carried out on the Muller-Hinton agar medium.

Optimization of culture conditions for the production of bioactive metabolite

Basal medium

Potato broth medium with or without was used as a basal medium to determine the optimum conditions for bioactivity exhibited by *T.harzianum*. Erlenmeyer flasks (500 ml) containing 250 ml Potato broth supplemented with 1% (w/v) of different carbon or nitrogen sources and sterilized.

Effects of carbon sources

Various carbon sources (Dextrose, Galactose, Glucose, Mannose, Starch, and Sucrose) have been amended separately into Tryptic Soy Broth medium at 1% (w/v) using 250 ml of medium in conical flasks. Each flask was inoculated with three discs (5 mm diam) taken from the fungal colony grown on PDA in a Petri dish. Cultures were incubated at 25°C for 10 days.

Effects of nitrogen sources

Different nitrogen sources (Asparagine, Peptone, Yeast extract (YE), Malt extract (ME), NaNO_3 , NH_4Cl , and $(\text{NH}_4)_2\text{SO}_4$) were separately amended into Tryptic Soy Broth medium at 1% (w/v) using 250 ml of medium in 500 ml flasks. The three discs (5 mm diam) taken from the fungal colony grown on PDA in the petri dish were inoculated. Cultivations have been incubated for 10 days at 25°C.

Effect of pH

Effect pH for detecting the bioactive product metabolite has been tested in the laboratory with liquid cultures containing different pH levels (4,5,6,7,8 and 9).

Effect of temperature

The fungus has been exposed to different temperatures to investigate the best temperature required for the bioactive metabolite (15, 20, 25, 30, and 35°C).

Determination of the incubation period

The impact of the incubation on the active metabolite was calculated using incubation periods ranging from 7 to 26 days.

Effect of NaCl concentration

Incubation in various NaCl concentrations, the effect of salinity on the bioactive metabolite of *T.harzianum* isolate has been performed, from 3-7% to 1% of carbon and nitrogen sources, while other parameters have remained optimum. The production of bioactive metabolite has been calculated and recorded for each level of sodium chloride.

Results and discussion

The present study results showed a significant effect on the bioactive production of *T.harzianum* metabolites in different microbiological cultural conditions. The production of antibacterial metabolites was determined by the disc diffusion assay method measuring of inhibition zone against two strains of reference bacteria, *E.coli* (ATCC 25922) and *S.aureus* (NCTC 6571). Further experiments on cultural optimization conditions to enhance bioactive metabolite production are therefore underway. In the previous decades, more attention has been paid to new bioactive compounds from fungi that have become a natural source²⁷. Cultural conditions such as pH, temperature, carbon, nitrogen sources, and NaCl concentration were optimized as they affect the metabolite of bioactivity in this study.

Effect of carbon source on antibacterial metabolite for *T.harzianum*. (Figure 1) Among the carbon sources, dextrose proved to be the best carbon source for antimicrobial metabolites produced by the fungus, with an inhibition zone 18.0 mm against *E.coli* and 20.0mm against *S.aureus*. Glucose also gave a similar pattern result followed by Sucrose, Starch, and Mannose, respectively, which agrees with (28). No antibiotic was produced when the medium was supplemented with galactose, and Carbohydrates are known to interfere with the production of secondary metabolites²⁹. In addition to CO₂, water, and energy, the production of intermediates, which produce primary and secondary metabolites is affected by simple carbohydrates like glucose and dextrose through metabolic pathways³⁰. The addition of glucose resulted in the highest fungal growth but significantly reduced the production of bioactive metabolites. A higher concentration of glucose affects suppressively the production of bioactive metabolites in many fermentation processes³¹. It should be noted that the filamentous fungi are ubiquitous organisms able to obtain energy from very different substrates³².

Figure 2; shows the effect of various nitrogen sources on *T. harzianum* production of bioactive metabolites. Maximum antimicrobial activity was obtained when media were supplemented with yeast extract with inhibition zone 18.5mm against *E.coli* and 20.5 mm against *S.aureus* followed by $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl , Asparagine, Peptone, and NaNO_3 . However, manipulation of nutrient factors has been stated to promote the biosynthesis by microorganisms of secondary metabolites³³.

The effect of pH on antimicrobial metabolite production by the fungus is presented in Figure 3. The optimum pH for antibacterial metabolite production was 7.0 with an inhibition zone of 18.0 mm against *E.coli* and 16.0 mm against *S. aureus*; (34) have noted that the most significant number of microorganisms can synthesize pH-based antimicrobial compounds between 5.5 and 8.5.

The influence of temperature is presented in Figure 4. The *T.harzianum*, showed a narrow range of bioactive metabolite incubation temperatures. The increase of the incubation temperatures from 25 to 30°C enhanced bioactive metabolite. Maximum inhibition zone 20.0mm against *E.coli* and 20.5 mm against *S.aureus* was recorded at 25C. This indicates the stra-

in was strictly mesophilic for secondary metabolites production. However, the lowest inhibition zone was observed at a low temperature of 15°C. These results are compatible with (14).

The influence of the incubation period on bioactive metabolite production of the isolate is presented in Figure 5. The production of metabolite increased and reached its maximum levels after 11 days and after that gradually decreased; this agrees with (14).

The influence of NaCl concentration on bioactive metabolite production of the isolate is presented in Figure 6. NaCl concentration of 6g/l was recorded as optimal for bioactive metabolite production.

Conclusions

This study showed good antibacterial activity against gram-positive and gram-negative bacteria produced by *T. harzianum* grown in optimized conditions.

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Conflicts of interest

The author declares that no conflict of interest exists.

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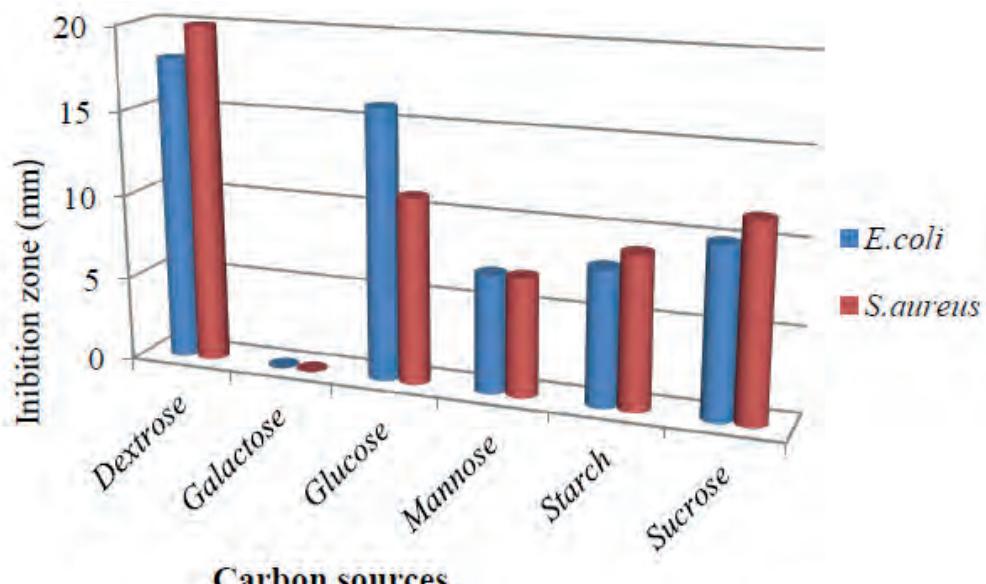


Figure 1. Effect of different carbon sources in the medium on bioactive metabolite by *T. harzianum*.

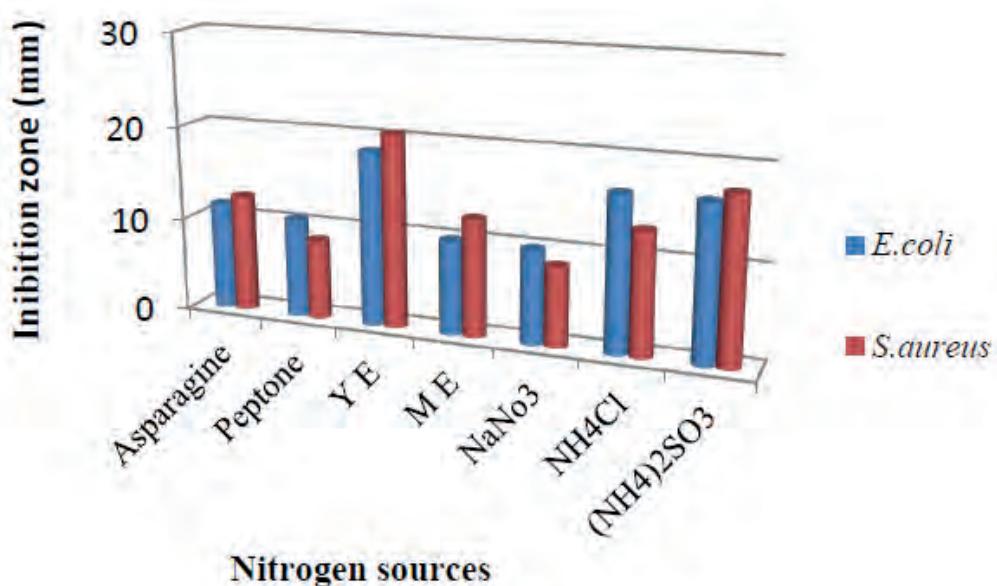


Figure 2. Effect of different nitrogen sources in the medium on bioactive metabolite by *T. harzianum*.

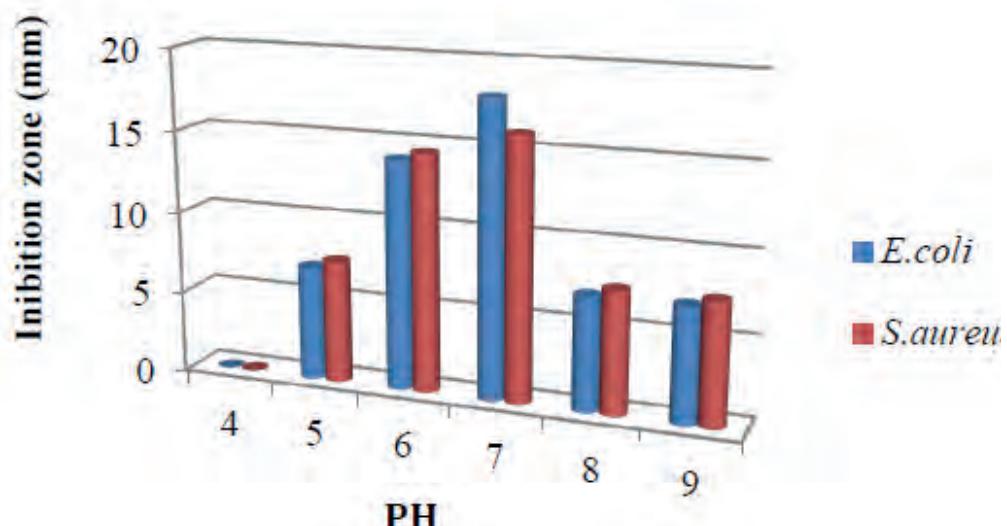


Figure 3. Effect of pH of the medium on bioactive metabolite by *T. harzianum*.

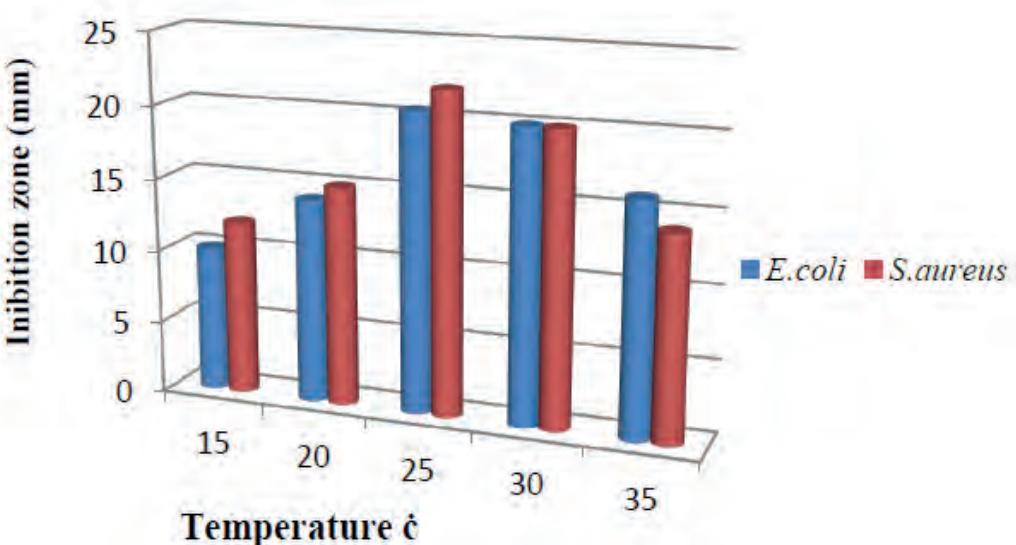


Figure 4. Effect of temperature on bioactive metabolite by *T. harzianum*.

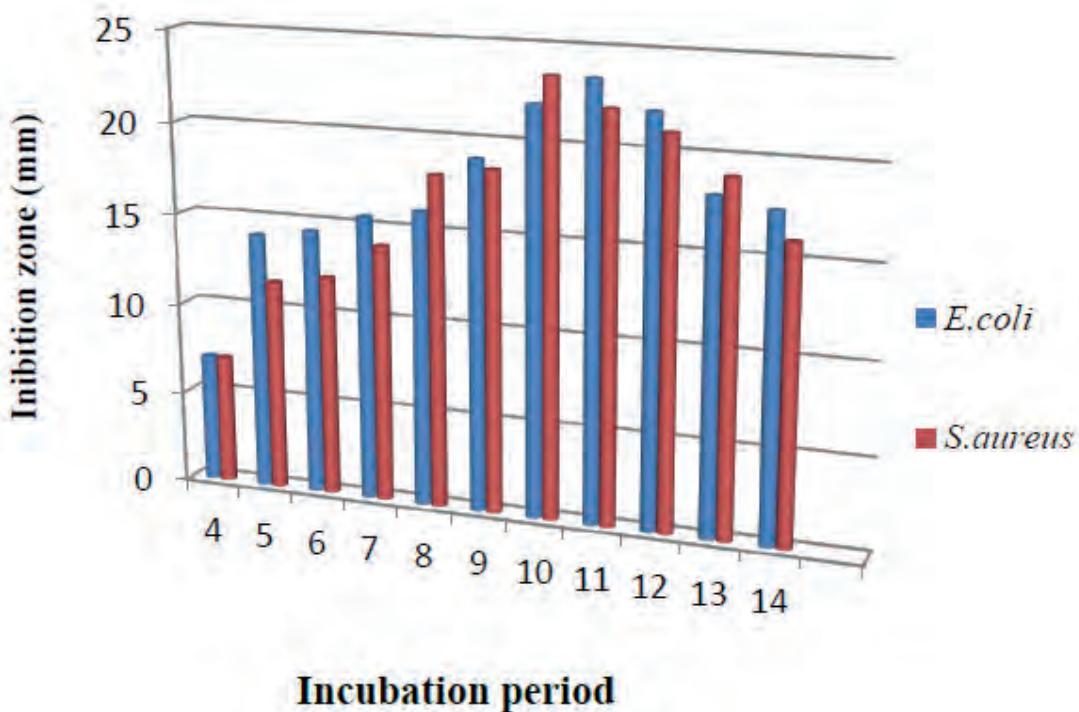


Figure 5. Effect of incubation period on bioactive metabolite by *T.harzianum*.

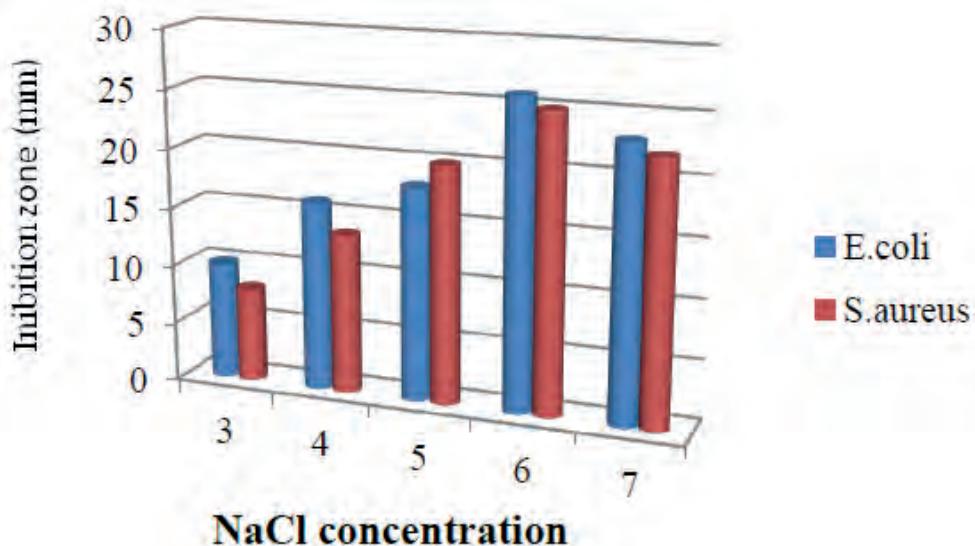


Figure 6. Effect of NaCl Concentration in the medium on bioactive metabolite by *T. harzianum*.

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RESEARCH / INVESTIGACIÓN

Potential effect of Imatinib on some sex hormones for male patients of Chronic Myelogenous Leukemia in Baghdad province

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Abstract: Imatinib Mesylate is an oral chemotherapy drug that has been used to treat Chronic Myelogenous Leukemia (CML). It works as an inhibitor of oncogene tyrosine kinase BCR-ABL1 as a target therapeutic agent. Despite the drug is well tolerated in most patients, impaired testosterone production and Gynecomastia after therapy might happen. The current study aims to evaluate the impact of Imatinib Mesylate on sex hormones of CML male patients in Baghdad province. Blood specimens were collected from (42) CML patients aged 23 to 68 years who used Imatinib drug for more than two years, and (45) normal persons aged 25 to 65 years as a control group. Exclusion criteria were performed for both control and CML patient's groups, including people with diabetes, hypertensive, and males complaining of infertility after taking medical history for every participant. The blood level of hemoglobin (Hb), white blood cells (WBC), platelet count, testosterone, LH, and FSH were evaluated and investigated. The obtained results showed a significantly lower level of testosterone (2.73 ± 0.97 ng/mL) than the control group (4.72 ± 1.02 ng/mL) with a p-value of 0.000. While LH (4.53 ± 2.1 mIU/mL) and FSH (5.12 ± 2.83 mIU/mL) were significantly higher than the control group (3.77 ± 0.8 mIU/mL and 3.85 ± 0.807 mIU/mL) with p-value of 0.026 and 0.005 respectively. Moreover, the outcomes revealed a moderate positive correlation ($r = +0.348$) between LH hormone levels with a duration increasing time of using Imatinib, while platelet showed a moderate negative correlation ($r = -0.321$) with time-consuming using that drug. In conclusion, Imatinib might harm testis functions and some hematological parameters that could increase using this drug.

Key words: Imatinib, Sex hormones, Myelogenous Leukemia, Patients.

2193

Introduction

During the last two decades, the treatment of Chronic Myeloid Leukemia (CML) has changed because of the introduction of tyrosine kinase inhibitor. The survival rate had been increased in patients with Leukemia and lymphoma¹. Besides that, the infertility complications were raised in parallel with that disease. The chance of getting CML can be increased at the 4th decade of life or a younger age group where reproductive functions are crucial in affected men^{2,3}. It has been identified many side effects about sexual function by using drugs for CML therapy. However, CML as a disease may adversely affect male fertility even before any therapy^{4,5}. The introduction of tyrosine kinase inhibitor has increased the survival rate significantly, highlighting issues related to the quality of life with an important area of fertility and paternity. The gonadal dysfunction induced by therapy depends in general on age dosages and types of the therapies used in CML treatment⁶.

Moreover, animal studies on Imatinib using standard dosages have shown non significantly impaired adult male fertility⁴⁻⁷. The effect of Imatinib on spermatogenesis seems to be dose dependably⁸. c-KIT is essential for developments of Leydig cells and migration survival and proliferation of spermatogonia. Also, Platelets derived growth factors (PDGF) and (PDGF-R) are very important for developing Myeloid and Leydig cells⁹. This study aimed to investigate the potential testicular effects of using imatinib drugs for CML patients (testicular Leydig cell function and androgen status) by measuring and characterizing reproductive hormones such as Follicle-stimulating Hormone (FSH), Luteinizing Hormone (LH), and testosterone.

Materials and methods

The investigation was a spin-off study for (42) male pa-

tients with CML that attended the National center for hematological diseases at Al-Mustanseria University. Participant patients have been using a standard dose of 400 mg of tyrosine kinase inhibitor (Imatinib) per day for equal or more than two years, and their ages ranged from 23 years to 68 years old. Patients in the above center that included in this spin-off protocol were invited to participate in this study only if they already agreed to be included. A healthy control group of (45) males aged 25 years to 65 was also involved. Exclusion criteria for both control and CML patients groups were performed for people with diabetes, hypertensive, and males complaining of infertility after taking medical history for each one. Blood samples were taken from both groups. Blood specimens were collected in EDTA tubes for Hb, white blood cells, platelets, and differential count for WBC analysis. The mentioned hematological parameters were estimated using an electronic counter (Mandray Company, France), and the blood films were performed on all patients and control groups using Giemsa stain. In addition, gel tubes were used to assess LH, FSH, and testosterone hormones concentrations by using the ELISA method through a micro plate reader (AWARENESS, USA). The ELISA kits were DRG from Germany.

Statistical Analysis

All statistics in the present study were performed by using SPSS Ver. 23 for windows. Quantitative values were presented as Mean and Standard deviation (SD) of all variables. An independent t-test was used to exhibit the significance of differences. The confidence interval (95%) p-value ≤ 0.05 was considered statistically significant, while the probability p-value > 0.05 indicates statistically not significant. Moreover, correlation analysis between parameters was assessed by Pearson's.

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Results and discussion

Nowadays, the new molecular design of cancer treatments such as tyrosine kinase inhibitors can be applied into therapy, and the strategy for young adults and children and the latent effect on testis functions should be considered. The role of these drugs in some of specific physiological signals cascade modulated may be varied with the maturation state and age, and the effectiveness and the side effects of these agents in young adults and children may be different. It is well known that the introduction of the tyrosine kinase inhibitor has revolutionized the therapy of CML that leads to prolonged survival and significant improvement in the quality of life that results in increasing the number of patients that wish to be the father. According to previous animal studies on the effect of tyrosine kinase inhibitor as treatment, there is a mild to moderate impairment of the male reproductive system¹⁰⁻¹².

In general, hormones act upon the phases of the spermatogenesis (like LH and FSH) act directly on the testis to stimulate somatic cells functions that support spermatogenesis¹³. It had been reported that FSH is mainly regulated spermatogonial development^{14,15}, while LH is working on Leydig cell to secrete testosterone. In the case of testosterone, it is the primary male steroid sex hormone and plays a crucial role in developing male reproductive tissue such as testis and prostate as well as secondary sexual characteristics¹⁶. Testosterone regulates the later phase of spermatogenesis, which takes about 74 days. Despite semen analysis not being performed in the current study, however, there is an effect of Imatinib on tail protein PY phosphorylation in human is related to sperm motility. The deficiency of this protein during therapy with tyrosine kinase inhibitor (Imatinib) may be associated with decreased motility of the sperm¹⁷⁻²⁰. For hematological edge, the present study showed that platelets count is significantly higher than the control group (13.81 ± 1.28) and (13.70 ± 0.79), respectively, with p-value of 0.03. Even though the patients and control group are within the normal range, our explanation regarding these results may be due to the small sample size. While for Hb and WBC account the obtained result exhibited that the is non-significant differences between health and the patient group were Hb 13.70 ± 0.79 (g/dL) and 13.81 ± 1.28 (g/dL), and 5.88 ± 1.21 , 6.81 ± 3.39 for WBC respectively), Table 1.

The results of the hormonal, biochemical assay showed in the case of testosterone that the mean level of the patient's group (2.73 ± 0.97) ng/mL is significantly lower than the con-

trol group (4.72 ± 0.72 ng/mL with p-value of (0.00) this finding is consistent with several studies^{14,15}. Also, the LH hormone in the patient's group is significantly higher than the control group 4.53 ± 2.1 mIU/mL and 3.77 ± 0.8 mIU/mL, respectively, with p-value 0.026. The same is true for FSH hormone; the patient's group is also significantly higher than the health group 5.12 ± 2.83 mIU/mL and 3.85 ± 0.807 mIU/mL, respectively, p-value 0.005. our results were different from that reported by (21). However, our data is consistent with that reported (22).

The correlation for all measured parameters with duration increasing time for Imatinib was assessed. Regarding that, the results showed a moderate positive correlation ($r = +0.348$) of LH hormone with increasing time duration of the mentioned treatment with p-value 0.026 as shown in table 3. while for platelet number, the results exhibited that there is a moderate negative correlation ($r = -0.321$) with increasing time consumed of the Imatinib drug with p-value (0.038). For all other parameters, the outcomes revealed that there is a non-significant correlation with duration consuming time, this is maybe due to our results dealing with a small number of samples. However, these results are consistent with that reported by (23) that stated Imatinib decreases the viability of normal Leydig cells in a manner not time-dependent. Moreover, Imatinib has an antiangiogenic effect that inhibits the vascular endothelial growth factor^{23,24}. Imatinib delays or may block migration of gonocytes from the center of the seminiferous cord to basement members to form a spermatogonial stem cells pool. Inhibition of migration was probably due to blockage of the c-kit receptor. The presence of c-kit antiserum in Sertoli cells inhibits migration of gonocytes^{25,26}. Imatinib interferes with several maturation processes in the rat-like spermatogonial stem cell and Leydig cells that produce testosterone and proliferation of differentiation type A spermatogonial. The low testosterone level is derived from the hypothalamus pituitary axis and LH; FSH levels will increase significantly secondary to low testosterone levels²⁷.

Conclusions

The therapy of tyrosine kinase inhibitor for male CML patients adversely affects testosterone levels with increased LH and FSH levels. However, it is unlikely to knock down the sperms production. It is advisable to have sperm banking in young adult patients before starting prolonged treatment by Imatinib

Parameters	Mean ± SD control	Mean ± SD patients	p-Value
Hb (g/dL)	13.70 ± 0.79	13.81 ± 1.28	Ns
WBC ($10^9/L$)	5.88 ± 1.21	6.81 ± 3.39	Ns
Platelet ($10^9/L$)	208.88 ± 39.72	231.71 ± 57.31	0.03

Ns : Non-significant

Table 1. Hematological parameters for patients and control health group

Parameters	Mean ± SD control	Mean ± SD patients	p-Value
FSH (mIU/mL)	3.85 ± 0.807	5.12 ± 2.83	0.005
LH (mIU/mL)	3.77 ± 0.8	4.53 ± 2.1	0.026
Testosterone (ng/mL)	4.72 ± 0.72	2.73 ± 0.97	0.000

Table 2. Hormonal biochemical parameters for patients and control groups

Parameters	Correlation coefficient (r)	p-value
LH	+0.348	0.026
Platelet	-0.321	0.038

Table 3. The correlation coefficient of some parameters with increasing consuming time of Imatinib drug.

that can be easily applied. However, the present study's limitation is the small sample size of CML patients included in the present data. A larger sample size is highly recommended to enforce those original results. Also, seminal fluid analysis was not done because most patients were unwilling to provide us with their semen.

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RESEARCH / INVESTIGACIÓN

Isolation and molecular identification of microorganisms isolated from soils contaminated with heavy metals in Mosul city

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2196

Abstract: This research is concerned with organisms isolated from soils contaminated with heavy metals in industrial and residential areas in Mosul, the center of Nineveh Governorate, and the diagnosis of these organisms using molecular biology techniques. Samples were collected from four locations in the city between the industrial area and residential neighborhoods. Soil samples were analyzed, and dilutions were prepared, then the dilutions were grown on potato extract and dextrose (PDA) medium for the development of fungi and Nutrient agar for bacterial development. The dilutions were planted by the casting method by three replications, then the process of purifying the fungal and bacterial colonies was carried out using the traditional methods. To diagnose these pure colonies using PCR technique, colonies of fungi were grown on the medium of PDA, and bacteria were grown on the medium of nutritious broth.

As a result, nine fungal species were diagnosed; two of them are new undiagnosed genera that have been registered in the gene bank, four of them contain genetic mutations, and three of them are known and previously diagnosed fungi. As for bacteria, two new strains were isolated and registered in the gene bank among the four diagnosed types. And some of these genera exhibited severe resistance to antibiotics, while others showed moderate resistance, in contrast to the control, which was very sensitive to antibiotics.

Key words: microorganisms, soils, contaminated, heavy metals.

Introduction

A "heavy metal" is an element with metallic properties (such as luster, electrical and thermal conductivity), and a relatively high density. Although essential heavy metals are required in many biological processes such as growth and function at low concentrations, they are harmful if they exceed the required concentration^{1,2}.

High concentrations of toxic metals in soils are a serious environmental problem worldwide, posing significant risks to human health through various exposure pathways. Oral ingestion, inhalation of volatile and particulate matter, and dermal contact are the most critical human exposure pathways to metal-contaminated urban soils. Children are more likely to be exposed to toxic metals in urban soils than adults because they may absorb through the skin, ingest or inhale large amounts of toxic metals during outdoor activities and play³. The most toxic heavy metals to plants such as lead (Pb), arsenic(As). Cadmium (Cd) and mercury (Hg) are metals found in the soil, which are highly mobile and are immediately absorbed by plants. Also, copper (Cu), chromium (Cr), zinc (Zn), selenium (Se), molybdenum (Mo), tin (Sn), and nickel (Ni) cause environmental pollution and health risks⁴. In his study, Rahman (2020) stated that pollution with heavy metals produced adverse effects such as mutagenic, teratogenic, and carcinogenic in humans and wildlife⁵.

Metal resistance is defined as the ability of an organism to survive and counteract metal toxicity by various mechanisms produced an indirect response to the metal species involved^{6,7} or that this resistance is due to changes to the genetic material through mutations or the addition of new genetic material⁸.

The term "antibiotics" was used for the first time in 1942 by the scientist Waksman, who defined antibiotics as metabolic substances produced by microorganisms that inhibit the growth of other microorganisms and do not affect the bacteria that produce them. Antibiotics target specific bacterial structures or processes and may act by inhibiting growth, directly killing the organism, or combining both mechanisms⁹⁻¹².

Most heavy metals are non-degradable and persist in the environment, and many species have evolved resistance mechanisms to combat metal toxicity. Heavy metals can cause selective pressure on microbial populations, leading to antimicrobial resistance through a "co-selection" mechanism^{13,14}.

This study aims to analyze soil samples contaminated with heavy metals to isolate and identify microorganisms (bacteria and fungi) thriving in this environment and investigate the sensitivity of isolated microorganisms to antibacterial and antifungal agents.

Methods

Sample collection sites

Samples were collected from four areas: the first and second from near a local electricity generator inside a residential area and within an industrial area, the third and fourth samples from near car repair and maintenance shops in a residential area and industrial area in the city, as shown in Figure (1). The sample collection process was carried out using a sterile drilling tool and sterile sample collection bags with a depth of (0-15) cm.

Isolation

The dilution method was used to isolate the organisms from the soil by taking 10 g of soil sample, placing it in a sterile glass beaker containing 90 ml of sterile distilled water, and shaking it well to obtain a dilution (10^{-3}). The dishes were incubated upside down at 28 °C for 7 days for PDA medium and 37 °C for one day for nutrient agar (NA) medium. Then the colonies were purified by repeated transfer on the culture media, and the purified cultures were kept on a slant, kept in the refrigerator, renewed every month, and used when needed¹⁵.

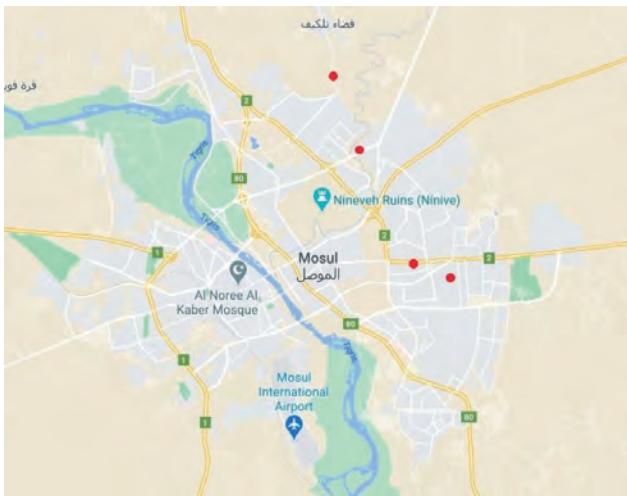


Figure 1. Isolation and identification of organisms from soil.

Diagnosis

Fungal and bacterial species isolated from soil samples were characterized by Polymerase Chain Reaction (PCR) by amplifying the Internal transcribed spacer (ITS) region preserved in the fungal DNA^{16,17}.

Study of Heavy metals Effect on the growth of living organisms (bacteria and fungi)

Two minerals were selected for the study, namely lead and zinc, the development of microorganisms on different concentrations of these minerals, and observation of the changes in them and their resistance to these concentrations. Fungi were cultured in PDA medium containing heavy metal separately at concentrations of (25, 50, 100, 150, 200, 300, 400, 500,

600, 700, 800, 900, 1000) ppm and incubated for 7 days. The fungus with the highest growth on the metal was taken and grown with different concentrations of the antifungal (nystatin) and compared with the fungus grown without metal as a control. Bacteria were cultured in nutrient broth and incubated for one day, then cultured on a petri dish containing NA by wiping the plate with a cotton swap on each plate, then drilling in the culture medium to apply the concentrations of minerals in it, using a micropipette with a volume of 100 µl, and incubated for one day.

Cultivation of living organisms with Antibiotics

A spore suspension of mushrooms was made and distributed on the plate containing the medium of the PDA, then digging was made, and the antifungal (nystatin) was placed in it at concentrations of (25%, 50%, 75%, 100%), and the dish was incubated in the fungi incubator for seven days. As for the bacteria, the plate containing the agar-nutrient medium was wiped with bacteria grown on the broth fed with cotton, then antibiotic tablets were distributed on the surface of the medium and incubated in the bacteria incubator for one day^{18,19}.

Results and discussion

The current study results of the organisms isolated from the soil showed the genera shown in Tables (1 and 2).

Study of resistance of microorganisms to heavy metals

Even though the importance of mineral elements to organisms for growth and function, such as potassium and magnesium and some trace elements such as manganese, iron, copper, zinc, and molybdenum, some high concentrations of some are toxic harmful^{1,20}, zinc and lead were chosen based on

Bacterial culture	strain	Bacterial genera
Isolated from soil	Already registered in the gene bank	<i>Bacillus cereus</i>
Isolated from soil	A new strain registered in the name of the 1 st author	<i>Bacillus sanna 1</i>
Isolated from soil	A new strain registered in the name of the 1 st author	<i>Bacillus sanna 2</i>
Isolated from water	New genes that have not been recorded	<i>Staphylococcus sp.</i>

Table 1. Isolated bacterial genera.

strain	Fungal genera
Already registered in the gene bank	<i>Aspergillus allahabadi</i>
Already registered in the gene bank	<i>Aspergillus niveus</i>
Already registered in the gene bank	<i>Aspergillus recurvatus</i>
Already registered in the gene bank	<i>Aspergillus sp. E30</i>
Already registered in the gene bank	<i>Aspergillus tubingensis</i>
Already registered in the gene bank	<i>Penicillium consobrinum</i>
A new strain registered in the name of the 1 st author	<i>Penicillium consobrinum SANA-3</i>
A new strain registered in the name of the 1 st author	<i>Fungal sp. SANA-4</i>
A new strain registered in the name of the 1 st author	<i>Fungal sp. SANA-5</i>

Table 2. Isolated fungal genera.

Concentrations \ Metals	50	100	200	300	400	500	600	700	800	900	1000	1200	1400	1600	1800	2000
Bacillus sp.sanna1	X	12	12	14	15	15	16	16	18	18	19	20	22	24	25	25
Bacillus sp.sanna2	X	12	13	15	16	16	17	18	18	20	20	21	22	22	24	26
Bacillus cereus	X	13	14	16	18	18	18	19	19	19	22	31	32	33	33	34
Staphylococcus sp.	X	8	10	10	11	12	13	14	13	15	15	16	18	22	22	23

Table 3. The diameter of the inhibition in millimeters when cultivating bacteria with zinc metal in the form of zinc sulfate.

Concentrations \ Metals	50	100	200	300	400	500	600	700	800	900	1000	1200	1400	1600	1800	2000
Bacillus sp.sanna1	X	X	X	X	X	X	X	X	X	X	X	X	11	11	11	12
Bacillus sp.sanna2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Bacillus cereus	X	X	X	X	X	X	X	X	X	X	X	X	X	12	15	15
Staphylococcus sp.	X	X	X	X	X	X	X	X	X	X	X	X	12	14	15	15

Table 4. The diameter of the inhibition in millimeters when cultivating bacteria with lead metal in the form of lead (II) nitrate $Pb(NO_3)_2$.

previous research on heavy metals in Mosul, the most recent of which was a study²¹. Low concentrations of the mineral, as shown in pink in Tables (3 and 4), through an increase in the diameter of the colony and an increase in its number, then the growth gradually decreased until the death of the organism. This is consistent with the study, which indicates that minerals at low concentrations are nutritious to the organism, but their toxicity appears by increasing these concentrations^{20,22}. Some species were slightly inhibited but remained resistant even at a concentration of 1000 ppm of metal, and this is in line with what was stated¹¹ where organisms can acquire resistance as a result of the presence of some metals in high concentrations of more than a few thousand mg/kg (ppm).

Fungi are highly resistant compared to many other microorganisms due to their high tolerance to high temperatures and acidity, low nutrients, and high levels of minerals. However, this adaptation and resistance are demonstrated by changing the abundance and structure of the fungal community, and this appeared in our study in a change in the color and density of hyphae and colonies for many species²³⁻²⁵. These studies also indicated that the fungi of the type Aspergillus and Penicillium were more resistant to heavy metals than the rest of the species, in addition to their ability to absorb some metals such as cadmium, lead biologically, and nickel, and their positive correlation with low levels of zinc and lead contamination, which is consistent with the results of the current study.

Heavy metals in trace amounts are necessary for the growth of bacteria, but they can be stress factors affecting the synthesis of proteins in them²³. Bacteria were cultured with different concentrations of heavy metals starting from 25 to 2000 ppm. The three bacterial species isolated from contaminated soil, in addition to bacteria isolated from the contaminated aquatic environment, were sensitive to zinc at small concentrations, starting from a concentration of 100 ppm, as shown in Tables (3 and 4). In contrast, to lead, which had a positive effect on it at low concentrations of the metal, which is consistent with most studies. However, it showed a negative effect starting from a concentration of 1400 ppm for three races. The fourth race remained resistant to the rise in metal concentration, and this may be a result in response to toxic metal stress and developing some survival mechanisms in their genome, such as producing a variety of enzymes and proteins that help them overcome this stress, as explained in his study²⁶.

In general, the bacterial species did not show strong resistance to metals. This may be because all bacterial species in this study are Gram-positive bacteria, and unlike Gram-negative bacteria, as stated in study²⁷, they tend to produce higher resistance levels. Compared to valued positive bacteria.

Cultivation of microorganisms with antibiotics

As shown in Figure (2), the current study results showed that the species *Aspergillus tubingensis*, *Penicillium concolor* SANA-3, *Aspergillus* sp E30 were almost identical moderately resistant during *Fungal* sp. SANA-5 was resistant without metal and in all antifungal concentrations, while it was sensitive to metal presence. Its explanation maybe what was stated in a study where heavy metals have adverse effects on the growth of microbes. Soil and fungi, the inhibition of fungal growth, come from heavy metals in the fungal cells²⁸.

Alternaria alternata isolate from a non-contaminated environment (as a control) was cultured with antifungal. The results showed that the fungus was more susceptible to the antifungal than the fungi isolated from the contaminated environment. This is consistent with what was stated in a previous study which reported that isolates from contaminated soil are more resistant than isolates from unpolluted environments²².

Concerning culturing bacteria with antibiotic tablets, the results showed resistance of all strains to amoxicillin and metronidazole, and *Streptococcus* bacteria resistant to tetracycline, gentamycin, and azithromycin as well. As shown in Table (5) and by comparing the damping diameter with the unique tables in some sources^{18,19,29}.

Streptococcus bacteria, which had the highest resistance among the bacterial genera under investigation, is a bacterium isolated from contaminated water. This resistance is explained by Dweba *et al.*(2018) in that these bacteria are characterized by their ability to develop antimicrobial resistance³⁰ rapidly. This resistance may also be that heavy metals' biological availability in water is more than their biological availability in soil. It was mentioned in a study that soil and its quality impact the biological availability of minerals and the presence of linking elements as well as bacterial and biological groups and the concentration of organic gases such as methane and ethane and the formation of complexes³¹.

The multiple antibiotic resistance (MAR) index was adopted to determine the severity of bacterial resistance to antibiotics, which came in the study^{12,32}. whereas

MAR = the number of antibiotics that the isolate showed resistance to / the number of total antibiotics exposed.

t is interpreted as

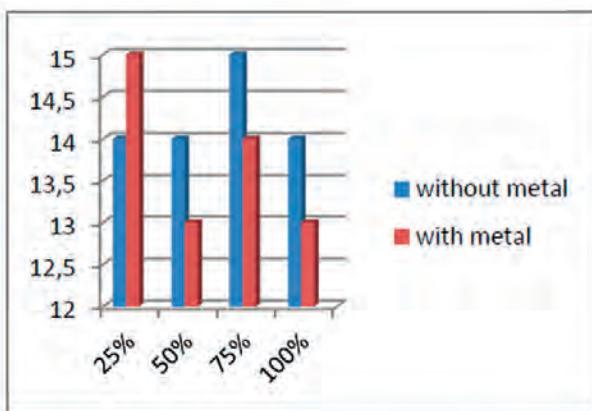
$MAR \geq 0.2$ as increased risk of antibiotic contamination.

$MAR < 0.2$ Low risk.

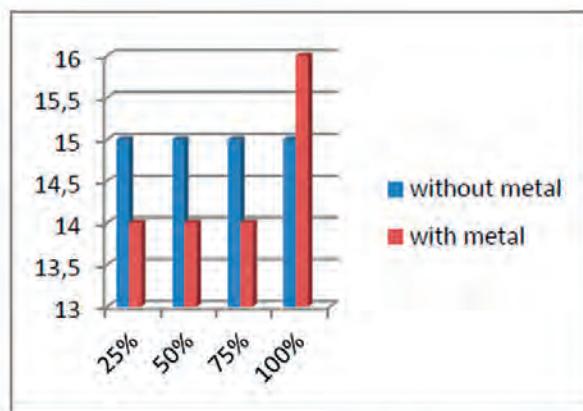
The results of the isolates were between (0.5-0.2), which indicates a high risk of antibiotic contamination, and that all these strains originated from a high-risk source of contamination.

Conclusions

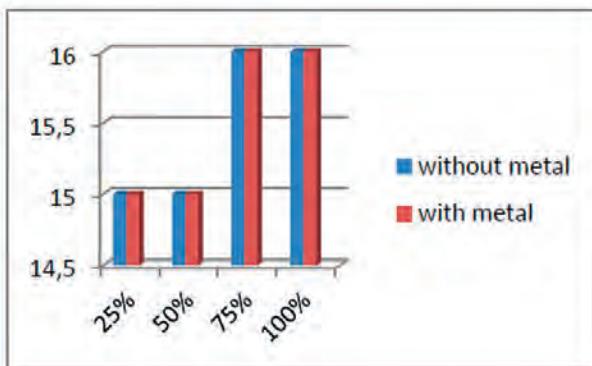
Heavy metals, a large part of them, are naturally present, but they are toxic only if they are soluble in water and bioavailable, and this comes as a result of human activities most of the time. In addition to the results of previous studies, the current results show an overlap between heavy metal contamination and its resistance by microorganisms and the resistance



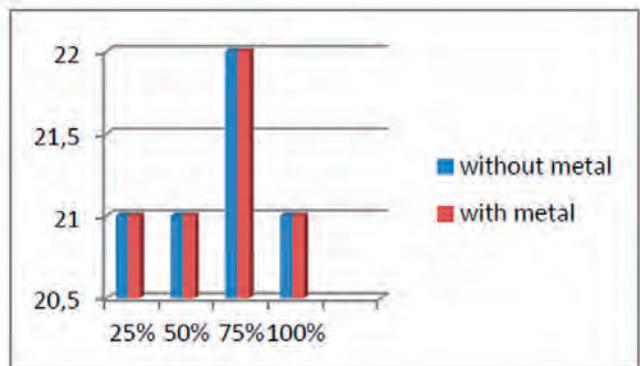
Aspergillus Tubingensis with nystatin



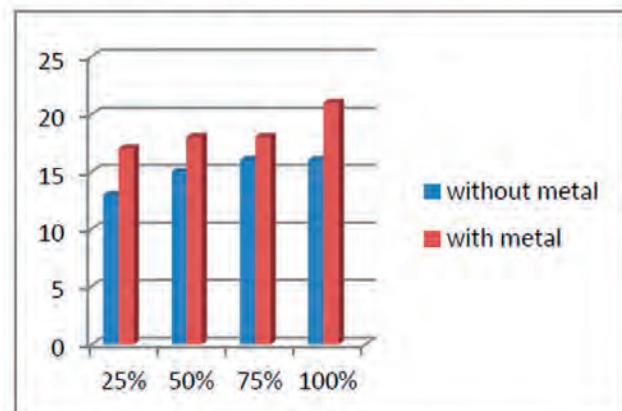
Penicillium Consobrinum SANA-3 with nystatin



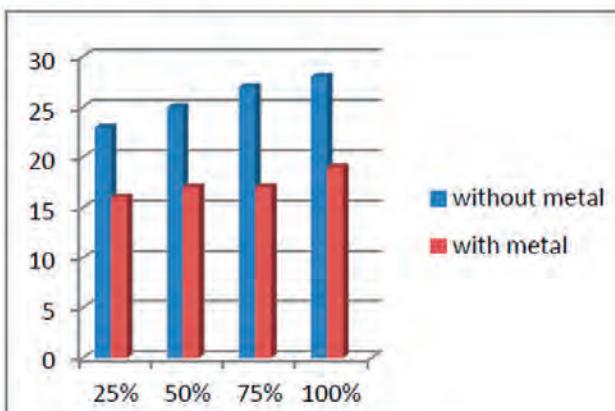
Aspergillus sp. E30 with nystatin



Fungal sp. SANA-4 with nystatin



Fungal sp. SANA-5 with nystatin



Aspergillus Niveus with nystatin

Figure 2. Diameter of inhibition when cultivating fungal species with antifungal.

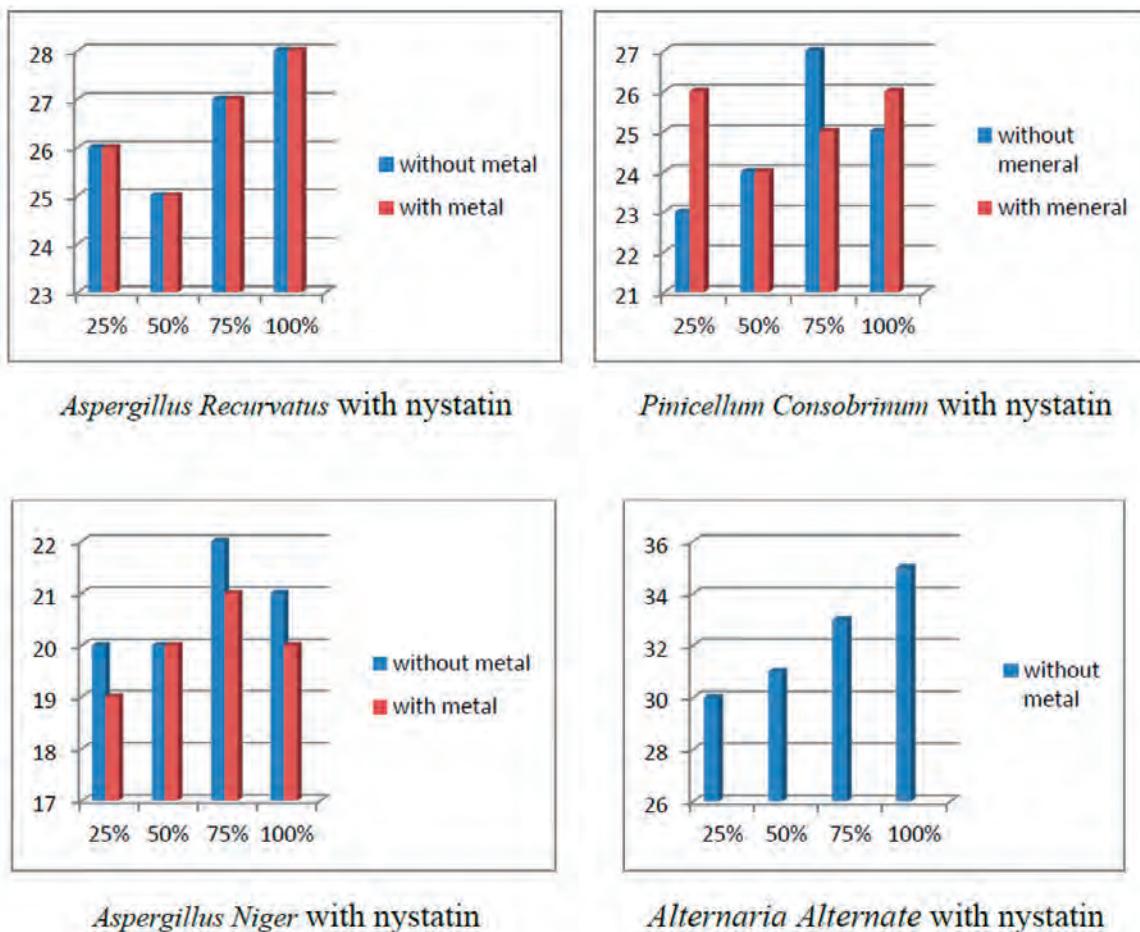


Figure 2. Diameter of inhibition when cultivating fungal species with antifungal.

Antibiotics			Diameter of inhibition zone of bacterial species			
Antibiotic	code	concentration	<i>Staphylococcus</i> sp	<i>Bacillus</i> sp.sanna2	<i>Bacillus</i> sp.sanna1	<i>Bacillus</i> cereus
Chloramphenicol	C	10 mcg	22	16	25	18
Ciprofloxacin	CIP	10 mcg	35	22	32	20
Amikacin	AK	10 mcg	14	18	16	20
Tetracycline	TE	10 mcg	X	6	14	15
Gentamicin	CN	10 mcg	18	18	22	20
Azithromycin	AZM	15 mcg	X	22	19	15
Streptomycin	S	25 mcg	18	20	30	25
Amoxicillin	AX	25 mcg	11	X	X	X
Metronidazole	MET	30 mcg	X	X	X	X
Vancomycin	VA	30 mcg	20	10	22	16

Table 5. Diameter of inhibition in mm of antibiotic tablets with bacteria. The dark orange color indicates the resistance of bacteria to the antibiotic, while the light pink color indicates medium resistance, and the white color indicates that the bacteria are sensitive to these antibiotics

tance of this organism to antibiotics, as heavy metals are an essential factor in making organisms resistant to antibiotics. The outcomes of this study will significantly contribute to the current body of knowledge of research towards public and veterinary health, especially in developing countries, and highlight the importance of fulfilling one-health attitudes to lower the ongoing spread of antimicrobial resistance.

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RESEARCH / INVESTIGACIÓN

Molasses as a new nutrition medium for *Scenedsmus quadricauda* growth and production of some bio compounds

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2202

Abstract: Algae comprise a large group of Thallophyta, which may be used as direct nutrition of human beings. Molasses is the by-product of the sugar manufacturing facility. In this study, a locally isolated *Scenedsmus quadricauda* from the environment of Mosul in the Shalalat region was obtained. Biomass of *Scenedsmus* was measurement by carried out and filtration then drying in an oven for 24 h and weighed. Estimation of chlorophyll and protein and carbohydrate content of *Scenedsmus*. The research has proved that the best growing period for *Scenedsmus quadricauda* is 15 days when using sugar factory waste as a carbon source, the growth reached (1.42 nm) as optical density, biomass (1525 mg /L), chlorophyll (green), pigment (18 mg /l) protein content (396 mg /l) and carbohydrates (501 mg / l). The research showed that the use of sugar factory waste as a nutritional medium for algal growth in the dark (11.5%) achieved good growth of *Scenedsmus quadricauda* (0.632 nm), biomass (820 mg / L), green pigment (Chlorophyll) (18 mg /L) protein content (235 mg / L) and carbohydrates (401 mg/L). while using phosphor (0.018%) of K₂HPO₄ in dark medium achieved highest growth rate (0.91 nm) , biomass (1110 mg / L) chlorophyll (22 mg/L) protein (301mg/L) and carbohydrate (461 mg/L) . It is noted too , that using IAA (0.5 g/L) in dark medium support best growth (0.888 nm) , biomass (1010 mg/L) chlorophyll (25 mg/L) , protein (230mg/L) and carbohydrate (440 mg//L) . The study showed that thiamine (1 g/L) in dark medium achieved highest growth (0.750 nm) biomass (218 mg/L), chlorophyll (29mg/L), protein (220 mg/L), carbohydrate (340mg/L). Therefore, using Molasses can enhance the growth, biomass, chlorophyll, protein, and carbohydrate content in the *S. quadricauda*.

Key words: *Scenedsmus quadricauda*, Chlorophyll contents, Molasses, Biomass.

Introduction

Algae can grow in different aquatic environments, whether fresh or salty water. These organisms are used as food for human beings to fulfill the increasing needs of nutrition due to the increase of population and malnutrition facing people in some regions of the world¹.

Also, many studies were carried out on the possibility of using algae for the natural environment to eliminate waste from factories, health centers, educational institutions. Algae can exploit the various wastes produced from the above sources and benefit from them in producing abundant algal growth, which is used as animal feed. Thus achieving two important objectives, ridding the local environment of the danger of industrial and productive pollutants, as well as exploiting these wastes as they are cheap basic materials to produce abundant algal growth^{2,3}.

Several research and references have indicated the possibility of switching algae from Autotrophic to Heterotrophic^{4,5}, indicated that some green algae could grow in heterotrophic medium, and (6), indicated that algae could exploit organic wastes as a medium for their growth. Molasses is a by-product of sugar manufacturing processes^{4,7}.

The current study aims to isolate and cultivate a local strain of green algae *S. quadricauda*. And its ability to exploit the wastes of the sugar manufacturing factory as a nutrition medium for growth.

Materials and methods

Isolation and preservation of algae

In this study, an isolate of *S. quadricauda* was obtained from the Iraqi environment (Shalalat region). *S. quadricauda*

was cultured in modified Ch10 medium⁸, that consists of the following components: 0.4 gm/L Ca (NO₃)₂, 0.1 gm/L K₂HPO₄, 0.2 gm/L Na₂CO₃, 0.25 gm/L MgSO₄·7H₂O, 0. 25 gm/L Na₂SiO₃, and 0.05 gm/L Ammonium Ferric-- figure (1,2).

The daily growth rate was measured using a spectrophotometer at (436 nm) wavelength, and the biomass was weighed for volume (100mL) of the culture⁹. The chlorophyll content, protein, and carbohydrates of alga were measured according to standard methods using spectrophotometry and specific wavelengths as described in the approved^{9,10}.



Figure 1. *S. quadricauda* microscopic picture magnification 40 X.

Results and discussion

The effect of growth periods in sugar factory wastes on the vitality of cells *Scenedsmus*. After seventeen days of cultivation (figure3), the best growth (1.45 nm) as optical densi-



Figure 2. Pure cultures of algae *S. quadricauda*.

ty, biomass (1525 mg/L), green pigment (18 mg /L), protein content (369 mg/L), and carbohydrate content (501 mg/L) of the alga were reported on the fifteenth day of transplantation. Perez *et al.*(2010) noticed that the fifteen-day was the best period to obtain high growth and yield from the cells of some green algae and indicated the direct relationship between cell growth and vital components; this was confirmed by (11) while studying the photosynthesis of some algae. This is also shown by (12) since they noticed that number of cells correlates positively with the growth value of *S. obliquus*.

The effect of different concentrations of sugar plant wastes on vitality *S. quadricuda* alga.

Algae depend on light in the manufacture of their food, and their conversion to feed throwing into the dark depends on the presence of carbohydrate sources^{11,12}. The results (figure 4) showed that the best growth of alga was achieved (1.601 nm) and the best biomass weight (890 mg/L) when using molasses at the concentration (11.5). The results showed that the best green pigment content was (18 mg/L), protein content (235 mg/L), and carbohydrate content (401 mg/L) when using concentrate (9.0%) from sugar production plant wastes (molasses). Shah (2012) recorded the best growth of *Chlorella* sp. achieved when using monosaccharides with (0.25 %) under dark conditions, whereas 13 also showed the ability of microalga *S. obliqua* to exploit sources of sugar such as glucose and acetate for heterogeneous nutrition. However, (14) explains that using common cultivation conditions (light and dark) and in the presence of a carbon source stimulates algae *Scenedesmus* to produce high carbohydrates and protein.

Effect of adding different concentrations of K₃HPO₄ on the vitality of alga *S. quadricuda*, which grows on sugar production plant wastes medium.

The results (figure 5) indicated that the best growth of alga *S. quadricauda* was reached (0.98 nm), biomass weight (1220 mg/L), green pigment (22 mg/L), protein content (299 mg/L), and carbohydrate content (461 mg/L) when using a substance potassium thiophosphate at a concentration (0.018 %). The positive effect of phosphorous on algae growth indicates the significant role of this element in building many cellular organelles and biomolecules¹⁵. These came together with biomass results, chlorophyll, protein, and carbohydrate content¹⁶⁻¹⁹.

Test the addition of different concentrations of IAA in molasses medium on the vitality of alga *S. quadricauda*

IAA is a natural plant hormone that can be synthesized

and has a vital role in the vitality and growth of plants in general, and many references indicated the ability of microalgae and cyanobacteria to produce IAA²⁰.

The results showed (figure 6) that the best values were obtained for the growth of alga *S. quadricauda* (0.891 nm), the biomass (1022 mg/L), the best photosynthetic pigment (25 mg/L), protein content (230 mg/L), and carbohydrate content (440 mg/L) when adding IAA (0.5 %) prove²¹, The *Scenedsmus* sp. and other microalgae is growth highest with the addition of IAA to the nutrient medium. Many studies have confirmed the relationship of IAA in stimulating algae growth, but the high concentration of IAA has an opposite focus on development and effectiveness²². A high concentration of IAA inhibits building up the photosynthetic pigment, protein, and carbohydrate content of algae *Chlorella*²³.

The effect of adding different concentrations of thiamine on the vitality of *S. quadricauda* in the dark.

Vitamins are essential in the growth and activity of algae in general; many studies indicate the importance of adding some vitamins in specific concentrations in algae development media to support their growth and biomass production^{24,25}.

The results indicated (figure 7) the best growth of *S. quadricauda* (0.766 nm), the best value for biomass (218 mg/L), the best content of the green pigment (28 mg/L), the best protein (220 mg/L), and carbohydrate content (340 mg/L) where achieved in (0.01) thiamine concentration. (24) confirm the importance of adding thiamine in the medium microalgae growth. (25) indicated that a minimum of thiamin, ascorbic acid (40 – 80 mg/L) have an essential role in increasing the content of the photosynthetic pigment, protein, and carbohydrate content. Vitamin thiamine has a strong position and relationship in building amino acids¹⁰.

Conclusions

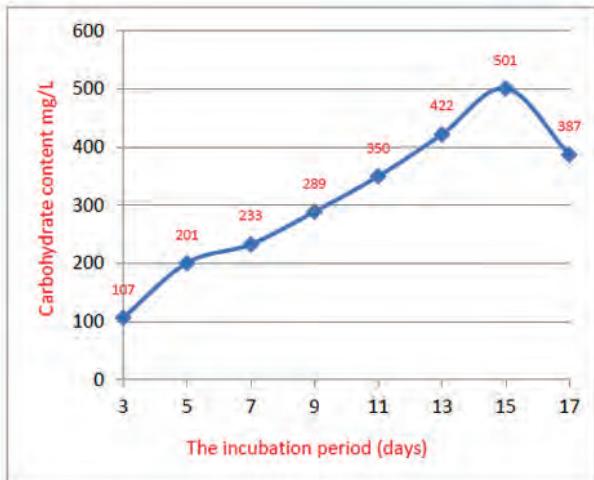
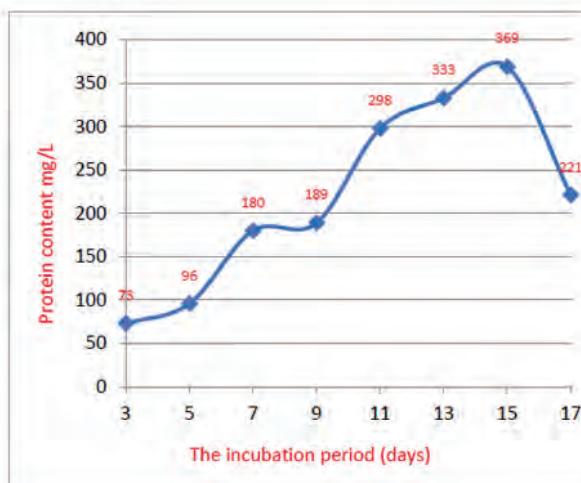
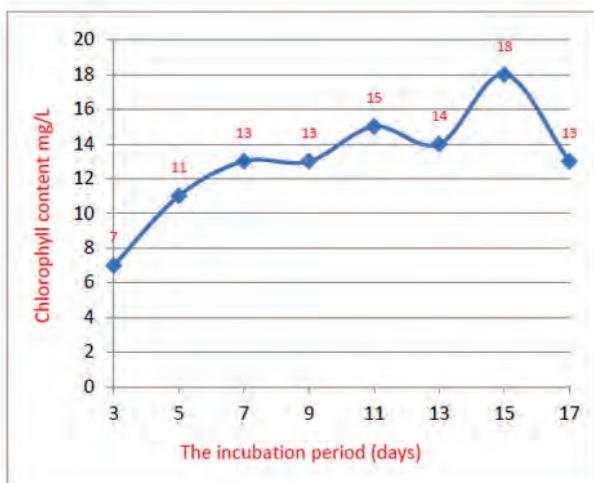
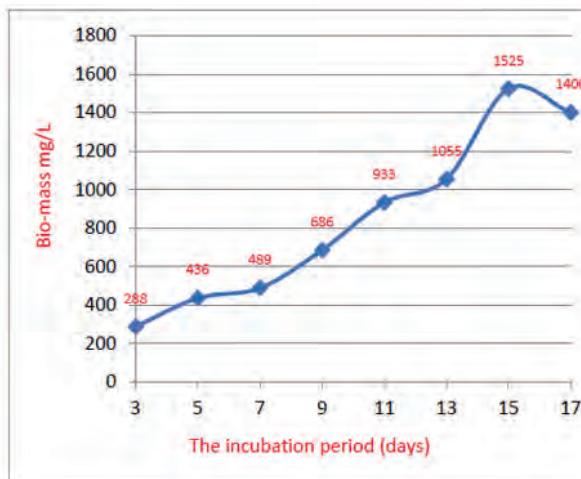
The study proved that the local isolate of *S. quadricauda* alga could exploit the industrial wastes of the sugar production plant (molasses) to grow and produce biomass and thus rid the environment of industrial pollution with these wastes.

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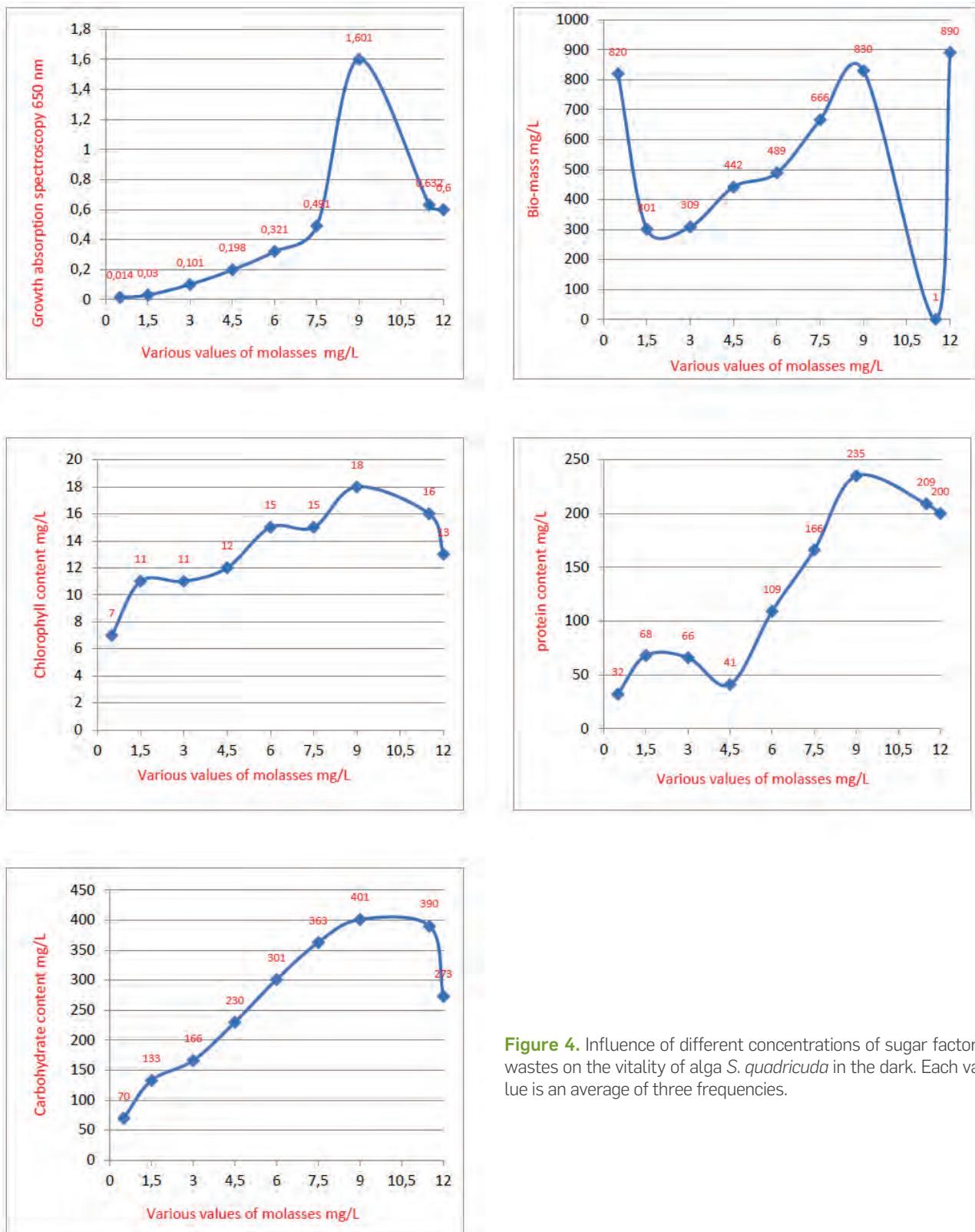


Figure 4. Influence of different concentrations of sugar factory wastes on the vitality of alga *S. quadricauda* in the dark. Each value is an average of three frequencies.

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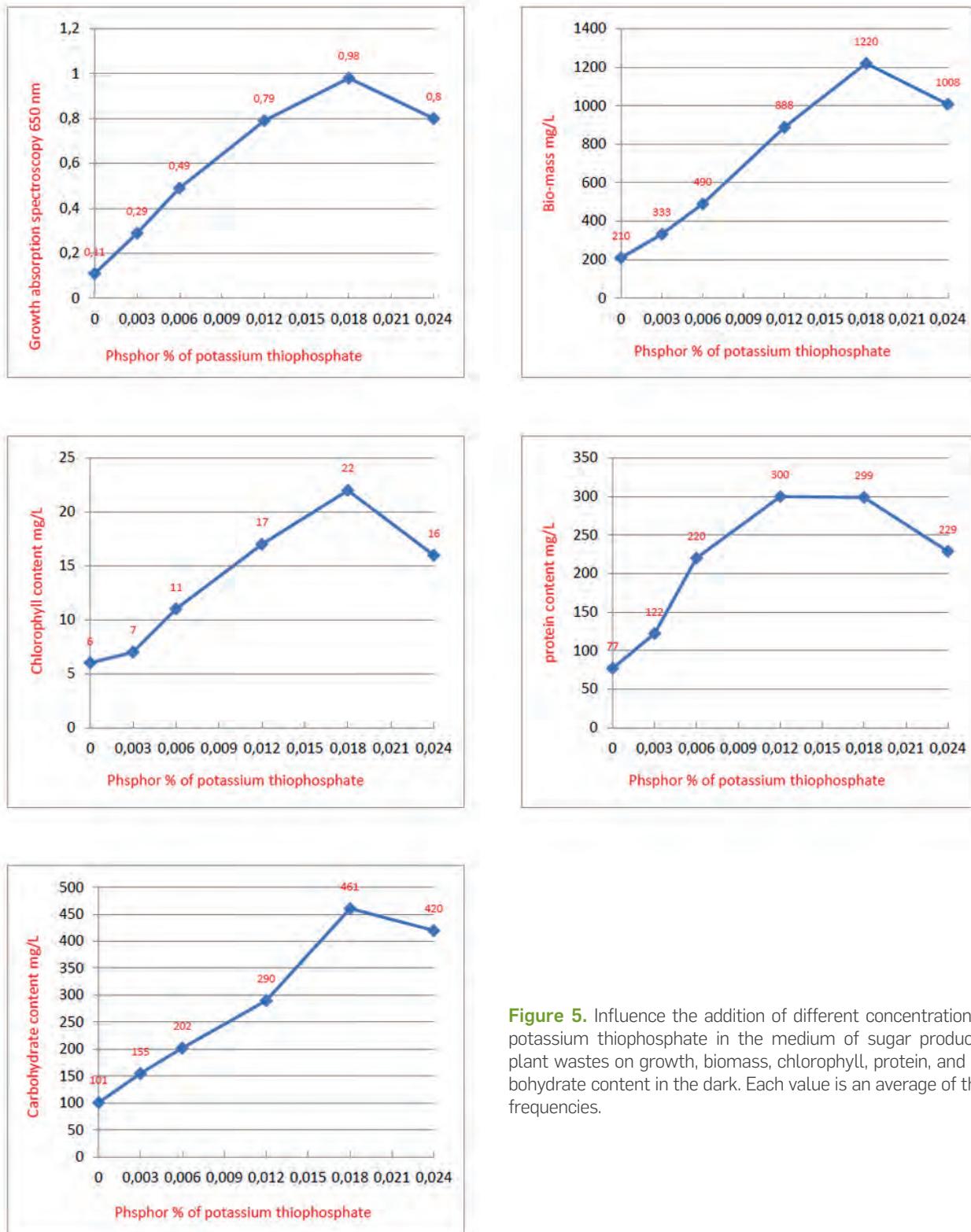


Figure 5. Influence the addition of different concentrations of potassium thiophosphate in the medium of sugar production plant wastes on growth, biomass, chlorophyll, protein, and carbohydrate content in the dark. Each value is an average of three frequencies.

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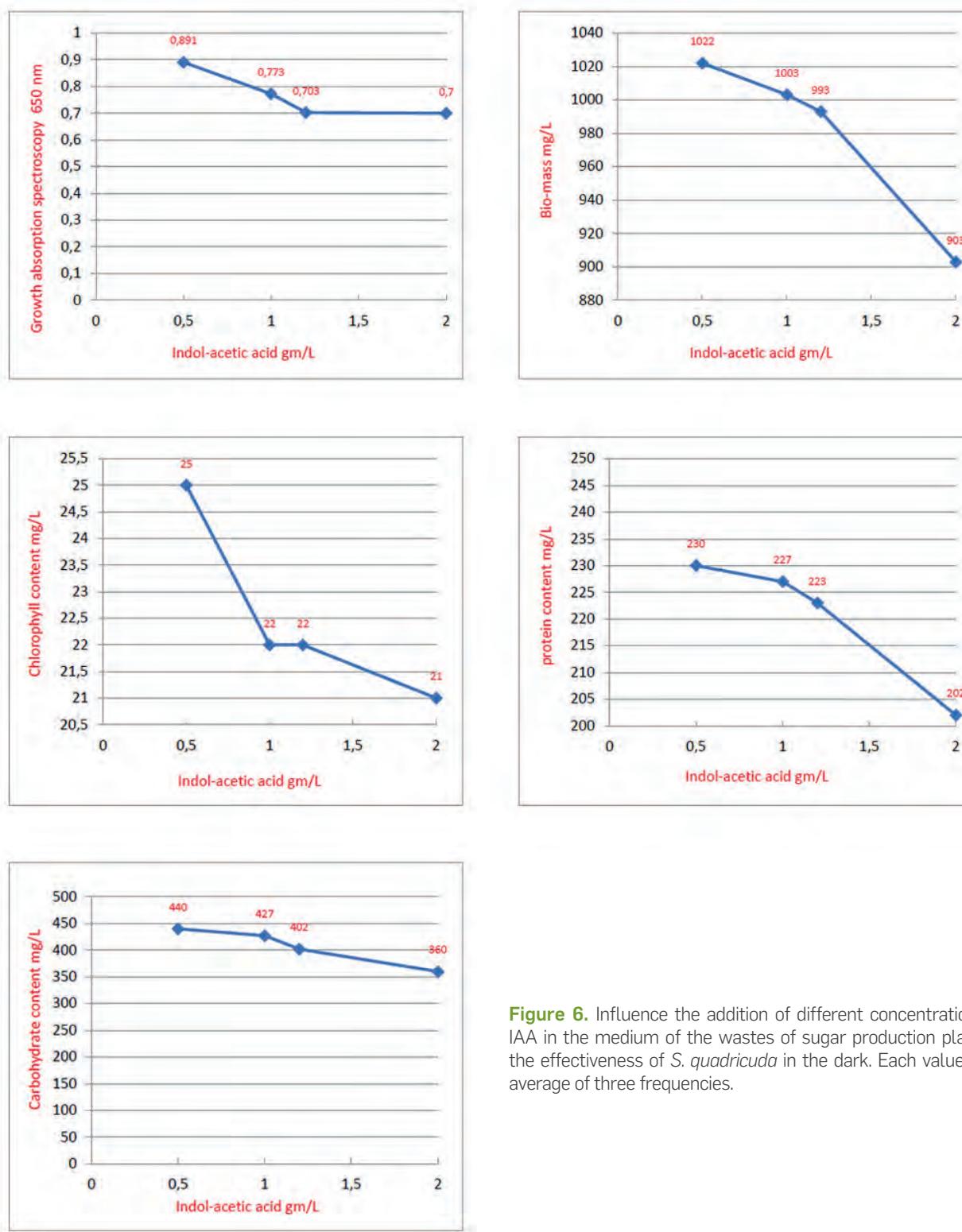


Figure 6. Influence the addition of different concentrations of IAA in the medium of the wastes of sugar production plant on the effectiveness of *S. quadricuda* in the dark. Each value is an average of three frequencies.

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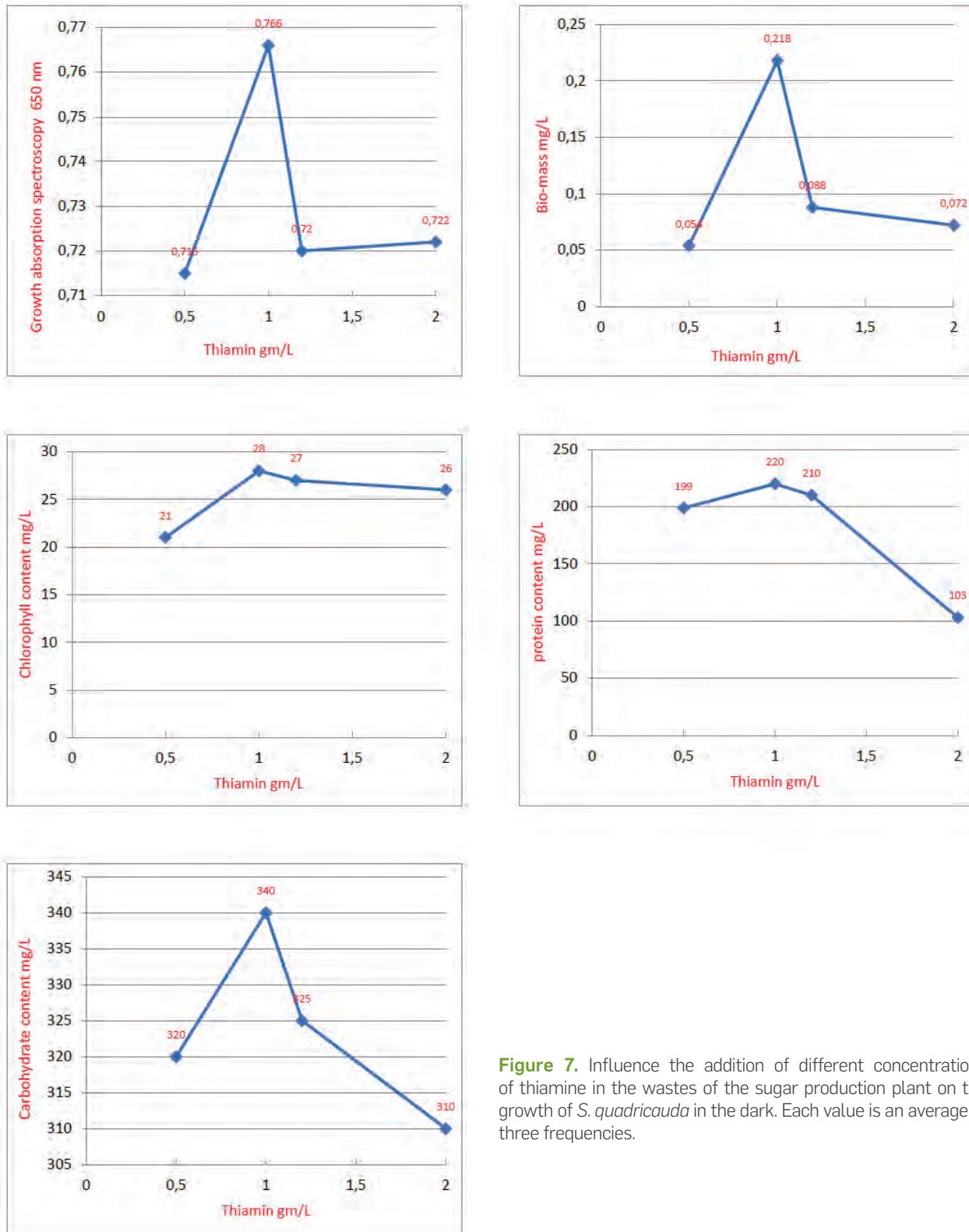


Figure 7. Influence the addition of different concentrations of thiamine in the wastes of the sugar production plant on the growth of *S. quadricauda* in the dark. Each value is an average of three frequencies.

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RESEARCH / INVESTIGACIÓN

Characteristics and quality of gray water and the possibility of reuse for irrigation purposes from the houses of some areas of the left side of the city of Mosul

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Abstract: The research dealt with assessing the quality of gray water generated from houses in some areas of the left side of the city of Mosul and determining the suitability of reusing it for irrigation; for this purpose, 90 samples of gray water were collected from fifteen neighborhoods of the left side for a period of six months during the period from November 2020 to April 2021. The research aims to conduct a monthly study to assess the quality and suitability of gray water for irrigation by conducting physical, chemical, and biological tests for gray water generated from houses Where the values of E.C. ranged (751-1621) $\mu\text{S}/\text{cm}$ and T.D.S. (483-684) mg/L, and pH ranged (6.3-8.2), and the concentration of sodium ions ranged between (7.8-147) mg/L as for the concentration of C.O.D. (Chemical Oxygen Demand) ranged between (69-200) mg/L While the C.O.D. values ranged between (69-200) mg/L, The S.A.R. values were between (0.18-2.98) meq/L, and the phosphate ions values ranged between (0.25-3.7) mg/L; finally, the average of fecal coliform bacteria was $(0.0-4) \times 10^5/100 \text{ ml}$. The study concluded that the water was classified under the category (C3-S1) high salinity - low sodium, which is suitable for irrigation of plants that are well tolerant to salinity and is suitable for use in soils that do not contain complex layers that prevent leaching because these soils will need to be washed when irrigated with this water. This study recommended diluting gray water by mixing it with fresh water and using it for irrigation.

Key words: Characteristics, Greywater, Reuse, irrigation, S.A.R. (Sodium adsorption ratio), C.O.D.

Introduction

Iraq has suffered from water scarcity in recent years, and this problem has steadily been exacerbated with the increase in the population. The per capita water supply will decrease annually unless alternative water sources are provided. This, in turn, pushes decision-makers and technicians to search for unconventional water sources, including gray water and treated wastewater for irrigation in environmentally safe ways, and the problems of the agricultural sector under the current water shortage conditions are a difficult task for decision-makers in Iraq due to the low share of the agricultural sector of water and the increased demand for water for other sectors. Therefore, it is necessary to reuse all the available water resources, including low-quality water sources, to face these problems¹.

Contaminated water sources such as gray water and treated wastewater are used for irrigation purposes in the dry and semi-arid countries of the region because of the lack of water in them, and their use reduces the demand for freshwater sources. The use of this type of water is either directly, that is, by adding this water to the soil directly, provided that not to be stored for a long time before use or indirectly by being thrown into rivers and streams, gray water is an essential source of water that can be used to irrigate crops and trees. It is divided into two types: the first is called (Blackwater); it includes water that is discharged from the toilet; this type of water contains a large number of pathogens. It also contains a high concentration of organic matter, in addition to containing nitrogen and phosphorous².

The second type is called (Gray water); it's the water collected from sewage discharge of cloth washers, showers, bathtubs, and sinks; it is called gray water because it is left for an extended period, its color will change to gray^{3,4}.

Gray water flowing from showers, washing machines, and bathroom sinks represents more than 60% of the total domes-

tic wastewater, indicating that gray water is an attractive source for reuse for irrigation purposes if it is properly exploited and used on correct environmental bases⁵.

Materials and methods

Study Area

The study area included several neighborhoods on the left side of the city of Mosul, and its included fifteen neighborhoods on the left side, which are:(Al-Muhandiseen, Al-Hadbaa, AL-Ziraal, Al-Mazarie, AL-Qadisyah 1, Al-Kafaat 1, AL-Shurta, AL-Zuhour, AL-Muthanna, Al-Noor, Al Baladyat, AL-Qahira, AL-Zahraa, Sumer, Aden). These areas are highly populated. The coordinates of the studied areas were fixed using the G.P.S. of the google earth program, as shown in table (1).

Sample collection

Water samples were collected from fifteen neighborhoods of Mosul from the left side of the city in the study area from November (2020) to April (2021) per month. The greywater samples were collected from the gray water drainage point of each house to conduct physical and chemical tests by using sterile polyethylene bottles with a capacity of 250 ml, and samples for bacterial tests were collected using sterilized bottles with sterilizers at a pressure of 1.5 pounds and degrees (121) °C for 15 minutes. The samples were kept in a refrigerated container away from light until they reached the laboratory⁶.

Field tests

It included measuring temperature using an alcohol ther-

Areas and locations		Coordinates	
		N	E
Al-Muhandiseen	1	36°22'05.6"N	43°08'08.9"E
Al-Hadbaa	2	36°23'42.8"N	43°09'10.7"E
AL-ZiraaI	3	36°21'16.6"N	43°08'37.7"E
Al-Mazarie	4	36°19'39.3"N	43°10'34.9"E
AL-Qadisiyah 1	5	36°22'32.9"N	43°11'25.1"E
Al-Kafaat 1	6	36°22'52.2"N	43°09'20.4"E
AL-Shurta	7	36°22'36.0"N	43°08'14.2"E
AL-Zuhour	8	36°22'54.5"N	43°11'11.7"E
AL-Muthanna	9	36°22'15.8"N	43°10'28.6"E
Al-Noor	10	36°21'58.0"N	43°11'06.3"E
Al Baladyat	11	36°23'07.4"N	43°09'44.5"E
AL-Qahira	12	36°23'50.9"N	43°11'29.5"E
AL-Zahraa	13	36°23'05.1"N	43°12'28.9"E
Sumer	14	36°18'00.9"N	43°12'17.9"E
Aden	15	36°21'07.0"N	43°12'46.7"E

mometer, measured in degrees Celsius, measuring total dissolved solids by T.D.S meter in mg/L, electrical conductivity in $\mu\text{S}/\text{cm}$ by using E.C. meter, and measuring the acidity function using a pH meter.

Laboratory tests

It included measurement of the chemical oxygen demand (C.O.D.) by Closed Reflux method, determination of sodium ion using the Flame photometer, determination of Phosphate ion by Stannous Chloride method, counting the number of fecal coliform bacteria by (Multiple Tube Method) and Most Probable Number method⁶ and finally calculation of the sodium adsorption ratio (S.A.R.). The chemical tests were conducted at the College of Environmental Engineering, while the biological tests were conducted at the College of Environmental Sciences and Technologies at the University of Mosul.

Results

The components of gray water depend mainly on the quality of the available water sources and family habits when cleaning and preparing food⁷.

Physical Characteristics

Temperature

Temperature is one of the critical environmental factors affecting the qualitative characteristics of water, as it affects water's chemical and physical reactions⁸. Table (2) and Figure (1) show that the temperature of the studied water samples fluctuated during the study period. The temperature range (15-32.3)°C and averaged (20-23.8)°C the highest temperature was in the site (8) in November 2020, and the lowest was in the site (5) in January 2021. This difference is attributed to the use of warm water when preparing food and for cleaning⁹.

Electrical Conductivity and Total Dissolved Solids (EC&T.D.S)

Electrical conductivity is a measure of the concentration of dissolved anions and cations. Electrical conductivity is used as an indicator of the water content of dissolved solids of salts and minerals. It can contain low concentrations of dissolved organic materials in water. The total dissolved solids are used as a primary indicator of water quality measurement^{10,11}.

The results are shown in table (2) and Figure (2) indicate

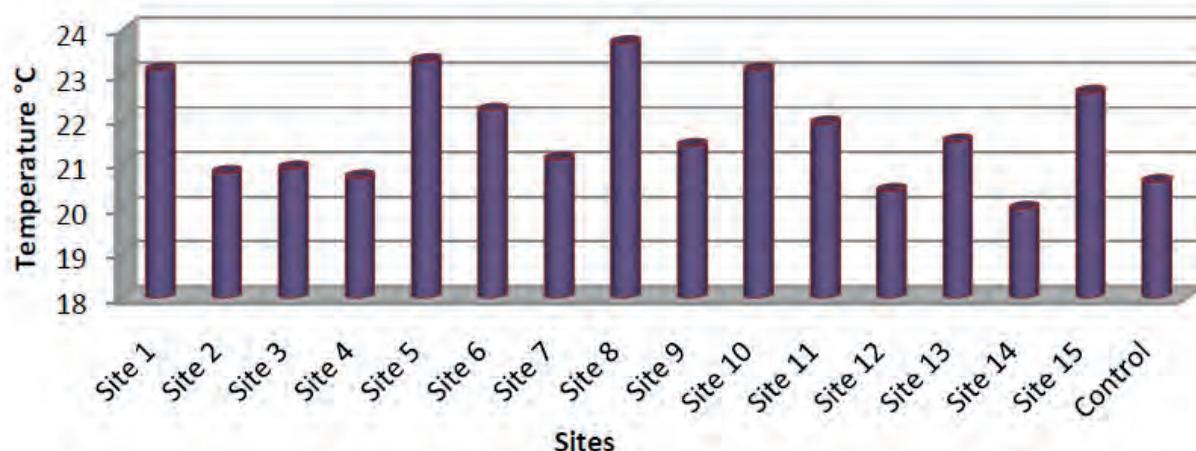


Figure 1. Shows the average temperatures of the study areas.

Table 1. The names of the areas and their locations.

Parameter Sites	Temp °C .	pH	E C μS/cm	T.D.S. mg/L	Na ⁺² mg/L	C.O.D. mg/L	PO ₄ ⁻³ mg/L	S.A.R. meq/L	Fecal coliform x10 ⁵ /100ml
Control	20.6	7.3	499	247	6	4.7	0.09	0.2	Nill
Site 1	23.1	7.4	1083	478	49	97	1.1	1.2	Nill
Site 2	20.8	7.2	1038	427	38	97	0.7	1	Nill
Site 3	20.9	7.4	986	452	44	92	1.4	1.1	1
Site 4	20.7	7.2	981	442	36	95	0.5	0.9	0.5
Site 5	23.3	7.5	106	512	78	105	0.8	1.8	0.7
Site 6	22.2	7.2	999	468	83	115	1.4	1.9	Nill
Site 7	21.1	7.3	944	436	34	117	0.8	0.8	0.5
Site 8	23.7	7.3	922	430	35	90	2	0.8	1.5
Site 9	21.4	6.8	927	430	32	85	0.9	0.8	1.5
Site 10	23.1	7.5	883	421	49	94	1.1	1.2	1
Site 11	21.9	7.6	965	452	36	99	1	0.8	Nill
Site 12	20.4	7.3	996	448	40	93	0.6	1	1
Site 13	21.5	7.5	917	425	67	98	1	1.7	0.7
Site 14	20	7.1	962	432	41	86	1.6	1.1	1
Site 15	22.6	7.4	994	470	32	121	0.8	0.8	1

Table 2. Physical and chemical, and biological characteristics of Mosul city greywater.

that there is a significant fluctuation in the electrical conductivity values, and the values ranged between (751-1621) μS/cm and averaged (883-1083) μS/cm the lowest value was in site (10) In November 2020 and the highest value was in site (2) in January 2021. The T.D.S. values shown in table (2) its ranged between (483-684) mg/l and averaged (421-512) mg/l where the highest value was in site (1) in November 2020 and the lowest value was in site (10) in December 2020 This increase is due to the presence of salts that are resulting from detergents such as sodium chloride and nitrates and phosphates, and the old water pipes contribute to increasing the electrical conductivity due to pipes rusting and then these materials leaching to the gray water^{12,13}.

The Gray water in this study was classified according to the average of electrical conductivity values within the category of highly saline water (C₃) according to the classification of the American Salinity Laboratory.

Chemical Characteristics

pH

The pH function expresses the activity and concentration of the hydrogen ion in water, the pH in the gray water depends mainly on the pH and alkaline of the water supply sources. The pH of gray water is directly related to certain chemicals such as softeners, bleaching agents, and disinfectants¹¹. The results shown in table (2) and Figure (3) showed that the pH values ranged between (6.3-8.2) and averaged (6.88-7.62), the highest value of the pH was in December 2020, January, February, and April 2021 in each of the site (3,4,7,10,11) while the lowest value was in March 2021 in site (4) and the pH of tap water was (7.30). Most of the samples tended to be alkaline this is due to the presence of alkaline substances used in detergents, this is due to the presence of alkaline substances used in detergents such as sodium hydroxide-based, where it was found that the main chemical components present in gray water are generated due to the use of these detergents in cleaning or washing activities. Detergents consist of surfactant

active substances that act as the primary active agent in most cleaning products¹⁴. in general, the values of the pH function for all areas were within the limits of international standards allowed for irrigation.

Sodium ions

The results are shown in table (2), and Figure (4) indicate that the concentrations of sodium ions ranged between (7.8-147) mg/l at an average of (32.1-83.5) mg/l. The highest value was in site(13) in November 2020 while The lowest value was in site (15) in February 2021, This rise is attributed to the fact that soap and detergents contain sodium salts.¹⁵ and the average values of sodium in tap water were (6.5) mg/l.

Phosphate ion

The results of the current study showed that phosphate concentrations ranged between (0.25-3.7) mg/L with an average of (0.5-2.1) mg/L, where the highest value was in site (14) in November 2020, while the lowest value was in site (11) in January 2021, The reason for these high values is due to the use of detergent products containing a high percentage of phosphates¹⁵.

Chemical Oxygen Demand (C.O.D.)

The results of the study shown in table (2) and Figure (6) indicated that the C.O.D. concentrations were high, ranging between (69-200) mg/L and an average of (82.5-121.3) mg/L. This rise is due to the presence of Biodegradable and non-biodegradable organic matter and detergents. Produced from washing powders and dishwashing liquids, in addition to the presence of anionic surfactants and other oil substances in gray water samples, which increase the concentration of COD¹⁶.

Sodium adsorption ratio (S.A.R.)

The sodium adsorption ratio represents the inverse relationship between sodium ions and calcium and magnesium

ions and the degree of the tendency of irrigation water to enter into cation exchange reactions in the soil¹⁷⁻¹⁹.

The results shown in table (2) and Figure (7) showed that the values ranged between (0.18-2.98) meq/L, where the lowest value was in (15) in February 2021, while the highest value was in (10) during November 2020. The reason for the decrease is due to the high concentrations of calcium and magnesium ions compared to sodium ions²⁰, and when comparing the results with the certified International Classifications for Irrigation The (S.A.R.) values of gray water for this study were within the S1 category, meaning that the water is of good quality for irrigation and there is no problem to affect the permeability and filtration²¹.

Biological Characteristics

Fecal Coliform Bacteria (FC)

It is a branched group of Total Coliform bacteria that is naturally present in huge numbers in the intestines of humans and warm-blooded animals, and the presence of these bacteria in the water is evidence of water contamination with feces^{22,23}.

The results are shown in table (2) and Figure (9) indica-

te that the number of fecal coliform bacteria ranged between $(0.0-4) \times 10^5/100$ ml, where the highest value was in site (13) and (5) in December 2020 and February 2021 respectively when the lowest value was in most of the gray water samples in the studied areas. The contamination of some water samples in this study is usually attributed to largely associated with poor personal hygiene and disposal of grey water that contains washed nappies or as a result of washing shoes, or due to the presence of children under the age of 12 years in the house, or it may result from washing the raw meat^{24,25}.

Conclusions

There is an increase in some studied parameters such as E.C., T.D.S., C.O.D., and sodium ions. Gray water in this study was classified according to the E.C. values within the category of high salinity water (C_3) according to the classification of the American Salinity Laboratory, where it is recommended to use it for irrigation of crops that tolerate high salinity. The S.A.R. values for all studied samples were within the appropriate limits for irrigation and were classified within the S1 category, meaning that the water has a good quality for irrigation.

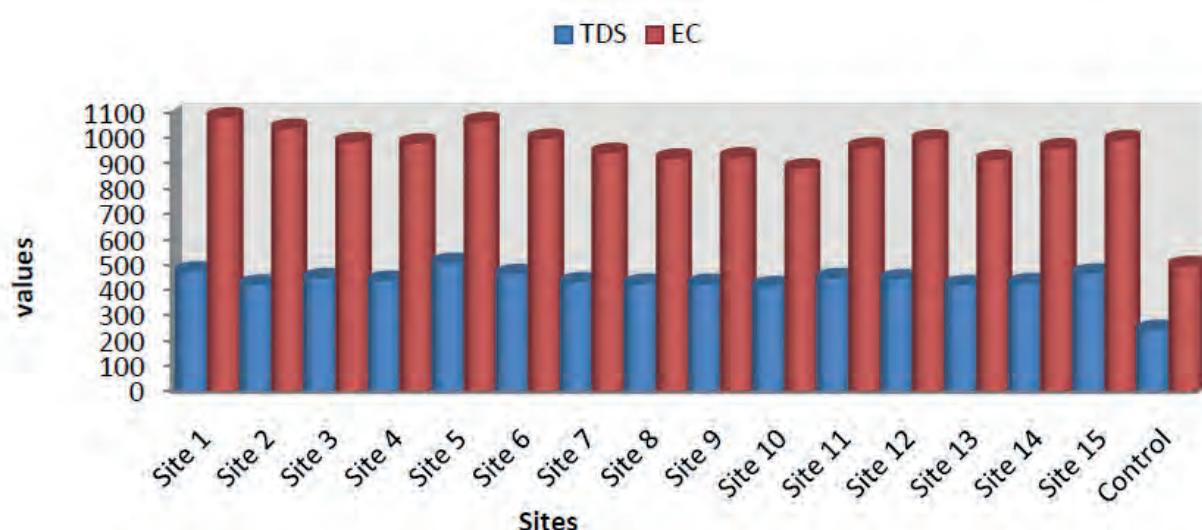


Figure 2. Average values of electrical conductivity and total dissolved solids concentration.



Figure 3. Shows the average values of the pH in the studied areas.

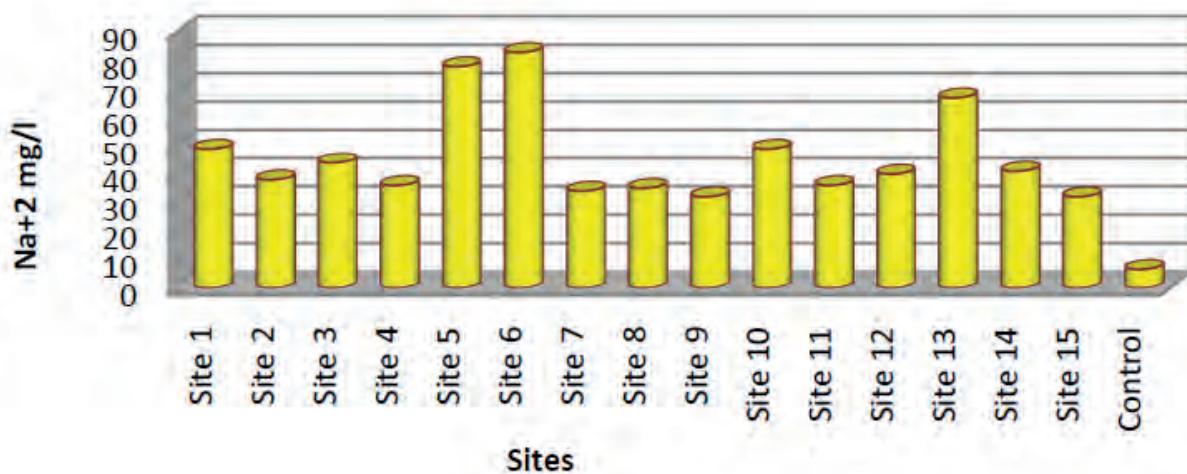


Figure 4. Shows the average concentration of sodium ions of samples.



Figure 5. Shows the average concentration of phosphate ions in the samples.

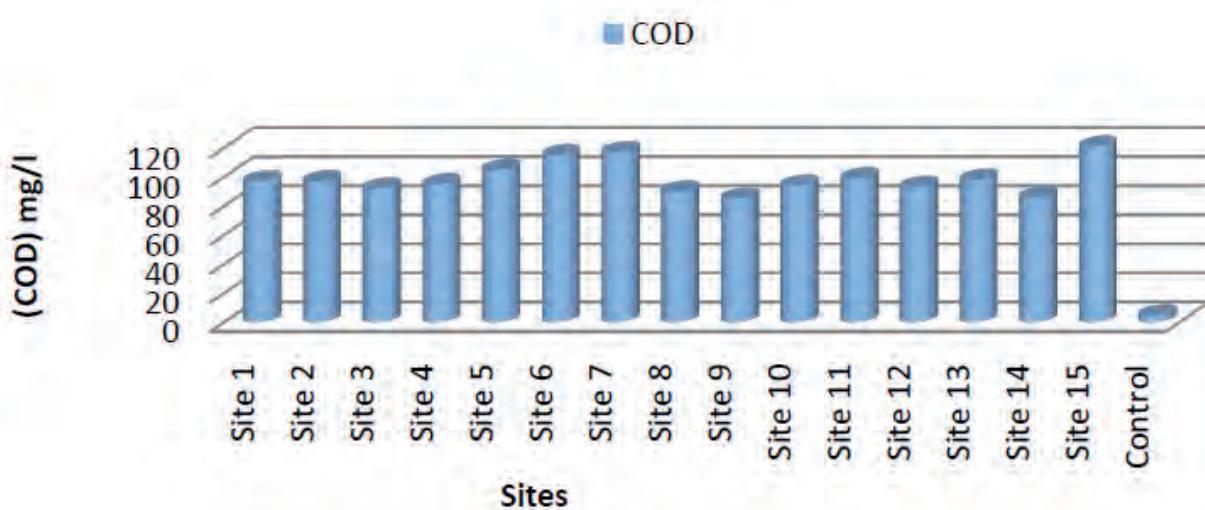


Figure 6. Shows the average C.O.D. concentration in the samples.

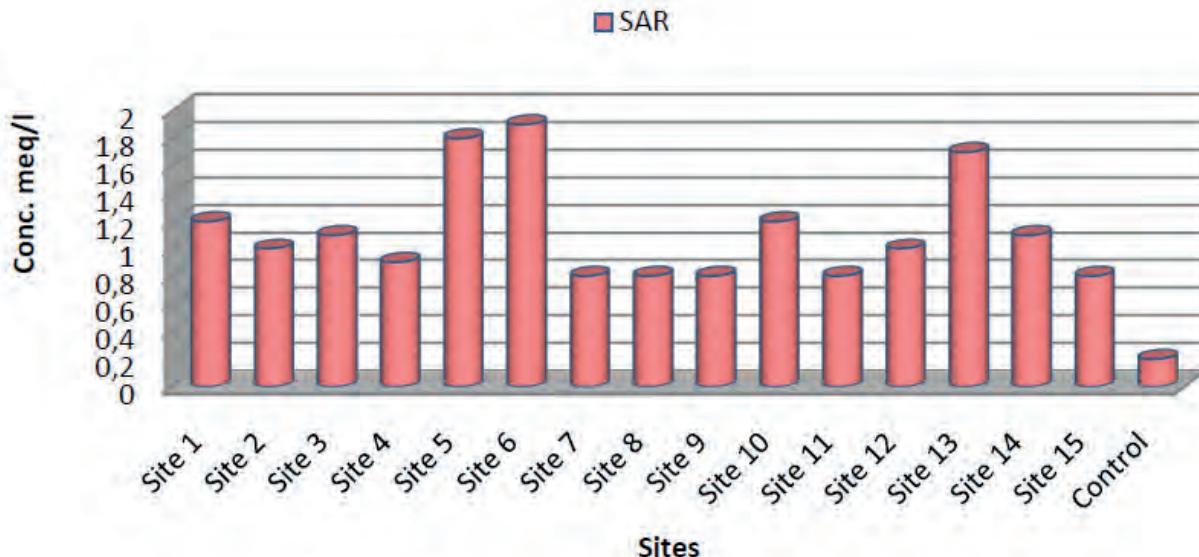


Figure 7. The average of S.A.R. for the water of the studied areas.

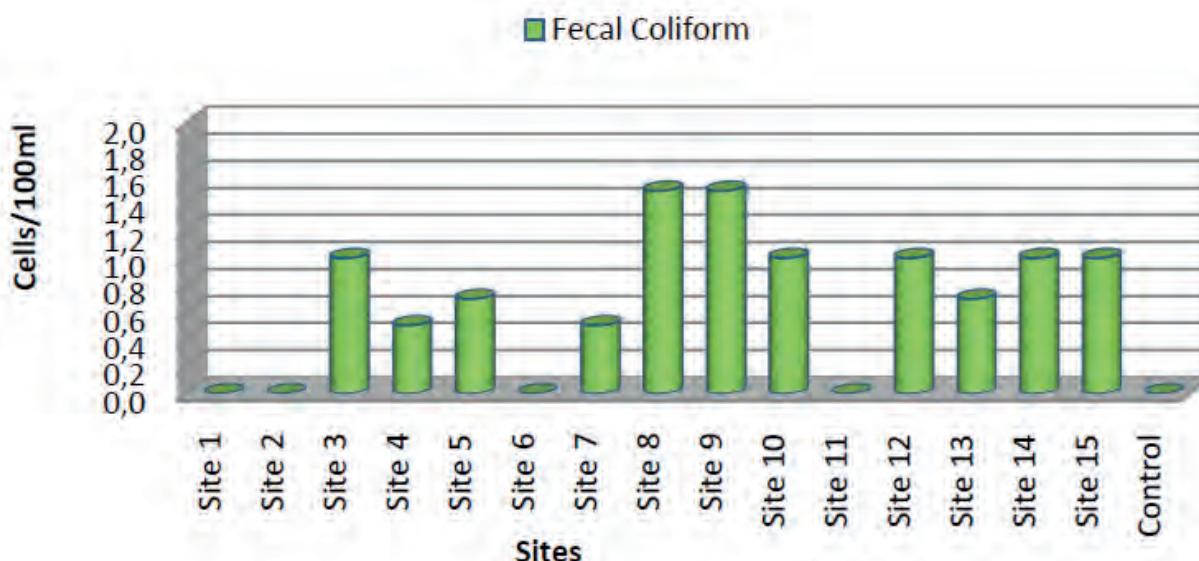


Figure 8. Shows the average number of fecal coliform bacteria in the cells of the sample $\times 10^5/100 \text{ ml}$.

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RESEARCH / INVESTIGACIÓN

Micropagación *in vitro* de naranja agria (*Citrus aurantium* L.) a partir de segmentos nodales

In vitro micropagation of sour orange (*Citrus aurantium* L.) from nodal segments

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Resumen: La naranja agria (*Citrus aurantium* L.) presenta un alto valor nutricional y gastronómico en el distrito de Huacho, Lima, Perú, pero en la actualidad se considera una especie en peligro de desaparecer del distrito y alrededores por problemas fitosanitarios. Para la recuperación y repoblación de esta especie se planteó emplear técnicas biotecnológicas para la obtención de plantas libres de patógenos. Por lo tanto, el objetivo de la presente investigación fue micropropagar *in vitro* naranja agria a partir de segmentos nodales. Los segmentos nodales de naranja agria fueron desinfectados en diferentes concentraciones de NaClO, luego se introdujeron en medio de cultivo MS (Murashige y Skoog) adicionado con BAP, KIN y AG3 para la fase de multiplicación, posteriormente se transfirieron a medios MS adicionado con IBA y ANA para la fase de enraizamiento. La evaluación del porcentaje de contaminación se realizó a los diez días, la evaluación de formación de brotes en fase de multiplicación se realizó a los 30 días y la evaluación de enraizamiento a los 30 días. En la fase de desinfección y establecimiento *in vitro* se logró obtener 0% de contaminación y 0% de oxidación de los explantes. En la fase de multiplicación *in vitro* los mejores resultados se obtuvieron en el medio de cultivo M8 generando 4,7 brotes por explante. Y finalmente en la fase de enraizamiento el medio E4 permitió obtener 94,7% de explantes enraizados, 23,4 mm de longitud de raíz y 2,2 raíces por explante.

Palabras clave: Desinfección, establecimiento, multiplicación, biotecnología, Huacho.

Abstract: Sour orange (*Citrus aurantium* L.) has a high nutritional and gastronomic value in Huacho, Lima, Peru, but is currently considered a species in danger of disappearing from the district and surrounding areas due to phytosanitary problems. For the recovery and repopulation of this species, biotechnological techniques were proposed to obtain pathogen-free plants. Therefore, the objective of the present research was to micro propagate sour orange *in vitro* from nodal segments. Sour orange nodal segments were disinfected in different concentrations of NaClO, then placed in MS culture medium (Murashige and Skoog) supplemented with BAP, KIN, and AG3 for the multiplication phase, and then transferred to MS medium supplemented with IBA and ANA for the rooting phase. The evaluation of the percentage of contamination was carried out after 10 days, the evaluation of shoot formation in the multiplication phase was carried out after 30 days, and the evaluation of rooting after 30 days. In the disinfection and *in vitro* establishment phase, 0% contamination and 0% oxidation of the explants were obtained. In the *in vitro* multiplication phase, the best results were obtained in the M8 culture medium, generating 4.7 shoots per explant. Finally, the E4 medium yielded 94.7% of rooted explants, 23.4 mm root length, and 2.2 roots per explant.

Key words: Disinfection, establishment, multiplication, biotechnology, Huacho.

Introducción

Los cítricos (*Citrus*) son un importante género de árboles frutales por presentar un alto valor nutricional y económico, encontrándose ampliamente distribuido por todo el mundo debido a su gran consumo^{1,2}. Con una producción de 124 millones de toneladas en promedio anualmente a nivel mundial, mientras que en Perú solo se llega a producir un millón de toneladas, de los cuales alrededor del 40% es producido por naranjas^{3,4}.

Dentro de las naranjas que produce el país se tiene a la naranja agria o amarga (*Citrus aurantium* L.), la cual es utilizada en la gastronomía, como saborizante, en la medicina natural y como portainjerto de gran número de especies de valor comercial, por presentar resistencia a condiciones abiotícas como salinidad y sequía, además de ser tolerante a fitopatógenos como *Armillaria mellea* y *Phitophthora* sp.⁵. Es así como se puede encontrar a la naranja agria dentro de la biodiversidad vegetal del distrito de Huacho, Provincia de Huaura, Departamento de Lima. La cual es utilizada como insumo para muchos platos típicos de la zona como el ceviche de pato y ceviche de pescado, pero actualmente su producción es escasa,

al punto de encontrar números reducidos de estos árboles frutales por lo que esta próxima de desaparecer del distrito y sus alrededores^{6,7}, esto debido a ser susceptible al virus de la tristeza de los cítricos (CTV), ocasionando escasa longevidad de las plantas, bajos rendimientos y deficiencias de calidad de la fruta^{8,9}, y además la preferencia de los agricultores locales a otros cultivos de mayor demanda económica^{10,11}.

Considerando a la naranja agria como una planta de gran importancia gastronómica a nivel local y nacional, se busca estrategias para la recuperación y repoblación de esta especie. En la actualidad se pueden aplicar herramientas de biotecnología vegetal, como el cultivo *in vitro* de tejidos vegetales^{12,13}. Resultando en clones uniformes a partir de una planta madre libre de enfermedades y de gran producción, conservando y garantizando los rasgos seleccionados¹⁴. Además, la propagación *in vitro* permite obtener material vegetal durante todo el año, de esta manera no habría un limitante como el suministro de semilla o plantines de invernaderos^{15,16}.

En tal sentido las aplicaciones de los cultivos *in vitro* no solo se limitan a las altas tasas de multiplicación como método de propagación asexual para la producción de plantas clonales, sino que también forma la base para gran variedad de estudios en la mejora genética vegetal¹⁷. Es así como se han reportado investigaciones en el cultivo *in vitro* de cítricos, donde se ha evaluado las diferentes respuestas morfogenéticas influenciadas por la especie, tipo de explante y la composición del medio de cultivo^{18,19}. Iniciando desde el establecimiento *in vitro* de semillas de naranja dulce (*Citrus sinensis* L.) y su posterior multiplicación a partir de segmentos maduros del tallo²⁰. De la misma manera²¹, trabajaron inicialmente con semillas de naranja agria y lima (*Citrus aurantifolia*) respectivamente, para luego multiplicar a las plántulas germinadas empleando diferentes concentraciones de reguladores de crecimiento²², trabajo con semillas de naranja y con varetas de limón, logrando establecer *in vitro* a ambas especies para realizar microinjertos, evaluando la influencia de las concentraciones de sacarosa en el medio de cultivo²³, realizaron experiencias con yemas de limón criollo (*Citrus limon*), para la formación de callos y brotes empleando picloram y ácido 2,4-diclorofenoxiacético (2,4-D)²⁴, trabajaron en la microporpagación de limón a partir de segmentos nodales²⁵, evaluó el efecto de las citoquininas 6-Bencilaminopurina (BAP) y kinetina (KIN) en la propagación *in vitro* de lima (*Citrus aurantifolia*) a partir de segmentos nodales. 26, establecieron un protocolo de microporpagación de citrumelo "Swingle" a partir de segmentos nodales, y lograron obtener tasas aceptables de multiplicación²⁷, evaluaron varias concentraciones de reguladores de crecimiento (citoquininas, auxinas y ácido giberélico) en el medio de cultivo MS para inducir la formación de brotes a partir de segmentos nodales de naranja agria y mandarina.

Por lo tanto, el objetivo del presente estudio fue lograr la microporpagación *in vitro* de naranja agria a partir de segmentos nodales, estableciendo un protocolo óptimo de propagación efectiva para la obtención de una fuente ideal de material aséptico y homogéneo para usar en programas de mejoramiento genético vegetal.

Materiales y métodos

La investigación se realizó en el Laboratorio de Biotecnología Vegetal de la Escuela Profesional de Biología con mención en Biotecnología, Facultad de Ciencias, Universidad Na-

cional José Faustino Sánchez Carrión, en el distrito de Huacho, provincia de Huaura, departamento de Lima, Perú.

Material vegetal

Se emplearon segmentos nodales de naranja agria procedentes del vivero que pertenece al Laboratorio de Biotecnología Vegetal, ubicada en la ciudad universitaria de la Universidad Nacional José Faustino Sánchez Carrión; situado a una altitud de 41 msnm, latitud sur 11° 7' 34" y longitud oeste 77° 36' 34", con temperatura media anual de 19,2°C.

Desinfección y establecimiento *in vitro* de segmentos nodales

Los segmentos nodales fueron llevados a laboratorio donde se lavaron con agua destilada durante 5 minutos y se continuó con el proceso de desinfección. Se trasladaron a cámara de flujo laminar y se sumergieron en etanol al 70 % durante 1 minuto, después fueron sumergidos durante 5 minutos en solución de hipoclorito de sodio (NaClO) al 1%, seguido se sometieron a cuatro tratamientos con variaciones en las concentraciones de hipoclorito de sodio: 1% (T1), 1,5% (T2), 2% (T3) y 2,5% (T4) durante 10 minutos cada uno en agitación continua y luego se realizaron tres enjuagues en agua destilada estéril para eliminar los residuos de hipoclorito de sodio, seguidos los segmentos nodales se cortaron por la parte inferior y superior hasta tener un explante de aproximadamente 1.5 cm de longitud. Finalmente fueron introducidos *in vitro* en tubos con medio de cultivo MS²⁸ a la mitad de su concentración, adicionado con 6 g/L de agar, 15 g/L de sacarosa y pH a 5,7. El medio de cultivo empleado fue previamente esterilizado en autoclave a 1,2 Bar de presión y una temperatura de 121°C durante 20 minutos.

Multiplicación *in vitro*

A los 30 días se realizaron cortes para obtener nuevamente segmentos nodales, los cuales fueron cultivados *in vitro* en el medio de cultivo MS suplementado con reguladores de crecimiento en diferentes tratamientos (Tabla 1). El medio de cultivo fue preparado con sales y vitaminas del medio de cultivo MS adicionado con 7 g/L de agar, 23 g/L de sacarosa y se ajustó el pH a 5,7. El medio de cultivo fue dispensado en 10 mL por frasco y luego se sellaron con papel aluminio y fueron esterilizados en autoclave a 1,2 Bar de presión y una temperatura de 121°C durante 20 minutos.

Tratamiento	KIN (mg/L)	BAP (mg/L)	AG ₃ (mg/L)
M1	0	0	0
M2	0,25	0	0
M3	0	0,25	0
M4	0	0	0,25
M5	0,25	0,25	0
M6	0,25	0	0,25
M7	0	0,25	0,25
M8	0,25	0,25	0,25

KIN = Kinetina; BAP = 6-Bencilaminopurina; AG₃ = Ácido Giberélico.

Tabla 1. Combinación de los reguladores de crecimiento en el establecimiento *in vitro* de naranja agria (*Citrus aurantium* L.).

Enraizamiento *in vitro*

Transcurrido 30 días los explantes fueron transferidos a frascos que contenían medio de cultivo MS suplementado con auxinas en distintos tratamientos (Tabla 2). El medio de cultivo fue preparado con las sales y vitaminas del medio de cultivo MS adicionado con 7 g/L de agar, 23 g/L de sacarosa y se ajustó el pH a 5,7. El medio de cultivo fue dispensado en 10 mL por frasco y luego se sellaron con papel aluminio y fueron esterilizados en autoclave a 1,2 Bar de presión y una temperatura de 121° C durante 20 minutos.

En las tres etapas de evaluación (desinfección e introducción, multiplicación y enraizamiento) los explantes fueron incubados durante 30 días a una temperatura constante de 27° C, con humedad relativa del 75±2% y fotoperíodo de 16 horas luz con intensidad lumínica de 1500 Lux. (Plant Growth Chamber, LGC – 5201 G, LabTech)

Tratamiento	IBA (mg/L)	ANA (mg/L)
E1	0	0
E2	1	0
E3	0	1
E4	1	1

IBA = Ácido Indol Butírico; ANA = Ácido naftalenacético

Tabla 2. Combinación de las auxinas para el enraizamiento *in vitro* de naranja agria (*Citrus aurantium* L.).

Diseño experimental, variables medidas y análisis estadístico

Se empleó un diseño completamente al azar con 10 frascos por tratamiento, cada frasco contenía un explante como unidad experimental y el mismo se repitió tres veces. En la fase de desinfección e introducción se evaluó el porcentaje de contaminación, la oxidación de los explantes y la sobrevivencia, en la fase de multiplicación se evaluó el número de brotes, longitud de los brotes, numero de nudos y numero de hojas por explante y finalmente en la fase de enraizamiento se evaluó el porcentaje de explantes enraizados, longitud de la raíz y número de raíces. Los datos se procesaron mediante Análisis de Varianza (ANVA) con los paquetes estadísticos agricolae y car del programa libre R (versión 4.1.0 para Windows), y la comparación entre las medias se realizó de acuerdo a la prueba de Tukey ($p \leq 0.05$).

Tratamiento	NaClO (%)	Contaminación (%)	Oxidación (%)	Sobrevivencia (%)
T1	1	35 a	0 b	65 b
T2	1,5	0 b	0 b	100 a
T3	2	0 b	0 b	100 a
T4	2,5	0 b	52 a	48 c

Medias con letras distintas por columnas difieren significativamente según prueba de Tukey para $p < 0.05$.

Tabla 3. Efecto de la concentración de hipoclorito de sodio en la desinfección de los segmentos nodales de naranja agria (*Citrus aurantium* L.).

Resultados

Desinfección y establecimiento *in vitro* de segmentos nodales

La evaluación de la desinfección superficial de los segmentos nodales se realizó a los diez días después de realizada la introducción *in vitro*. La Tabla 3 muestra 0% de contaminación en los tratamientos T2, T3 y T4. La diferencia entre estos tratamientos se evidenció en el porcentaje de oxidación de los segmentos nodales, obteniéndose 0% de oxidación en los tratamientos T2 y T3.

Multiplicación *in vitro*

La evaluación de los brotes se realizó a los 30 días de cultivo (Figura 1). En la Tabla 4 los resultados indicaron la influencia de combinar dos citoquininas (BAP y KIN) y AG3 en el tratamiento M8 lo que permitió obtener un mayor número de brotes (Figura 2). En el tratamiento M4 se obtuvo un promedio de longitud mayor a los demás tratamientos (Figura 3), mientras que en el promedio de número de nudos los tratamientos M4 y M6 fueron los que no presentaron diferencia significativa, y finalmente para el promedio de número de hojas los tratamientos M3 y M5 fueron los que presentaron los valores más altos, pero no difiriendo significativamente entre ellos.

Enraizamiento *in vitro*

La evaluación del enraizamiento se realizó a los 30 días de cultivo (Tabla 5), alcanzando los mejores resultados en el tratamiento E3 compuesto por el medio MS adicionado con 1 mg/L de IBA y 1 mg/L de ANA.

Discusión

Los tratamientos de desinfección de los segmentos nodales de naranja agria procedentes del invernadero permitieron su establecimiento en condiciones *in vitro*. Con las concentraciones de 1,5% (T2) y 2% (T3) de NaClO se obtuvieron explantes con 0% de contaminación y oxidación, por lo cual se les considera los mejores tratamientos. La desinfección del material vegetal en la presente investigación se inició con etanol al 70% y se continuó con soluciones de hipoclorito de sodio, es de gran importancia en la desinfección superficial de los explantes emplear etanol para eliminar contaminantes bacterianos y para los contaminantes fúngicos determinar concentraciones adecuadas de hipoclorito de sodio para no dañar el material vegetal^[29].

Tratamiento	Promedio de Nº de brotes por explante	Promedio de longitud de brotes (mm)	Promedio de Nº de nudos por explante	Promedio de Nº de hojas por explante
M1	1,3 d	17,7 d	1,2 d	5,4 b
M2	3,3 b	27,6 c	1,6 d	5,7 b
M3	2 c	37,4 b	3,4 b	6,5 a
M4	1,6 cd	46,6 a	4,5 a	5,6 b
M5	2,2 c	33,1 b	3,2 b	6,2 a
M6	2,4 c	30,4 bc	4,3 a	3,8 d
M7	1 d	36,8 b	3,1 b	5,2 c
M8	4,7 a	29,4 bc	2,3 c	4,1 d

Medias con letras distintas por columnas difieren significativamente según prueba de Tukey para $p < 0.05$.

Tabla 4. Efecto de los reguladores de crecimiento en el establecimiento *in vitro* de naranja agria (*Citrus aurantium* L.).

Tratamiento	Explantes enraizados (%)	Promedio de longitud de raíces (mm)	Promedio de Nº de raíces por explante
E1	0 d	0 c	0 c
E2	22,3 c	26,6 a	1,3 b
E3	66,8 b	13,7 b	1,2 b
E4	94,7 a	23,4 a	2,2 a

Medias con letras distintas por columnas difieren significativamente según prueba de Tukey para $p < 0.05$.



Figura 1. Plántulas *in vitro* de naranja agria (*Citrus aurantium* L.), a los 30 días de cultivo en la etapa de multiplicación.

Tabla 5. Efecto en la combinación de las auxinas para el enraizamiento *in vitro* de naranja agria (*Citrus aurantium* L.).

En la fase de desinfección y establecimiento *in vitro*, los tratamientos utilizados en la presente investigación presentan mejores resultados en comparación de otros autores que han trabajado con especies del mismo género, como (25), que utilizó hipoclorito de sodio al 0,6% durante 15 minutos para la desinfección de segmentos nodales de *Citrus aurantiifolia*, logrando un porcentaje de contaminación del 56%. Mientras que (23), lograron porcentajes de contaminación del 0% al emplear hipoclorito de sodio al 1% durante 20 minutos, pero además los autores utilizaron un segundo lavado transcurrida las 24 horas y adicionalmente los sumergió por una solución de sulfato de cobre al 2% en la desinfección de yemas apicales de *Citrus limon*.

En la multiplicación *in vitro* de los segmentos nodales de naranja agria el tratamiento con medio de cultivo MS suplementado con 0,25 mg/L KIN, 0,25 mg/L BAP y 0,25 mg/L AG3 permitió obtener el mayor número de brotes con 4,7 brotes por explante. Este resultado está en relación a la adición de los tres reguladores de crecimiento, mientras que en los demás tratamientos que se adicionaron dos reguladores de crecimiento se vieron significativamente afectados en la disminución del número de brotes por explante en comparación a los tratamientos donde solo se adicionó un regulador de crecimiento, coincidiendo con (26), quienes obtuvieron una disminución en la formación de brotes de citrumelo (*Poncirus trifoliata* (L.) Raf. × *Citrus paradisi* McFadden) con tratamientos adicionando BAP y KIN, en comparación donde solo utilizaron BAP. Resultados similares obtuvo (25) quien no encontró diferencia significativa en el uso de BAP asociado con KIN que al utilizar únicamente BAP. Mientras que (27) emplearon un medio de cultivo MS suplementado con 2 mg/L de BAP y 0,6 mg/L de AG3 para la formación de brotes de naranja agria, donde obtuvieron dos

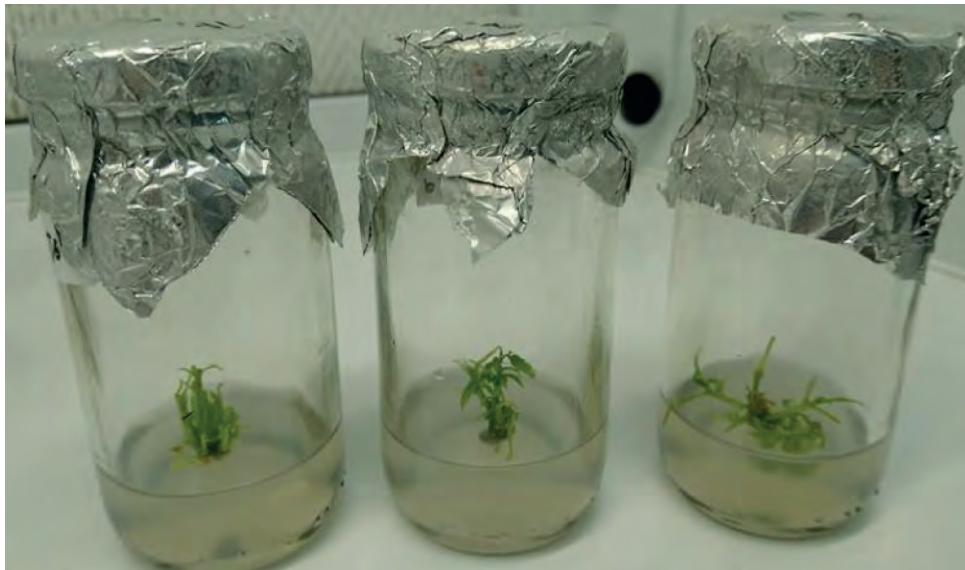


Figura 2. Efecto de la combinación de BAP, KIN y AG3 en el cultivo *in vitro* de naranja agria (*Citrus aurantium* L.), a los 30 días de cultivo.



Figura 3. Efecto del AG3 en el cultivo *in vitro* de naranja agria (*Citrus aurantium* L.), a los 30 días de cultivo.

brotes por segmento nodal, resultado muy cercano al obtenido con el tratamiento M6 en la presente investigación.

El tratamiento M4 fue el que presentó mayor longitud de los brotes con 46,6 mm, esto es debido a que este medio de cultivo solo estaba suplementado con AG3. (30) señalan que en *Citrus* se utiliza generalmente AG3 para aumentar la longitud de los brotes. Pero al adicionar AG a los demás tratamientos con BAP y KIN no tuvo mayor efecto en la longitud de los brotes que en los tratamientos donde solo se adicionó una de las citoquininas. Estos resultados guardan relación con estudios realizados en evaluación del uso de BAP en adición con AG3 en *Citrus aurantium*²⁷ y *Citrus limon*³¹, donde concluyeron que no había diferencia significativa en comparación con los resultados obtenidos con los tratamientos donde solo se adicionó BAP en el medio de cultivo.

El mayor número de hojas se logró obtener con el medio de cultivo MS suplementado con 0,25 mg/L de KIN, con un promedio de 6,5 hojas por explante. Este resultado coincide con (23), quienes obtuvieron 6 hojas en promedio por brote al utilizar el medio MS adicionado con 0,25 mg/L de KIN. Además, se observaron hojas atrofiadas en los tratamientos con mayor número de brotes y combinación de reguladores de crecimiento, similar a lo obtenido por (27), que al utilizar el medio de cultivo con 2 mg/L de BAP combinado con 1 mg/L AG3 dieron

lugar a nuevos explantes atrofiados con hojas estrechas e hipohidridad de naranja agria.

En la fase de enraizamiento el tratamiento E4 con medio MS al cual se le adicionó 1 mg/L de IBA y 1 mg/L de ANA fue el que presentó mejores resultados, obteniendo 94,7 % de explantes con raíces. Estos resultados reafirman los logrados por (32), quienes comprobaron que ANA induce porcentajes más altos de enraizamiento que IBA en varias especies del género *Citrus*. Además, los resultados de nuestro trabajo son similares a los obtenidos por (27) en naranja agria, con casi 95% de explantes enraizados cuando combinaron en el medio de cultivo 1 mg/L de IBA con 1 o 2 mg/L de ANA.

Con lo demostrado se puede regenerar plantas de naranja agria a partir de segmentos nodales en condiciones *in vitro*, de esta manera al tener mayor número de brotes por explante permitirá del mismo modo obtener una mayor tasa de multiplicación. Además de que se logra porcentajes de enraizamiento mayor al 94%, lo cual asegura que las plantas puedan desarrollarse adecuadamente. Con las plantas producidas por este método se pretende recuperar y repoblar la campiña de Huacho al ser una especie de gran importancia en la gastronomía local. Finalmente, también se podrá emplear el germoplasma en condiciones *in vitro* para programas de mejoramiento genético vegetal.

Conclusiones

Se logró micropropagar *in vitro* naranja agria a partir de segmentos nodales. Los mejores resultados en la fase de desinfección en los explantes se obtuvieron al emplear 1,5% y 2% de hipoclorito de sodio. En la fase de la multiplicación *in vitro* los mejores resultados se obtuvieron en el medio de cultivo con 0,25 mg/L de BAP, 0,25 mg/L KIN y 0,25 mg/L de AG3, el cual se recomienda emplear por permitir obtener mayor número de brotes. Y finalmente en la fase de enraizamiento el medio suplementado con 1 mg/L de IBA y 1 mg/L de ANA permitió obtener el mayor porcentaje de explantes enraizados y número de raíces.

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RESEARCH / INVESTIGACIÓN

Determining conditions for best pollen quality of red-purple tree tomato (*Solanum betaceum* Cav.) germplasm

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2222

Abstract: The germination and viability of pollen are characteristics required for fecundation when individuals of different or the same species are crossed. For this reason, assessing these parameters in selected individuals to be used in breeding programs will increase the chances for the obtainment of new progeny. In this study, pollen from different accessions of the red-purple tree tomato (*Solanum betaceum* Cav.) was used: local cultivar (Morado Puntón), two commercial varieties (Large Red and Oratia Red) and six segregants [(*Solanum unilobum* x *Solanum betaceum*) x *Solanum betaceum*]. Three types of flowers were taken (A-day of anthesis, B-one day after anthesis, and C-two days after anthesis). The pollen was conserved in two temperatures (4° and 22° C) and four storage times (0, 5, 10, 20 days). The percentage of germination and pollen viability of the selected individuals were evaluated. It was observed that the commercial materials showed higher germination percentages than the segregants in flower A and B at a temperature of 4 ° C at all storage times, except for the segregants GT7P47 and GT7P48 at the same temperature on day 0. In addition, high percentages of viability were obtained both in flowers A and B, at both temperatures and at all storage times. However, the immediate use of pollen after it is collected is recommended because better germination is achieved. This study is helpful to improve breeding procedures in the initial stages of directed crosses.

Key words: Pollen quality, red-purple, tree tomato, *Solanum betaceum*, germplasm.

Introduction

Tree tomato (*Solanum betaceum* Cav.), native to the Andean region of South America¹, belongs to the Solanaceae family, is popular in this region for its consumption in juices and as fresh fruit. This fruit is characterized by its slightly bitter, sour, and astringent taste with a particular aroma^{2,3}. In Ecuador, the cultivated area is around 2,000 ha and produces 28,512 tons with a yield of 13.79 t ha⁻¹⁴). The cultivation of this fruit is carried out by small and medium producers⁵, but this fruit has excellent possibilities of positioning in the world market due to its excellent organoleptic characteristics, exotic aroma and flavor, and nutraceutical properties⁶.

In Ecuador, tree tomato production is based mainly on local cultivars, which are the product of natural crosses⁷. However, breeding processes have generated segregants, originating genotypes with significant phenotypic heterogeneity reflected in agronomic traits, fruit quality, and productivity^{5,8}. Ecuadorian cultivars are not kept pure due to cross pollination that occurs in the production plots, generating fruits of different color, shape and size; being the most representative cultivars: Gigante Anaranjado, Anaranjado Puntón, Rendondo Anaranjado, Gigante Morado y Morado Puntón⁹; however, the cultivar Gigante Anaranjado is the one with the highest demand, consumption and production⁷.

Breeding on this fruit crop is carried out because tree tomato presents yield limitations mainly due to the attack of pests^{10,11}. On the other hand, this fruit has excellent nutritional and commercial value, causing desired relevant demand in the national and international market¹². Furthermore, from the nutritional point of view, tree tomato fruit is an excellent source of vitamins A, B6, C, and E, and minerals such as iron; it also has a low carbohydrate content and less than 40 calories per 100 g¹³; it is a valuable source of pectins that favor the preparation of jellies and jams¹⁴. In addition, it has compounds with antioxidant capacity such as lycopene, anthocyanins, and high content of polyphenols such as 3-O-caffeoylequinic acid and rosmarinic acid^{3,14}.

For those mentioned above, the Fruit Program of the National Agricultural Research Institute (INIAP) has evaluated tree tomato segregants with different levels of resistance and fruit quality (physical and chemical traits)^{5,8}, to select elite materials to be used as parents in future crosses. Plant breeding through directed crosses is essential for generating new genotypes with better characteristics; consequently, pollen quality studies are essential in artificial hybridization techniques^{15,16} to guarantee fertilization and the generation of new progeny.

Determining the quality of pollen is of great importance in breeding¹⁷ because it has a tremendous impact in the efficacy of the genetic improvement practices¹⁸. This characteristic is essential to define the direction of a cross and have bases for the success of controlled hybridizations that guarantee the generation of new hybrids¹⁹, originating superior individuals with better productivity, fruit quality, and obtain resistance or tolerance pests.

Currently, the Fruit Program has identified tree tomato individuals with superior characteristics associated with fruit quality, including soluble solids content and red-purple mucilage, a characteristic related to a more significant amount of antioxidant compounds³. These individuals will be used as parents in future crosses in the breeding program; therefore, it is necessary to study the pollen quality to guarantee an appropriate starting material to continue with the breeding of this fruit crop successfully.

Methods

The research was carried out in the Laboratory of the Tumbaco Experimental Farm. The pollen belonged to 1 local cultivar (CMP4 -Morado Puntón), 2 commercial varieties (NZL-RP5 - Large Red and NZORP7 - Oratia Red), and 6 segregants of tree tomato (GT7p47, GT7p48, GT9p18, GT20p2, GT20p7, and GT33p5) coming from the cross [*Solanum unilobum* x

Solanum betaceum] x *Solanum betaceum*^{5,8}. These individuals were selected for their fruit quality characteristics (Table 1, Figure 1).

The plants used for this study were grafted onto *Nicotiana glauca*, with an age of 4 years. They were sown in the Tumbaco Experimental Farm of the INIAP at an altitude of 2348 masl, with maximum temperatures of 27 ° C and minimum of 5 ° C, precipitation of 800 mm per year, average relative humidity of 70.86%, and geographical location of latitude: 00° 13' 00" South and longitude: 78° 24' 00" West.

The pollen was stored in two temperatures (4 ° C and 22 ° C) and four periods of time (0, 5, 10, and 20 days) for its conservation. For the 0-day storage period, storage was performed for 8 hours at the two temperatures. Pollen extraction was carried out in the morning (8:00 - 10:00 am), taking fully open flowers in 3 states (Figure 2): where flower A (day 0) is the day of anthesis, flower B (day 1) one day after anthesis and flower C (day 2) two days after anthesis. To extract the pollen, the tips of the anthers were cut, and the pollen of the flower was obtained using light strokes and shaking.

A 1 mg sample of pollen was placed in Eppendorf tubes for each treatment. For the evaluation of pollen viability, the staining technique based on acetocarmine glycerol gelatin²⁰ was used. This test measures the integrity of the cytoplasm; that is, the pollen grains turn red when the cytoplasmic membrane is intact²¹. Viable pollen was considered to be those grains that did not show deformations and had intense staining. While for the evaluation of pollen germination, the pollen was sown for 24 hours in a sucrose medium described by Rodríguez and Dafni²². Germinated pollen grains were considered to be those that showed the pollen tube with a length greater than or equal to the pollen diameter²³⁻²⁵. In both cases, 250 pollen grains were counted using an optical microscope (Olympus, SX40).

A completely randomized design was used, with a 9 x 2 x 4 x 3 factorial arrangement with a total of 216 treatments with 5 observations. An analysis of variance was performed, and the 5% Tukey test was used to determine differences between means.

Results and discussion

Research carried out on pollen quality is of great importance for the success of breeding programs aimed at the generation of hybrids with better agronomic characteristics that allow increasing the efficiency of genetic improvement through directed crosses¹⁷.

Germination

To simulate pollen development in the gynoecium *in vivo*, the germination tests of pollen grains *in vitro* are established; this is achieved by placing the grains in a germination solution that must present similar conditions to the stigma of the female organ²⁶. In terms of germination percentage (Figure 3), significant differences were observed in flower type and temperature as storage days progressed (Table 2, 3, and 4). In the tree tomato, the size and production of pollen grains varies⁷, and the type of flower to be collected is of utmost importance before pollen collection²⁷. In this research, it was observed that flowers A and B showed values greater than 55% both at 22 ° C and 4 ° C on day 0, while in subsequent conservation times, values greater than 40% were observed only at 4 ° C. On the other hand, in flower C, low values (less than 13%) were obtained in the day 0 and the percentages were considerably reduced later until reach 0% in various individuals at 10 and 20 days. Consequently, while the storage time increases, the germination percentage decreases. This trend was also reported by González *et al.*¹⁶ who found that in potato (*S. tuberosum*), pollen stored at a temperature of 17 ° C loses its germination percentage rapidly, but if the pollen is stored at 4 ° C it had up to 20% germination. Araméndiz *et al.*¹⁷ also reported this behavior in a study carried out on eggplant (*S. melongena*), where the highest germination percentage was obtained at 0 days of storage and it decreased as the storage time progressed and the temperature increased.

Statistical differences between the tree tomato individuals were observed, highlighting the commercial individuals CMP4, NZLRP5, and NZORP7 with values in a range of 54 to

Material	Weight of fruit (g)	Pulp Color	Color of mucilage	Content of soluble solids (° Brix)	Acidity (%)	Firmness (N)
CMP4 Morado Puntón	92,56	Orange	Red	10,6	1,70	2,08
NZLRP5 Large Red	61,14	Orange	Red	11,74	1,31	2,77
NZORP7 Oratia Red	70,28	Orange	Red	9,92	1,26	1,34
GT7p47	50,49	Orange	Purple	11,03	2,47	2,19
GT7p48	59,31	Orange	Red	11,02	1,78	1,87
GT9p18	66,97	Orange	Purple	12,64	1,31	1,83
GT20p2	84,24	Orange	Purple	11,92	2,52	1,25
GT20p7	80,17	Orange	Purple	12,03	2,37	1,38
GT33p5	41,10	Orange	Purple	11,10	1,25	3,10

Table 1. Characteristics of the tree tomato germplasm.



Figure 1. Tree tomato materials. Variety Large Red (top left), cultivar Morado Puntón (top right), and segregants (bottom left and right).



Figure 2. Flower types, where flower day 0 is the day of anthesis, flower day 1 is one day after anthesis, and flower day 2 is two days after anthesis.

59% at 22° C and 53 to 64% at 4° C at 0 days of storage in flower A. The germination percentage was not optimum (less than 70%), even at 4° C, which is a temperature commonly used to conserve pollen grains in some species. Later these values decreased, but CMP4 and NZLRP5 showed higher percentages, around 42%, at 20 days (Table 2). Only the GT7P47 and GT7P48 segregants showed germination percentages higher than 53% at 22° C and 4° C at 0 days and around 42% at 5 days at 4° C; while at 20 days, their germination decreased to values of 16 and 26% respectively (Table 2). For flower B, the trend was similar to that observed in flower A with germination percentages in a range between 50 and 59% at 0 days and decreasing considerably until 20 days (Table 3). In flower C, only the segregant GT2OP7 had values of 12% (22° C) at 0

days and 9% (4° C) at 5 days, while the rest of the individuals showed shallow values or did not have germination (Table 4). According to Revelo *et al.*²⁸, this response is because commercial cultivars or varieties are better adapted to climatic and soil conditions, showing a more significant number of fruit set per inflorescence, corroborating that pollen from commercial materials has a superior germination capacity.

Pollen viability

The determination of pollen viability allows making reliable fertility estimates, besides being used for incompatibility studies in crosses²⁹. In fruit crops, staining with 2% acetocarmine and observation by optical microscope are used to de-

termine the viability of the pollen grain²⁶ (Figure 3). Similar to germination, significant differences were observed for the type of flower and temperature as the storage time advanced.

Flower A and B showed high percentages of viability (greater than 95%) (Table 2 and 3) in the commercial materials CMP4, NZLRP5, and NZORP7, as well as in the segregants GT7P47 and GT7P48, both at 22 °C and at 4 °C and at all storage times (0, 5, 10 and 20 days). While in flower C, only the segregating GT20P7 obtained 12% viability at 22 °C at 0 days and 8% at 4 °C at 5 days, while the rest of the individuals showed shallow values, most of which did not have viability (Table 4). The results obtained in flower C corroborate what Montaner *et al.*³¹ found that pollen loses its viability after its anthesis period.

In other Solanaceae species, using the same method of this research, viability percentages have been reported chiefly over 80% and reaching up to 98%^{26,30}. On the other hand, in a study of pollen quality in chirimoya (*Annona cherimola*), it was reported that in storage at 7 °C, higher viability percentages are achieved as the conservation time advances³². This study obtained stable percentages throughout the pollen storage time at the two temperatures evaluated (Table 2 and 3).

The commercial materials (CPM4, NZLRP5, and NZORP7) showed high percentages of viability and relatively acceptable germination percentages (greater than 40%) in flower A and flower B at different storage times. On the other hand, the segregants GT7P47 and GT7P48 had a similar result only on day 0 of storage; after that, although they had high viability percentages, their germination was less than 40% even at 4 °C. When pollen is preserved correctly, it maintains its hydration capacity and regular morphology. However, it loses its potential to germinate over time, so morphological stains are not a good technique for measuring actual fertility. It is an excellent tool to visualize the quality of pollen²⁷ because the staining technique only allows observing the pollen morphology related to its viability²². In addition, there are differences between the needs of pollen grains to germinate, and it is necessary to establish the environmental conditions (primarily temperature and humidity) that favor the development of the pollen tube^{26,30}. In pollen studies carried out in *Physalis peruviana*, it has been reported that some accessions did not show pollen germination despite having good viability²⁶.

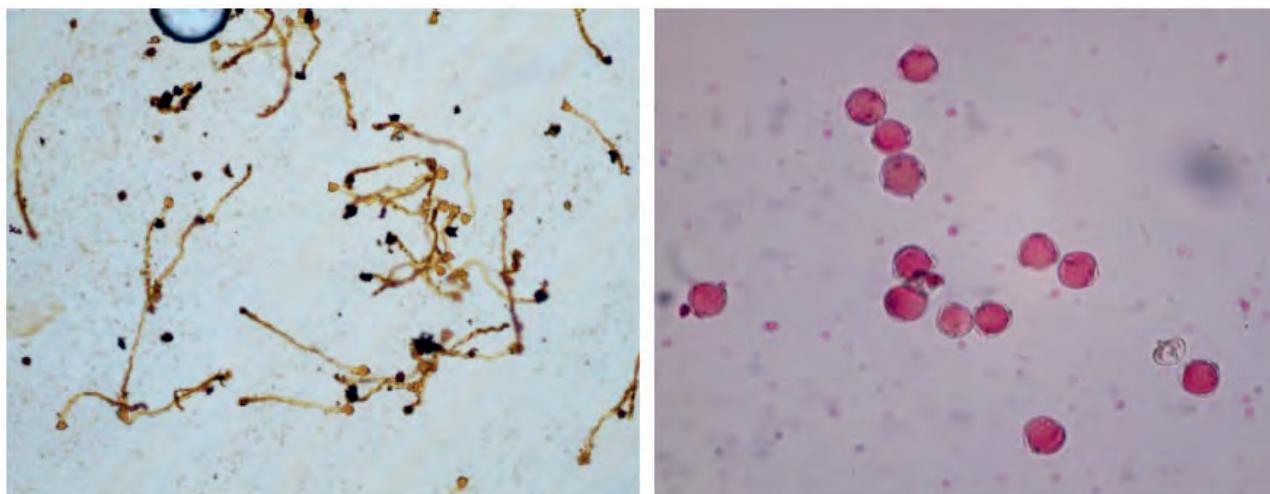


Figure 3. Flower A, day 0, temperature 22 °. Germination test, arrow indicates germinated pollen tube (left). Pollen viability test by acetocarmine staining (right).

Number of days	0d		5d		10d		20d	
	22°C	4°C	22°C	4°C	22°C	4°C	22°C	4°C
Germination (%)								
CMP4	59.76 ± 0.69 a	64.61 ± 1.24 a	8.98 ± 0.89 b	58.63 ± 1.50 a	3.09 ± 1.09 c	55.71 ± 0.96 a	0.24 ± 0.36 b	41.53 ± 2.46 a
GT20P2	1.71 ± 2.14 d	0.32 ± 0.34 d	0.95 ± 1.10 c	1.35 ± 0.83 d	0.40 ± 0.28 d	0.64 ± 0.36 f	0.00 ± 0.00 b	0.32 ± 0.33 f
GT20P7	34.82 ± 2.05 b	18.31 ± 2.16 c	16.18 ± 2.18 a	22.68 ± 2.71 c	8.16 ± 1.39 a	14.98 ± 1.34 e	4.95 ± 0.88 a	5.97 ± 2.20 e
GT33P5	21.42 ± 1.28 c	14.90 ± 1.22 c	14.29 ± 1.77 a	20.62 ± 1.47 c	3.68 ± 2.69 bc	13.11 ± 1.51 e	0.56 ± 0.46 b	0.80 ± 0.80 f
GT7P47	56.4 ± 3.14 a	57.46 ± 1.22 ab	7.64 ± 1.79 b	41.57 ± 1.68 b	2.06 ± 0.94 cd	35.94 ± 1.81 d	0.24 ± 0.22 b	16.68 ± 1.63 d
GT7P48	55.52 ± 3.78 a	53.43 ± 3.31 b	7.04 ± 1.58 b	43.6 ± 3.23 b	3.63 ± 0.76 bc	40.50 ± 1.33 c	0.56 ± 0.22 b	26.19 ± 1.73 c
GT9P18	3.63 ± 3.54 d	1.02 ± 0.57 d	0.16 ± 0.21 c	1.25 ± 0.91 d	0.32 ± 0.33 d	0.56 ± 0.36 f	0.00 ± 0.00 b	0.31 ± 0.32 f
NZLRP5	55.44 ± 2.02 a	62.37 ± 8.39 a	8.12 ± 1.94 b	42.13 ± 4.21 b	2.69 ± 1.41 cd	52.96 ± 2.11 a	0.24 ± 0.22 b	43.36 ± 1.54 a
NZORP7	54.71 ± 2.73 a	58.67 ± 6.47 ab	9.72 ± 1.00 b	54.29 ± 2.61 a	5.95 ± 0.92 ab	44.70 ± 1.84 b	0.72 ± 0.33 b	36.16 ± 0.48 b
Viability (%)								
CMP4	97.68 ± 1.43 a	97.12 ± 1.04 a	97.04 ± 1.40 a	98.80 ± 0.49 a	97.04 ± 1.51 a	97.92 ± 1.51 a	97.12 ± 1.07 a	97.92 ± 1.51 ab
GT20P2	1.35 ± 0.92 c	0.17 ± 0.23 c	0.80 ± 0.80 c	1.05 ± 0.86 e	0.24 ± 0.22 c	0.48 ± 0.44 c	0.00 ± 0.00 b	0.16 ± 0.22 d
GT20P7	33.51 ± 3.17 b	17.63 ± 1.99 b	12.15 ± 2.09 b	24.46 ± 3.16 c	4.80 ± 1.95 b	13.76 ± 2.63 b	0.48 ± 0.52 b	6.21 ± 2.37 c
GT33P5	0.00 ± 0.00 c	14.97 ± 2.99 b	0.00 ± 0.00 c	19.39 ± 1.41 d	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 b	0.00 ± 0.00 d
GT7P47	97.76 ± 1.43 a	97.52 ± 1.25 a	97.44 ± 1.46 a	97.92 ± 1.34 ab	98.08 ± 1.04 a	97.60 ± 1.36 a	98.08 ± 1.43 a	97.92 ± 1.51 ab
GT7P48	98.16 ± 1.08 a	97.04 ± 1.28 a	98.40 ± 1.26 a	98.00 ± 1.10 a	97.60 ± 1.33 a	97.36 ± 1.28 a	97.76 ± 1.43 a	97.60 ± 1.33 ab
GT9P18	2.03 ± 2.45 c	1.56 ± 1.03 c	0.40 ± 0.49 c	0.70 ± 0.64 e	0.64 ± 0.54 c	0.16 ± 0.21 c	0.24 ± 0.22 b	0.16 ± 0.21 d
NZLRP5	96.48 ± 1.58 a	98.24 ± 1.73 a	98.40 ± 1.26 a	95.60 ± 0.85 b	97.20 ± 1.36 a	98.24 ± 1.28 a	98.40 ± 1.26 a	98.48 ± 1.34 a
NZORP7	97.76 ± 1.31 a	98.48 ± 15.93 a	97.44 ± 1.61 a	99.36 ± 0.61 a	98.00 ± 1.41 a	95.60 ± 0.85 a	97.92 ± 1.73 a	95.76 ± 0.96 ab

Table 2. Pollen germination and viability in Flower A as a function of tree tomato plant material, number of days and temperature.

Number of days	0d		5d		10d		20d	
Temperature	22°C	4°C	22°C	4°C	22°C	4°C	22°C	4°C
Germination (%)								
CMP4	55.52 ± 3.78 a	58.04 ± 1.52 ab	7.35 ± 2.01 a	54.99 ± 1.18 a	3.63 ± 0.76 b	50.76 ± 1.04 a	0.56 ± 0.22 ab	39.57 ± 1.77 a
GT20P2	0.72 ± 0.33 c	0.24 ± 0.22 e	0.24 ± 0.35 b	0.56 ± 0.36 e	0.16 ± 0.22 c	0.24 ± 0.36 f	0.00 ± 0.00 c	0.48 ± 0.33 e
GT20P7	21.83 ± 1.93 b	10.63 ± 2.03 d	9.40 ± 1.35 a	12.4 ± 1.89 d	0.24 ± 0.22 c	5.34 ± 1.05 e	0.00 ± 0.00 c	0.24 ± 0.22 e
GT33P5	4.21 ± 3.41 c	1.04 ± 1.19 e	0.00 ± 0.00 b	1.65 ± 2.04 e	0.00 ± 0.00 c	2.57 ± 2.33 ef	0.00 ± 0.00 c	0.72 ± 0.33 e
GT7P47	54.71 ± 2.73 a	55.23 ± 1.07 ab	8.07 ± 1.46 a	36.11 ± 0.62 c	2.06 ± 0.94 b	32.32 ± 1.22 d	0.24 ± 0.22 bc	14.37 ± 0.93 d
GT7P48	55.44 ± 2.02 a	50.21 ± 3.35 c	9.72 ± 1.00 a	38.86 ± 1.20 c	2.69 ± 1.41 b	36.08 ± 1.17 c	0.72 ± 0.33 a	24.17 ± 5.67 c
GT9P18	0.00 ± 0.00 c	0.24 ± 0.22 e	0.00 ± 0.00 b	0.16 ± 0.21 e	0.00 ± 0.00 c	0.00 ± 0.00 f	0.00 ± 0.00 c	0.00 ± 0.00 e
NZLRP5	55.89 ± 3.48 a	59.06 ± 1.25 a	7.64 ± 1.79 a	37.66 ± 2.19 c	2.06 ± 0.94 b	48.59 ± 2.72 a	0.24 ± 0.22 bc	41.75 ± 1.66 a
NZORP7	54.71 ± 2.73 a	53.58 ± 4.69 bc	9.72 ± 1.00 a	49.22 ± 1.07 b	6.25 ± 0.83 a	40.48 ± 1.14 b	0.72 ± 0.33 a	34.43 ± 0.64 b
Viability (%)								
CMP4	98.16 ± 1.08 a	97.12 ± 1.04 a	98.40 ± 1.26 a	98.80 ± 0.49 a	98.00 ± 1.41 a	97.92 ± 1.51 a	97.12 ± 1.07 a	97.92 ± 1.51 a
GT20P2	0.56 ± 0.45 c	0.24 ± 0.22 c	0.16 ± 0.22 c	0.24 ± 0.36 d	0.08 ± 0.18 b	0.32 ± 0.18 c	0.16 ± 0.22 b	0.08 ± 0.18 c
GT20P7	21.08 ± 1.29 b	10.67 ± 2.53 c	6.88 ± 2.18 b	6.53 ± 1.38 c	0.16 ± 0.22 b	0.48 ± 0.44 c	0.00 ± 0.00 b	0.08 ± 0.18 c
GT33P5	0.88 ± 0.71 c	0.76 ± 0.59 c	0.00 ± 0.00 c	2.20 ± 2.32 d	0.00 ± 0.00 b	1.51 ± 1.42 c	0.00 ± 0.00 b	0.40 ± 0.49 c
GT7P47	97.76 ± 1.43 a	98.48 ± 15.93 a	97.44 ± 1.46 a	98.00 ± 1.10 a	97.60 ± 1.33 a	95.60 ± 0.85 b	97.76 ± 1.43 a	97.92 ± 1.51 a
GT7P48	97.76 ± 0.43 a	97.52 ± 1.25 a	97.44 ± 1.61 a	99.36 ± 0.61 a	98.08 ± 1.04 a	97.36 ± 1.28 ab	97.92 ± 1.73 a	98.48 ± 1.34 a
GT9P18	0.48 ± 0.43 c	0.32 ± 0.18 c	0.16 ± 0.22 c	0.48 ± 0.44 d	0.08 ± 0.18 b	0.08 ± 0.18 c	0.00 ± 0.00 b	0.00 ± 0.00 c
NZLRP5	96.48 ± 1.58 a	98.24 ± 1.73 a	98.40 ± 1.26 a	95.60 ± 0.85 b	97.20 ± 1.36 a	98.24 ± 1.28 a	98.40 ± 1.26 a	95.76 ± 0.96 b
NZORP7	97.68 ± 1.43 a	97.04 ± 1.28 a	97.04 ± 1.40 a	97.92 ± 1.34 ab	97.04 ± 1.51 a	97.60 ± 1.36 ab	98.08 ± 1.43 a	97.60 ± 1.33 ab

Table 3. Pollen germination and viability in Flower B as a function of tree tomato plant material, number of days, and temperature.

Number of days	0d		5d		10d		20d	
Temperature	22°C	4°C	22°C	4°C	22°C	4°C	22°C	4°C
Germination (%)								
CMP4	0.00 ± 0.00 b	5.79 ± 7.93	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
GT20P2	0.16 ± 0.22 b	0.16 ± 0.22	0.08 ± 0.18 b	0.16 ± 0.22 b	0.00 ± 0.00 b	0.40 ± 0.49	0.08 ± 0.18	0.00 ± 0.00
GT20P7	12.06 ± 2.02 a	7.29 ± 1.19	6.45 ± 1.71 a	8.99 ± 1.09 a	0.24 ± 0.22 a	0.16 ± 0.22	0.00 ± 0.00	0.00 ± 0.00
GT33P5	0.40 ± 0.28 b	0.16 ± 0.22	0.00 ± 0.00 b	0.24 ± 0.22 b	0.00 ± 0.00 b	0.40 ± 0.89	0.00 ± 0.00	0.00 ± 0.00
GT7P47	0.00 ± 0.00 b	5.92 ± 8.14	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
GT7P48	0.00 ± 0.00 b	5.82 ± 5.49	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
GT9P18	0.46 ± 0.46 b	0.24 ± 0.22	0.00 ± 0.00 b	0.23 ± 0.21 b	0.00 ± 0.00 b	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
NZLRP5	1.96 ± 2.69 b	2.52 ± 5.63	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
NZORP7	2.79 ± 3.84 b	5.07 ± 6.95	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Viability (%)								
CMP4	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00
GT20P2	0.24 ± 0.22 b	0.08 ± 0.17 b	0.16 ± 0.22 b	0.32 ± 0.33 b	0.08 ± 0.18 b	0.16 ± 0.22 ab	0.16 ± 0.22 a	0.00 ± 0.00
GT20P7	11.96 ± 2.25 a	7.00 ± 1.12 a	5.84 ± 2.13 a	7.73 ± 1.51 a	0.24 ± 0.22 b	0.24 ± 0.22 a	0.00 ± 0.00 b	0.00 ± 0.00
GT33P5	0.48 ± 0.44 b	0.23 ± 0.21 c	0.00 ± 0.00 b	0.16 ± 0.22 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00
GT7P47	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00
GT7P48	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00
GT9P18	0.24 ± 0.22 b	0.32 ± 0.18 b	0.00 ± 0.00 b	0.32 ± 0.53 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00
NZLRP5	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00
NZORP7	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00 b	0.00 ± 0.00

Table 4. Germination and viability in Flower C of pollen as a function of plant material of tree tomato, number of days and temperature.

Conclusions

According to the results obtained in the pollen quality tests, it was observed that in flowers A and B, the commercial materials (CMP4, NZLRP5, and NZORP7) showed higher germination percentages than the segregants at a temperature of 4 °C in the different storage times. Only the segregants GT7P47 and GT7P48 showed germination values similar to those obtained by commercial materials at 22 °C and 4 °C after 0 days of storage. Regarding viability, high percentages (greater than 95%) were observed in flowers A and B, at both temperatures and at all storage times. Consequently, the materials mentioned above would be the best options for parents in future crossing plans in the tree tomato breeding program.

On the other hand, it is concluded that tree tomato pollen can be collected in flower A or flower B and stored at 4 °C for up to 5 days. However, it is recommended to use the pollen to make crosses immediately after it's obtained from the flower because, in this stage, it has a higher percentage of germination and high viability.

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RESEARCH / INVESTIGACIÓN

Comparative Study for Carrot Juice and Selenium Supplement in Many Physiological and Biochemical Parameters in Patients with Rheumatoid Arthritis in Kirkuk City

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Abstract: Carrot juice is a critical source of vitamins, selenium, and β-carotene, which is suggested to protect from Rheumatoid Arthritis (RA). The present study aimed to show the effect of carrot juice supplementation compared to selenium tablet supplementation, so our study includes (44) blood samples belonging to young men with RA. All patients aged (20-45) years, 44 blood samples were obtained before treatment at week (0), twenty-four men with RA were supplied with fresh carrot juice. The other group of 20 patients was given an artificial selenium supplement for 21 days as well, and the results were analyzed. The samples were collected from Kirkuk hospital, and external specialized clinical from October/2019 to September /2020; experimental groups were divided into three groups: Group 1 : (44) men Rheumatoid arthritis (RA) before treatment, Group 2: (24) men with RA+ Carrot juice, Group 3: (20) men with RA +Selenium Tab, We reach to following results: the patients who have RA consumption Carrot juice and patients take up Se tab. show significant decrease respectively in RBCs, WBCs, ERS, and RF compared with the Rheumatoid arthritis group, so as the results show a significant decrease in Leptin, IL-6, C-Reactive Protein, and TNF-α concentrations in comparison with the Rheumatoid arthritis group. In contrast, we found a significant increase in GSH, Selenium concentrations, and VD3 in men with RA administration carrot juice and patients' consumption Se tab. Respectively compared with the RA group and, finally, our finding shows no difference in Ceruloplasmin in experimental groups.

Key words: Rheumatoid Arthritis, Carrot Juice, antioxidant agents, Selenium, VitD3, and Leptin.

Introduction

Rheumatoid arthritis (RA) is an autoimmune, chronic, and inflammatory disease that affects ~1% of the global population¹. It is characterized by excessive production of inflammatory mediators, including cytokines (IL-1β, IL-6, TNF-α, IL-17), chemokines, and autoantibodies that lead to an exacerbated activation of immune system cells. If not treated promptly and adequately, this disease can lead to irreversible joint damage². It has been considered a multifactorial disease resulting from the interaction of genetic, hormonal, and environmental factors that contribute to the loss of immune tolerance³⁻⁵. Among the environmental risk factors, the diet has been reported to play an essential role in regulating RA disease activity as specific foods can aggravate or attenuate inflammation⁶. Recently, it has been established that nutrition is essential for the proper development of the immune system, which has led to the study of the relationship between both of them⁷.

Fruits and vegetables are rich sources of nutrients that contain phytochemicals (also known as bioactive compounds), which are recognized for their nutraceutical effects and health benefits. The cultivated carrot (*Daucus carota L.*) is one of the most essential vegetable plants globally because of its high yield potential and use as a fresh or processed product⁸. Although pharmacological treatment of RA patients is currently more effective, not all treatments achieve the reduction of the disease but immune-nutrients in the diet of RA patients could be an alternative to improve some disease conditions such as pain alleviation, reduced count of tender joints, and shortening of the morning stiffness duration, which can also influence on the attenuation of the disease clinical activity⁹. The importance of the adequate consumption of a diet rich in micronutrients is that they have a fundamental role in the immune system throughout life. Among the micronutrients, vitamins A, C, D, E, and B12 and minerals such as iron, zinc, and selenium are

involved in activating and functioning the immune response^{10,11}.

Selenium (Se) is an essential micronutrient that is important for various aspects of human health, including proper thyroid hormone metabolism, cardiovascular health, prevention of neurodegeneration and cancer, and optimal immune responses. Very low (depleted) or very high (toxic) levels of Se intake can be detrimental or possibly fatal. Extreme deficiency or toxicity is not commonly found in humans, but selenosis has been reported in cases of miscalculated supplement formulations, suicides, accidental overdose, or intentional poisoning¹². That said, less overt changes in Se status within an individual may still affect inflammation and immune responses. The biological effects of Se are mainly exerted through its incorporation into selenoproteins, and selenoproteins are involved in the activation, proliferation, and differentiation of cells that drive innate and adaptive immune responses. Dietary Se and selenoproteins are not only important for initiating or enhancing immunity but are also involved in immunoregulation, which is crucial for preventing excessive responses that may lead to autoimmunity or chronic inflammation. It should be noted that most studies in the literature involve modifications to dietary Se, and insights into mechanisms often are not clear, but roles for individual selenoproteins and mechanisms are discussed when data are available.

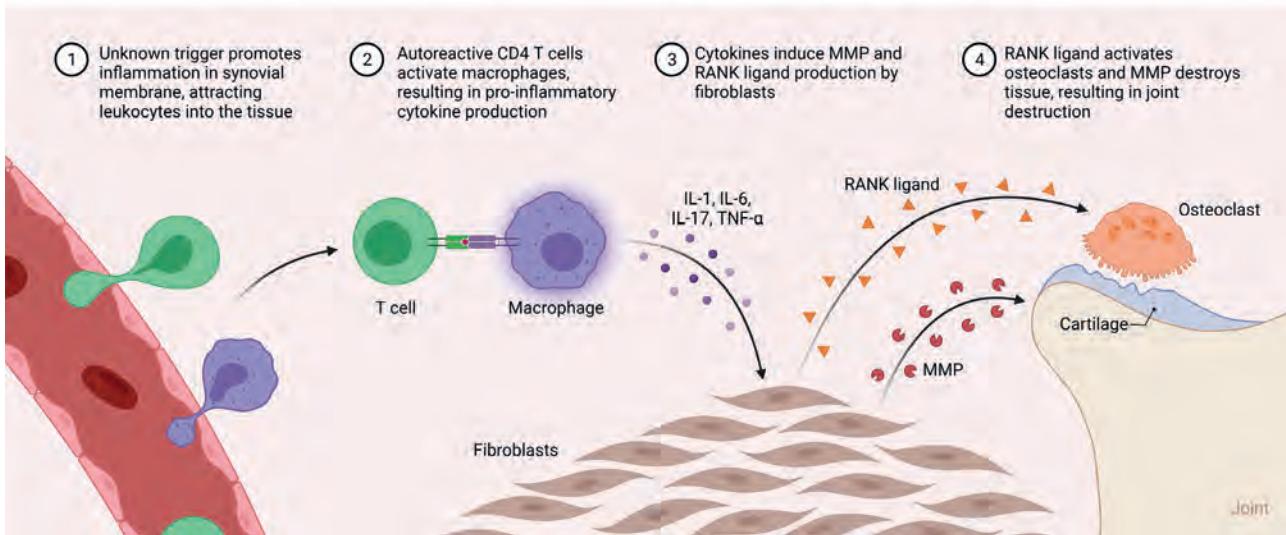
Leptin is a cytokine-like 16 kDa peptide produced mainly through adipose tissue and regulates food intake, basal metabolism, and the β-oxidation of fatty acids. When binding to its receptor(s) located in hypothalamic nuclei occurs, leptin is an essential trigger of adaptive mechanisms during starvation leading to downregulation of thyroid and reproductive functions and stimulation of the hypothalamus. In healthy subjects, leptin levels in the blood are proportional to the body fat mass. Leptin has recently been recognized as a modulator of inflam-

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Pathogenesis of Rheumatoid Arthritis



2229

Figure 1. Rheumatoid arthritis (RA) is an autoimmune, chronic, and inflammatory disease. It is characterized by excessive production of inflammatory mediators, including cytokines (IL-1 β , IL-6, TNF- α , IL-17), chemokines, and autoantibodies that lead to an exacerbated activation of immune system cells.

matory and immune responses. Indeed, leptin participates in bone formation by stimulating osteoblastic cell proliferation and the formation of mineralized nodules in primary osteoblasts and osteosarcoma cells. Leptin facilitates the proliferation of human endothelial cells supporting angiogenesis and neovascularization. Leptin has a dual role in inflammation.

On the one hand, it activates monocyte/macrophage cells and potentiates the production of the pro-inflammatory cytokines, tumor necrosis factor α (TNF α), interleukin (IL)6, and directs T cell differentiation to Th1 phenotype, expressing interferon γ ¹³. Vitamin D is a secosteroid hormone involved in bone and calcium metabolism. It is involved in regulating calcium homeostasis, as it regulates calcium absorption from the gastrointestinal system. The hormone is synthesized in the skin by ultraviolet irradiation. Vitamin D has extraskeletal effects as well¹⁴. The non-classical actions of vitamin D are currently under discussion. Vitamin D has been found to have immunomodulatory actions; Vitamin D deficiency has been shown to be correlated with the appearance of autoimmune diseases, such as diabetes mellitus type 1 and multiple sclerosis; Vitamin D deficiency may increase the risk for the development of RA¹⁵. Recently, the role of vitamin D deficiency in the pathogenesis of RA and the relationship between vitamin D deficiency and the activity of RA is discussed. RA is an inflammatory disease characterized by flares and remissions, flares being characterized by pain. Vitamin D deficiency is also known to be associated with diffuse musculoskeletal pain¹⁶.

The current study aims to clarify the effect of Carrot juice administration rich with vitamins, antioxidants, and minerals as selenium to treat Rheumatoid arthritis in men in comparison with artificial selenium supplement main role in increasing the production and release of the antioxidants and minerals and reducing inflammation factor.

The study also aims to elucidate the role of natural components for carrots in improving human bone and cartilage abilities through its effect as a natural inducer, dispensing as much as possible with medication or excessive treatment without consulting a doctor. This is because these supplements

are an essential natural requirement for the human body in general and particularly for patients in Kirkuk city.

Methods

Experimental Design

Our study include (44) blood samples belonging to young men with RA, we withdraw (44) blood samples before treatment in a week (0), twenty-four men with RA volunteered to consumption fresh carrot juice rich naturally with antioxidants agents and minerals (Fe and Selenium) for twenty-one days, while the other (20) volunteered has taken selenium tablets as an artificial supplement, With age (20-45) year, the samples were collected from Kirkuk hospital and clinical from October /2019 to September /2020 the samples were given within a specific measurement and a regular schedule at a rate of 250 ml per sample in Every morning for 21 days. experimental groups were divided into three groups: Group 1 : (44) men Rheumatoid arthritis(RA) before treatment, Group 2: (24) men with RA+ Carrot juice, Group 3: (20) men with RA +Selenium Tablet.

The beginning fasting was done for the samples for 8 hours, after which blood samples were drawn through the vein in the arm with the use of the tanka to show the vein wall; the blood was placed in Test tubes and placed in the incubator at a temperature of 37 ° for 30 minutes, after which a centrifuge was used Central at a speed of 3000 cycles per minute duration is 15 minutes. Separate the serum from the other ingredients using a micropipette and place it at a temperature of -20 ° C until the Physiological and biochemical tests are performed.

Determination of parameters

Estimation total number of RBCs, WBCs, the results were calculated automatically by Hematology Analyzer. In contrast, the results of Erythrocytes sedimentation rate (ESR) calcu-

lated by Westergren method is considered the standard method for measuring ESR as a nonspecific indicator of disease activity. Clinicians often use it in assisting the diagnosis and follow-up of many inflammatory disorders¹⁷. Rheumatoid factor (RF) the most commonly used serological method is the latex agglutination test. RF is an IgM class of antibody directed against the Fc portion of the IgG molecule; it is detected by its ability to agglutinate the latex particles coated with the IgG molecule.

Estimation of antioxidant agents -Selenium concentration in blood serum¹⁸ estimation Glutathione and Ceruloplasmin, Estimation of VitD3 Concentration in blood serum for Experimental groups: the assay principle combines enzyme immunoassay competition method with a final fluorescent detection ELFA (Enzyme-Linked Fluorescent Assay) technique by using mini vidas device¹⁹.

Estimation of Leptin concentration, IL-6 , C-Reactive Protein and TNF- α level in blood serum.

Statistical analysis

The one-way analysis of variance (ANOVA) is used to determine whether there are any statistically significant differences between the means of two or more groups. The arithmetic averages of the coefficients were tested using the Dunkin' multiple range test with a level of significance (0.05) to determine the significant differences between the groups.

Results

Effect of consumption Carrot juice in RBCs, WBCS, ESR, and Rheumatoid factor (RF)in Patients with Rheumatoid Arthritis

Our study shows a significant decrease in RBCs count in patients with Rheumatoid arthritis after consuming fresh carrot juice and selenium group 4.0250, 4.5120 respectively in comparison with Rheumatoid arthritis without treatment 6.7962. The results proved that in Table (1) the WBCs was high to 14100 we reduced it to 9171.2, 11660 in groups 2 & 3 So as the ESR also decreased from 43.212 to 29.295, 33.0114 respectively. Our study shows a significant decrease in RF level

in patients with Rheumatoid arthritis after consuming fresh carrot juice 6.4011, 6.4482 respectively, compared with the Rheumatoid arthritis group 8.4963.

Effect of consumption Carrot juice in Leptin, IL-6, C-Reactive Protein and TNF- α in Patients with Rheumatoid Arthritis

Our study shows a positive effect for consuming fresh carrot juice in leptin concentration in men suffering from Rheumatoid Arthritis, so our study shows a significant decrease in Leptin level In patients with RA after consuming fresh carrot juice Selenium tab. 23.550, 43.440. Respectively compared with RA with out treatment 56.111, Our study shows a significant decrease in IL-6 concentration in patients with Rheumatoid arthritis after consuming fresh carrot juice and selenium group 2.22 &4.22 respectively compared with Rheumatoid arthritis without treatment 9.67. Our study shows a significant decrease in CRP in patients with Rheumatoid arthritis after consuming fresh carrot juice and selenium group 2.10 & 2.05 respectively compared with Rheumatoid arthritis without treatment 8.73. At the same time, TNF- α concentration witnessed a high decrease in patients with Rheumatoid arthritis after consuming fresh carrot juice and selenium group 20.55& 25.2.77 respectively compared with Rheumatoid arthritis without treatment 48.53.

Effect of consumption Carrot juice in Selenium, Glutathione Ceruloplasmin and VitD3 in Patients with Rheumatoid arthritis

The recent result in our study in table 3 shows a highly significant increase in Selenium (Se) concentration in patients RA +Selenium tablets group, and in RA +carrot juice 86.0021, 66.5400 respectively in compare with Rheumatoid Arthritis without treatment 26.5647 As well as Glutathione(GSH) concentration observed a significant increase in patients with Rheumatoid arthritis consume fresh carrot juice and in RA+ Se tab. Group respectively 3.902, 3.013 as compared with Rheumatoid arthritis without treatment 1.011 So as in VitD3 in patients with Rheumatoid arthritis consume Se tab. And in patients RA +carrot juice 77.0141, 74.2907 respectively as compared with Rheumatoid arthritis without treatment 19.831 While Ceruloplasmin concentration did not have any change in our experimental groups as shown in Tablet 3.

Parameters Group	RBCs cells/mcL	WBCs Cells/mcL	ESR mm/hr	RF IU/ml
Rheumatoid group	8.4963±0.6046	43.212±19.9770	14100±32.6390	6.7962±0.1321
Rheumatoid group+Carrot juice	6.4482±0.4542	29.295±7.00890	9171.2±14.4271	4.0250±0.1159
Rheumatoid group + Selenium tab.	6.4011±0.40110	33.0114±10.1210	11660±16.0022	4.5120±0.1200

Table 1. The concentration of RBCs, WBCS, ESR, and Rheumatoid factor (RF) in experimental groups.

Parameters Group	TNF- α pg/ml	C-reactive protein mg/dl	IL-6pg/ml	Leptin ng/dl
Rheumatoid group	48.53± 7.00	8.73±1.22	9.67±2.28	56.111±17.44
Rheumatoid group+Carrot juice	20.55±2.052	2.10±1.00	2.22±0.15	23.550±7.500
Rheumatoid group + Selenium tab.	25.2.77±2.55	2.05±1.24	4.22±2.11	43.440±12.22

Table 2. The concentration of Leptin, IL-6, C-reactive Protein, and TNF- α in experimental groups.

Parameters Group	VitD3 IU/L	Ceruloplasmin $\mu\text{mo/L}$	Glutathione $\mu\text{mo/L}$	Selenium ng/ml
Rheumatoid group	19.831 \pm 8.7321	195.95 \pm 2.88	1.011 \pm 0.370	26.5647 \pm 11.0467
Rheumatoid group + Carrot juice	74.2907 \pm 14.564	209.36 \pm 2.92	3.902 \pm 0.813	66.5400 \pm 15.7306
Rheumatoid group + Selenium tab.	77.0141 \pm 14.591	196.16 \pm 2.81	3.013 \pm 0.800	86.0021 \pm 11.0122

Table 3. The concentration of Selenium, Glutathione, Ceruloplasmin, and VitD3, in experimental groups.

Discussion

Our study found a significant decrease in RBCs, WBCs count in patients with Rheumatoid arthritis after consuming fresh carrot juice; the previous studies showed that autoimmune disorders and infections could result in a high white blood cell count, but a diet rich in vegetables can help reduce its levels reach to normal value White blood cells are important because they fight bacteria and viruses that could cause illness. A reduced count under average level may depress your immunity. A multivitamin, taken in conjunction with plenty of vegetables, can help you get the recommended daily intake of white blood cell-boosting nutrients. On a cellular level, dietary may influence various leukocytic effector functions, including adherence, migration, phagocytosis, and cytokine secretion. Several members of the selenoprotein family regulate or are regulated by cellular redox tone, a crucial modulator of immune cell signaling and function²⁰. So our study agreed with Shweta, and he follows (2017) they found that a fasting of 7–10 days with the partial nutrient intake of vegetable broth, herbal teas, parsley, garlic, and decoction of juice extracts from carrots, celery; and a controlled daily energy intake followed by 1 year of a vegan diet as compared to omnivorous diet was studied in different trials, Together these studies observed a remarkable decrease in swollen and tender joints, pain, erythrocyte sedimentation rate (ESR), and C-reactive protein²¹.

Dietary selenium (Se), mainly through its incorporation into selenoproteins, plays an essential role in inflammation and immunity. Adequate levels of Se are essential for initiating immunity, but they are also involved in regulating excessive immune responses and chronic inflammation. Evidence has emerged regarding roles for individual selenoproteins in regulating inflammation and immunity, and this has provided important insight into mechanisms by which Se influences these processes. Se deficiency has long been recognized to negatively impact immune cells during activation, differentiation, and proliferation. This is related to increased oxidative stress; RA is a chronic disease requiring long-term intake of drugs, including anti-rheumatics and non-steroidal anti-inflammatory drugs.

Patients with RA are prone to drop out of drug treatment due to the adverse effects. In RA, free radicals are associated with joint inflammation and damage. Antioxidant supplements and diets have long been advocated for the treatment and prevention of RA due to their protective role against free radicals²²; it is well-established the role of leptin as a growth factor for the monocytes, promoting phagocytic function and proliferation of circulating monocytes, inducing the production of pro-inflammatory cytokines (TNF- α , IL-6, and IL-12) and stimulating the oxidative burst as well as the chemotactic responses mediating the inflammatory infiltrate. On the other hand, ROS production in HIV-infected patients indicates pro-

grammed cell death in monocytes. Even though vitamins and selenium with antioxidant properties have been demonstrated to be beneficial to RA in a cellular study, there are contradicting results concerning the effects of antioxidant vitamins on the development of RA in animal and clinical studies. In the present study, the effects of selenium on the inflammatory cytokine networks were observed. When treated with a diet rich with selenium compared with selenium supplement, the levels of leptin, TNF- α , and IL-6 were significantly reduced in addition to reduce the C-RP and TNF- α levels of were significantly reduced increased. This suggests that vitamins suppress the inflammatory reaction in RA by increasing the levels of anti-inflammatory cytokines and reducing inflammatory cytokines. Due to the complicated cytokine networks in RA, cytokines interact by several signal transduction pathways. Therefore, the simulative effects of different vitamins and trace elements on inflammatory factors levels may be counteracted by the interaction between cytokines in RA^{23,24}.

Our study agreed with the previous study, showing that antioxidant agents were significantly higher in the selenium-treated sheep than in control in the samples taken 14 days after lambing and 30 days after lambing^{25,26}. Another paper showed that treatments of Selenium supplementation affected an increase in whole blood antioxidant agents and antioxidant enzyme glutathione peroxidase (GSH-Px) as well as increased plasma Selenium(Se) concentrations in experimental groups. On the other hand, the liver Se exhibited a dose-response relationship to treatment, but kidney Se concentrations were unaffected by treatment. Some dietary meat rich with Se concentrations can therefore be increased by supplementation and could contribute to increased human dietary intakes of the element²⁷, as well as carrot juice good source of carotenoids which act as antioxidants and help detoxify the system because antioxidants fight free radicals and reduce oxidative stress so they help stimulate metabolism^{28,29}. The data support a role for vitamin D deficiency in the development and progression of autoimmune inflammatory conditions in general, particularly RA. Earlier animal models indicate that the 1,25(OH)₂D₃ metabolite and its analogs may suppress collagen-induced arthritis. Other data suggest that vitamin D receptor agonists may also prevent and suppress established collagen-induced arthritis; however, data show that vitamin D may be negatively affected in acute response, that is, its levels may decrease in the setting of inflammation, such as inactive RA.³⁰⁻³².

Despite that, treatment with rituximab in RA did not affect vitamin D levels. However, it decreased indices of inflammation Supplementation with selenium induces a level of vitamin D in plasma blood which has been proposed to induce immune tolerance and thus prevent the development of autoimmune di-

seases³³. Recently, the combination of anti-rheumatic and the drugs of dietary supplement of selenium induce and increase the level of vitamin D has been suggested for RA Patients with RA are prone to osteoporosis as well as for its possible effects on disease activity^{34,35}.

Conclusions

Consumption of natural juice rich with selenium, vitamin D, and several antioxidants agents more beneficial in the treatment of Rheumatoid arthritis than taken artificial supplements should be taken with caution given its possible toxic effects that exceed its recommended consumption limits.

Conflict of Interests

The authors of this paper declare that it has no financial or personal relationships with individuals or organizations that would change unacceptably bias the content of this paper and therefore declare that there is no conflict of interests.

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RESEARCH / INVESTIGACIÓN

Perfil bacteriano del shock séptico en una unidad de cuidados intensivos de la altitud del seguro social del Perú

Bacterial profile of septic shock in an intensive care unit of the Peruvian social security altitude

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Resumen: Conocer el perfil bacteriano del shock séptico permitirá una adecuada elección de antibióticos empíricos. Objetivos: a) Describir el perfil bacteriano del shock séptico en una unidad de cuidados intensivos de la altitud. b) Conocer la localización de los cultivos positivos. c) Identificar la sensibilidad y el mecanismo de resistencia bacteriana. d) Encontrar diferencias de los perfiles bacterianos de la altitud. Estudio retrospectivo transversal. Realizado en una unidad de cuidados intensivos a 3,250 "msnm". Se incluyeron los cultivos positivos y antibiogramas de residentes de la altitud con shock séptico extraídos antes del inicio de los antibióticos durante 7 años. 1,212 muestras. *Escherichia coli* (18.48%). Las bacterias gramnegativas presentaron sensibilidad para colistina (94-99%) el principal mecanismo de resistencia fue betalactamasa de espectro extendido (43-91%). *Staphylococcus aureus* (22.19%). Las bacterias grampositivas presentaron sensibilidad para tigecilina, linezolid (100%) y vancomicina (36-100%) el principal mecanismo de resistencia fue ampicilina/sulbactam resistente productor de betalactamasa (50-97%) y meticilino resistente (87-100%). En Conclusión. - a) *Escherichia coli* la gramnegativa más frecuente y *Staphylococcus aureus* el grampositivo. b) El cultivo más frecuente provenía del tracto respiratorio inferior. c) De las gramnegativas, *Pseudomonas aeruginosa* mostró elevada sensibilidad para colistina, el resto también para tigecilina. El mecanismo de resistencia más frecuente fue betalactamasa de espectro extendido. Las Bacterias grampositivas tienen una elevada sensibilidad para tigecilina, linezolid y vancomicina. Su mecanismo de resistencia más frecuente fue ampicilina/sulbactam resistente. d) No encontramos diferencias de los perfiles bacterianos informados en la altitud. Recomendamos confirmar los resultados de sensibilidad "in vitro" de Tigeciclina.

Palabras clave: Altitud, bacteria, Perú, sepsis, shock, unidad de cuidados intensivos.

Abstract: Knowing the bacterial profile of septic shock allowed an adequate choice of empirical antibiotics. Objectives: a) To describe the bacterial profile of septic shock in an intensive care unit at altitude. b) Know the location of positive cultures. c) Identify the sensitivity and the mechanism of bacterial resistance. d) Find differences in the bacterial profiles of altitude. Retrospective cross-sectional study. Done in an intensive care unit at 3,250 "masl". Positive cultures and antibiograms from high-altitude residents with septic shock taken before antibiotics for 7 years were included. 1,212 samples. *Escherichia coli* (18.48%). Gram-negative bacteria showed sensitivity to colistin (94-99%), the primary resistance mechanism was extended-spectrum beta-lactamase (43-91%). *Staphylococcus aureus* (22.19%). Gram-positive bacteria showed sensitivity to tigecillin, linezolid (100%), and vancomycin (36-100%); the primary resistance mechanism was resistant ampicillin/sulbactam producer of beta-lactamase (50-97%) and resistant methicillin (87-100%). In Conclusions.- a) *Escherichia coli* is the most frequent gram-negative and *Staphylococcus aureus* the gram-positive. b) The most frequent culture came from the lower respiratory tract. c) Of the gram-negative ones, *Pseudomonas aeruginosa* showed a high sensitivity for colistin, the rest also for tigecillin. The most frequent resistance mechanism was extended-spectrum beta-lactamase. Gram-positive bacteria have a high sensitivity for tigecillin, linezolid, and vancomycin. Its most common resistance mechanism was resistant ampicillin/sulbactam. d) We did not find differences in the reported bacterial profiles at altitude. We recommend confirming the "in vitro" sensitivity results for Tigecycline.

Key words: Altitude, bacteria, Perú, sepsis, shock, intensive care units.

Introducción

El shock séptico tiene una prevalencia del 17.9%, una incidencia del 11.64 al 13.5 % y una mortalidad del 34.5 % del total de ingresos a la "unidad de cuidados intensivos" (UCI)^{1,2}. Del total de cultivos de los pacientes infectados 70 % son positivos³. Los cultivos identifican el patógeno y dirigen el tratamiento antibiótico, se recomienda su obtención antes de iniciar antibióticos⁴. Son cultivos positivos; las muestras de pacientes con cuadro clínico compatible con bacteriemia, Si la bacteria no es causa habitual de la contaminación se necesitará al me-

nos un cultivo y si la bacteria es causa común de la contaminación se necesitará al menos dos cultivos⁵. El inicio precoz de antibiótico empírico adecuado y eficaz en el shock séptico evita el aumento de su morbi-mortalidad⁶. La elección del antibiótico empírico depende de los antecedentes, del estado clínico del paciente, la localización de la infección y del perfil bacteriológico local que nos informan la prevalencia, susceptibilidad y mecanismo de resistencia⁴.

Definimos: Shock séptico; al estado de hipoperfusión tis-

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lar asociado a sepsis caracterizado por hipotensión o requerimiento de vasopresores para mantener una "presión arterial media" (PAM) ≥ 65 mmHg y un lactato sérico mayor de 2 mmol/L^{4,7}. Perfil bacteriano; al reconocimiento de la bacteria responsable de la infección diagnosticado por cultivo. Sensibilidad antimicrobiana; respuesta de la bacteria a los antibióticos determinada a través del antibiograma. Resistencia bacteriana; mecanismo mediante el cual la bacteria puede disminuir la acción de los antibióticos y se estudia a través del antibiograma⁸. Residente de la altitud; todo poblador que se encuentra viviendo en forma constante durante un año como minino por encima de los 1,500 "metros sobre el nivel del mar" (msnm)⁹.

A nivel mundial, Vincent et al (2009) evaluó 14,414 pacientes en 1.265 "UCIs" de 75 países, aislando "bacterias gram-negativas" (BGN) (62%), "bacterias grampositivas" (BGP) (47%) y hongos (19%). Los gérmenes más comunes fueron *Staphylococcus aureus*, *Pseudomonas*, *Escherichia coli*, *Klebsiella*, *Staphylococcus epidermidis* y *Acinetobacter species*³.

En Latinoamérica, en "UCIs" ubicados por debajo de 1,500 "msnm" se encontraron cuatro estudios, ellas reportaron una mayor frecuencia de "BGN". Molina et al. (2011- Colombia) estudio 826 pacientes y Gómez-Gonzales et al. (2018 – Pereira Colombia) estudio 62 pacientes encontrando como bacterias más habituales: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* y *Enterobacter cloacae*. En Perú Paz Rojas et al. (2008 – Lima) aisló 1,322 bacterias y Fernández-Mogollón et al. (2016 – Chiclayo) estudio 125 muestras reportando que las bacterias más frecuentes fueron *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Klebsiella pneumoniae*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Enterococcus faecium* y *Stenotrophomonas maltophilia*. *Klebsiella pneumoniae* fue sensible a imipenem, ertapenem, meropenem amikacina, trimetoprim/sulfametoazol y cefepime y resistente a ampicilina/sulbactam, piperacilina/tazobactam y cefazolina. *Pseudomonas aeruginosa* fue sensible a meropenen, y tobramicina y resistencia para ceftazidima, cefepime, amikacina y piperacilina/tazobactam. *Escherichia coli* fue sensible a ertapenem, meropenem, amikacina y tobramicina y resistente a ampicilina, cefazolina, piperacilina/tazobactam y trimetoprim/sulfametoazol. *Acinetobacter Baumanii* fue sensible a imipenem, meropenem y ampicilina/sulbactam¹⁰⁻¹³.

En Latinoamérica, en "UCIs" ubicados por encima de los 1,500 "msnm" se encontraron tres estudios encontrando que las bacterias más frecuentes eran las "BGN". Briceño et al. (2006 – Mérida; Venezuela) 1,630 "msnm", estudio 241 muestras, Quintanilla Chanez et al. (2011- Cochabamba, Bolivia) 2,558 "msnm", estudio 40 pacientes y Álvarez et al. (2006 – Bogotá, Colombia) 2,640 "msnm", estudio 18,407 microorganismos. Reportando que las bacterias más frecuentes fueron *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Escherichia coli* y *Staphylococcus epidermidis*. *Klebsiella pneumoniae* fue sensible a imipenem y resistente a ciprofloxacina, gentamicina y ceftazidima. *Pseudomonas aeruginosa* fue sensible a imipenem y resistente a ampicilina/sulbactam, ceftriaxona, cefotaxime, ceftriaxone, gentamicina y amikacina.; *Acinetobacter baumanii* fue sensible a imipenem y ampicilina/sulbactam y resistente a ciprofloxacina y amikacina. *Staphylococcus Aureus* fue sensible a vancomicina y resistente a gentamicina, cefotaxima, eritromicina, oxacilina y clindamicina. *Escherichia coli*; fue sensible a trimetoprim/sulfametoazol, ampicilina/sulbactam, cefazolina, ciprofloxacina, levofloxacina, y resistente a ampicilina^{14,15}. En Latinoamérica no ubicamos estudios ejecutados en "UCIs" por encima de los 3000 "msnm".

Nuestros objetivos son: a) Describir el perfil bacteriano de los pacientes con shock séptico en una unidad de cuidados intensivos de un hospital de la altitud del "seguro social del Perú" (EsSalud). b) Conocer la localización de la muestra más frecuente de los cultivos positivos. c) Identificar la sensibilidad y el mecanismo de resistencia bacteriana a los antibióticos más frecuente y d) Encontrar diferencias de los perfiles bacterianos de la altitud y del nivel del mar.

Materiales y métodos

Estudio observacional, descriptivo, retrospectivo y transversal. Realizado en la "UCI" del "Hospital Nacional Ramiro Priale Priale" de "EsSalud" con seis camas, ubicado en Huancayo a 3,250 "msnm" con una presión barométrica de 535 mmHg. Se analizaron los resultados de los cultivos y antibiogramas de un periodo de 7 años (2012-2018).

Se incluyeron los informes de los cultivos positivos y antibiogramas de los pacientes mayores de 18 años residentes de la altitud con shock séptico extraídos antes del inicio de los antibióticos, al ingreso a la "UCI". Se excluyeron los pacientes con otros tipos de shock asociados, informes de muestras repetidas y los informes incompletos. Para determinar cultivo positivo y susceptibilidad de los antibiogramas se siguieron las recomendaciones de la "Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica" (SEIMC)⁵. Las variables estudiadas fueron: shock séptico, perfil bacteriano, sensibilidad antimicrobiana y resistencia bacteriana. El procedimiento de recolección se inició con la identificación y confirmación de los casos que ingresaron a la "UCI" con shock séptico, luego de aplicar los criterios de inclusión y exclusión se revisaron las historias clínicas de los casos seleccionados para encontrar y analizar los informes de los cultivos y antibiogramas. Para el estudio de los cultivos y del antibiograma se utilizó un equipo VITEK 2-COMPAC de BIOMERIEUX con una autoclave, una incubadora y una refrigeradora.

El tamaño de la muestra calculada para siete años fue de 1,253 y fue determinado con una población de Huancayo de 545,615, una población con "EsSalud" de 147,470 que el promedio de ingresos a la "UCI" por año fue 354, que el shock séptico tiene una prevalencia puntual del 17,9% y una incidencia calculada del 13,5%, con un nivel de confianza del 95% y precisión del 5%¹². Se realizó un muestreo no probabilístico de casos consecutivos.

El análisis estadístico descriptivo univariado, evaluamos la normalidad de las variables cuantitativas numéricas usando métodos gráficos y estadísticos (shapiro wilks y Kolmogorov Smirnov) Las variables con normalidad se analizaron con media y desviación estándar, las variables sin normalidad se analizaron con mediana y rangos. Las variables cualitativas categóricas fueron analizadas utilizando frecuencias y porcentajes. El programa estadístico utilizado fue stata 14 oficial.

El estudio no generó riesgos, no se tuvo contacto directo con los pacientes, se basó en el análisis retrospectivo de resultados de laboratorio. Se codificó las historias clínicas y los resultados de laboratorio asegurando la confidencialidad y anonimato de los sujetos que ingresaron al estudio. Para la ejecución de la investigación se obtuvieron los permisos institucionales y aprobación de los comités de ética del Hospital Nacional Ramiro Priale Priale y de la Facultad de Medicina Humana de la Universidad Peruana Los Andes. Los autores auto-financiaron el estudio.

Resultados

Se identificaron 1,526 cultivos positivos con antibiograma de pacientes con shock séptico, luego de aplicar los criterios de exclusión quedaron 1,212 muestras de 826 pacientes. El principal motivo de exclusión fue muestra repetida. Se recolecto los cultivos durante el segundo semestre del 2019. Del 2012 al 2018 se observó un incremento progresivo del 39% de cultivos positivos, hay una proporción mayor del sexo masculino, una media de 70 años. Las "BGN" fueron las más frecuentes (58.25%). La bacteria gramnegativa más frecuente fue *Escherichia coli* (18.48%). La "BGP" más frecuente fue *Staphylococcus aureus* (22.19%). Las muestras más frecuentes provenían del tracto respiratorio inferior (aspirado traqueal y bronquial) (46.2%). Las muestras de cavidad abdominal (6.19%) provenían del estudio de líquido peritoneal, absceso intraabdominal, punción de páncreas y punción de hígado. Las muestras de piel (4.62%) provenían del estudio de herida operatoria obtenida durante la cura quirúrgica. (Tabla 1).

En el tracto respiratorio inferior las bacterias más frecuentes fueron *Staphylococcus aureus* (28.4%), *Acinetobacter Baumannii* (18 %) y *Escherichia Coli* (14.8 %). En Sangre (Hemocultivo) los más frecuentes fueron *Staphylococcus aureus* (22.9 %), *Staphylococcus epidermidis* (21.8 %) y *Escherichia Coli* (15.3 %). (Tabla 2).

Las "BGP" (a excepción de los enterococos) presentaron sensibilidad mayor al 90% para tigecilina, linezolid y vancomicina. Las "BGN" presentaron sensibilidad mayor al 90% para colistina.

Del total de muestras de *Staphylococcus aureus* (269): se estudiaron 266 para tigecilina, encontrando 100% de sensibilidad, se estudiaron 257 para linezolid encontrando 100 % de sensibilidad y se estudiaron 269 para vancomicina encontrando 99 % de sensibilidad. Del total de muestras de *Escherichia coli* (224) se estudiaron 95 para tigecilina encontrando 99 % de sensibilidad, se estudiaron 95 para colistina encontrando 99 % de sensibilidad y se estudiaron 222 para imipenem encontrando 95 % de sensibilidad (Tabla 3 y 4).

Los mecanismos de resistencia más frecuentes de las "BGP" están relacionado a la producción de enzimas hidrolíticas inhibitorias en el caso de la resistencia a la ampicilina/sulbactam resistente y a la producción de PBP (Penicilin Binding Protein) en el caso de la resistencia a la meticilina. (Tabla 5).

Los principales mecanismos de resistencia de las "BGN" son los relacionados a las betalactamasas de espectro extendido (Tabla 6).

Discusión

Lo reportado mundialmente no cambian a una altitud mayor a 3,000 "msnm", el mayor porcentaje son "BGN"¹⁶ *Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* y *Staphylococcus epidermidis* son responsables del 80% de las infecciones. La infección del tracto respiratorio fue el más frecuente.

Staphylococcus aureus

"BGP" más frecuente. La prevalencia en 1,400 "UCIs" europeas fue del 30% y de estos 70% fueron causados por "*Staphylococcus aureus* meticilino resistente" (MRSA). Nuestra prevalencia fue 22.19 % de *Staphylococcus aureus* y de estos 87 % son "MRSA". La Prevalencia de "MRSA" en "UCI" oscila entre 8% y 14%, nuestra prevalencia fue de 19% del total de 1,212

muestras. La incidencia de "MRSA" en la "UCI" han aumentado desde el 1961. En Estados Unidos entre 1989 y 2002, la proporción de "MRSA" en "UCI" aumentó del 29% al 60% convirtiéndose en un patógeno común en las "UCI" a nivel mundial. Weiner y Tanriover encontraron una mayor frecuencia del *Staphylococcus aureus* en infecciones en: catéter venoso central (51%), tracto urinario (52%), neumonías asociadas al ventilador (42%) y del sitio quirúrgico (43%). Nosotros encontramos frecuencias elevadas en el tracto respiratorio inferior pero mucho menor en sangre y catéter venoso central. Vancomicina sigue siendo un antibiótico eficaz, en los últimos años, fenotipos de resistencia adicionales han llevado a la introducción acelerada de agentes como linezolid y tigeciclina que muestran una mayor tasa de curación microbiológica e inhibición. Nuestros resultados mostraron una elevada sensibilidad para tigecilina, linezolid, vancomicina y sulfametoxzazol/trimetropim lo cual los mantiene como buenas opciones terapéuticas. Ciprofloxacino no es una buena opción por su muy baja sensibilidad contrario a lo reportado en otros estudios. Encontramos que el mecanismo de resistencia más frecuente fue ampicilina/sulbactam resistente, también reportamos dos casos de "*Staphylococcus aureus* intermedio a vancomicina" (VISA)^{17,18}.

"*Staphylococcus Coagulasa Negativo*" (ECN)

Encontramos a *Staphylococcus epidermidis*, *Staphylococcus hominis* y *Staphylococcus haemolyticus*, similar a reportes anteriores. Son frecuentes en pacientes inmunosuprimidos con afectación de la continuidad de la piel por accesos vasculares como catéter venosos centrales, su presencia en los hemocultivos está muy relacionado con contaminación en el momento de la punción. Nuestros resultados muestran elevada sensibilidad a tigecilina, linezolid y vancomicina similar a lo reportado. Encontramos mecanismos de resistencia elevados para ampicilina/sulbactam y meticilino resistente, superior a otros estudios similares que van del 50 al 80%^{19,20}.

Enterococos

Encontramos *Enterococo faecium* y *Enterococo faecalis* similar a reportes latinoamericanos. Los encontramos con mayor frecuencia en orina, informes anteriores lo informan con mayor frecuencia en cavidad abdominal. Nuestros resultados muestran elevada sensibilidad a tigecilina y linezolid similar a estudios anteriores, convirtiéndolos en buenas opciones terapéuticas sobretodo para "*Enterococcus* resistente a la vancomicina" (VRE). Los mecanismos de resistencia encontrados son elevada resistencia a meticilina, ampicilina/sulbactam (mayor a los antecedentes) y vancomicina. A nivel mundial se reportan enterococos vancomicina resistentes de hasta el 72% e incremento en la resistencia a la ampicilina^{12,21,22}.

Las "BGN" se ubicaron con mayor frecuencia en el tracto respiratorio inferior y la orina. Las "BGN" no fermentadores encontrados fueron *Acinetobacter Baumannii* y *Pseudomonas aeruginosa*. Excepto la *Pseudomonas aeruginosa* (que tuvo buena sensibilidad para colistina), los otros también tuvieron buena sensibilidad para tigecilina y meropenem. Su mecanismo de resistencia más frecuente fue "Betalactamasa de espectro extendido" (BLEE) y las carbapenemas.

Escherichia coli

"BGN" más frecuente. Su presencia en el tracto respiratorio inferior concuerda con el aumento de microorganismos no habituales en la neumonía adquirida en la comunidad. En orina es frecuente. Reportamos elevada sensibilidad para tigecilina,

	Frecuencia (#)	Porcentaje (%)
EDAD	70	18-96
AÑO		
2012	87	7.18
2013	155	12.79
2014	53	4.37
2015	142	11.72
2016	212	17.49
2017	222	18.32
2018	341	28.14
SEXO		
Femenino	326	39.47
Masculino	500	60.53
TIPO DE MUESTRA		
Tracto respiratorio inferior	560	46.2
Sangre	170	14.03
Orina	165	13.61
Catéter Venoso central	120	9.9
Cavidad abdominal	75	6.19
Piel	56	4.62
Líquido cefalorraquídeo	35	2.89
Líquido pleural	15	1.24
Heces	13	1.07
Otros	3	0.25
GRAM		
Negativo	706	58.25
Positivo	506	41.75
BACTERIAS		
<i>Staphylococcus aureus</i>	269	22.19
<i>Escherichia coli</i>	224	18.48
<i>Acinetobacter baumannii</i>	150	12.38
<i>Klebsiella pneumoniae</i>	134	11.06
<i>Pseudomonas aeruginosa</i>	109	8.99
<i>Staphylococcus epidermidis</i>	85	7.01
<i>Enterococcus faecium</i>	36	2.97
<i>Staphylococcus hominis</i>	32	2.64
<i>Enterococcus faecalis</i>	31	2.56
<i>Staphylococcus haemolyticus</i>	29	2.39
Otros	113	9.33
n= 1212		

Table 1. Características generales del perfil bacteriano.

Bacteria	TRI	S	O	CVC	CA	P	LCR	LP	H	Ot	Total
	#	#	#	#	#	#	#	#	#	#	#
	%	%	%	%	%	%	%	%	%	%	%
<i>Staphylococcus</i>	159	39	5	32	8	16	5	3	1	1	269
<i>Aureus</i>	28.4	22.9	3.0	26.7	10.7	28.6	14.3	20.0	7.7	33.3	22.2
<i>Escherichia</i>	83	26	61	8	25	13	2	1	5	0	224
<i>Coli</i>	14.8	15.3	37.0	6.7	33.3	23.2	5.7	6.7	38.5	0.0	18.5
<i>Acinetobacter</i>	101	10	14	8	2	10	3	2	0	0	150
<i>Baumannii</i>	18.0	5.9	8.5	6.7	2.7	17.9	8.6	13.3	0.0	0.0	12.4
<i>Klebsiella</i>	76	8	16	8	11	7	2	1	3	2	134
<i>Pneumoniae</i>	13.6	4.7	9.7	6.7	14.7	12.5	5.7	6.7	23.1	66.7	11.1
<i>Pseudomonas</i>	64	3	20	7	8	4	3	0	0	0	109
<i>Aeruginosa</i>	11.4	1.8	12.1	5.8	10.7	7.1	8.6	0.0	0.0	0.0	9.0
<i>Staphylococcus</i>	3	37	1	26	6	2	6	4	0	0	85
<i>Epidermidis</i>	0.5	21.8	0.6	21.7	8.0	3.6	17.1	26.7	0.0	0.0	7.0
<i>Enterococcus</i>	8	2	14	5	4	0	3	0	0	0	36
<i>Faecium</i>	1.4	1.2	8.5	4.2	5.3	0.0	8.6	0.0	0.0	0.0	3.0
<i>Staphylococcus</i>	5	20	0	3	2	0	1	1	0	0	32
<i>Homini</i>	0.9	11.8	0.0	2.5	2.7	0.0	2.9	6.7	0.0	0.0	2.6
<i>Enterococcus</i>	5	1	19	3	2	0	1	0	0	0	31
<i>Faecalis</i>	0.9	0.6	11.5	2.5	2.7	0.0	2.9	0.0	0.0	0.0	2.6
<i>Staphylococcus</i>	4	10	3	8	1	1	2	0	0	0	29
<i>Haemolyticus</i>	0.7	5.9	1.8	6.7	1.3	1.8	5.7	0.0	0.0	0.0	2.4
Otros	52	14	12	12	6	3	7	3	4	0	113
	9.3	8.3	7.3	10.0	8.0	5.4	20.0	20.0	30.8	0.0	9.3
Total	560	170	165	120	75	56	35	15	13	3	1212
	100	100	100	100	100	100	100	100	100	100	100

TRI: Tracto respiratorio inferior, **S:** Sangre, **O:** Orina, **CVC:** catéter venoso central, **CA.** Cavidad abdominal, **P:** Piel, **LCR:** Líquido cefalorraquídeo, **LP:** Líquido pleural, **H:** Heces, **Ot:** Otros.

Tabla 2. Frecuencia de las bacterias según tipo de muestra.

colistina, imipenem, meropenem y ertapenem. Los carbapenémicos se reservan para el shock séptico por *Escherichia coli* productor de "BLEE". El principal mecanismo de resistencia fue "BLEE" muy superior a lo reportado^{10,23,24}.

Klebsiella pneumoniae

Su presencia en el tracto respiratorio inferior y en el tracto urinario está relacionado a cepas hipervirulenta. Reportamos una elevada sensibilidad para tigecilina, colistina, imipenem, meropenem y ertapenem. Tigecilina antimicrobiano bacterios-tático de amplio espectro se usa en infecciones complicadas de piel, tejidos blandos, neumonía adquirida en la comunidad y de infecciones intrabdominales debidas a *Klebsiella pneumoniae* carbapenemasa. Su uso en infecciones del tracto urinario es limitado. Colistina es una opción frente a una *Klebsiella pneumoniae* carbapenemasa de resistencia extendida. ceftazidima o cefepime no son opciones empíricas por su elevada resistencia. El mecanismo de resistencia más detectado fue

"BLEE" muy superior a los reportados. En el nivel de resistencia, encontramos *Klebsiella pneumoniae* con "resistencia extendida" (XDR) que son inferiores a otros estudios^{10,25,26}.

Acinetobacter Baumannii

Su presencia en tracto respiratorio inferior está relacionada con edad avanzada, enfermedad pulmonar crónica, inmunosupresión y uso de antimicrobianos. En orina están asociados a catéteres urinarios permanentes. Reportamos una elevada sensibilidad para tigecilina, importante en el tratamiento de peritonitis secundaria comunitaria o nosocomial por *Acinetobacter baumannii* "multirresistentes" (MDR) y para colistina que nos permite un tratamiento eficaz para *Acinetobacter baumanii* "MDR". Se informan elevadas resistencias a gentamicina, esto no concuerda con nuestros resultados^{27,28}. Gentamicina por vía inhalatoria alcanza buenas concentraciones en el árbol bronquial con menores efectos secundarios que por vía sistémica^{27,28}. Meropenem aún es una buena opción terapéutica.

Bacteria	Total de Muestras	TIG	LIN	COL	VAN	MER	ERT	IMI	GEN	MOX	AMI
		#	#	#	#	#	#	#	#	#	#
		S%									
<i>Staphylococcus aureus</i>	269	266	257	266	269	269	269	266	269	269	224
<i>Escherichia Coli</i>	224	95	0	95	0	221	221	222	222	223	221
<i>Acinetobacter baumannii</i>	150	71	0	71	0	15	15	141	141	54	0
<i>Klebsiella pneumoniae</i>	134	80	0	80	0	134	134	134	132	134	129
<i>Pseudomonas aeruginosa</i>	109	49	0	49	0	64	64	106	100	91	100
<i>Staphylococcus epidermidis</i>	85	84	83	84	85	85	85	85	85	85	61
<i>Enterococcus faecium</i>	36	31	32	31	36	33	33	31	0	0	36
<i>Staphylococcus homini</i>	31	24	25	24	31	31	31	31	0	0	29
<i>Faecalis</i>	29	26	28	26	29	29	29	29	29	29	25
<i>Haemolyticus</i>	100	100	0	100	7	7	7	0	72	0	

TIG: Tigeciclina, **LIN:** Linezolid, **COL:** Colistina, **VAN:** Vancomicina, **MER:** Meropenem, **IMI:** Imipenem, **GEN.** Gentamicina, **MOX.** Moxifloxacino, **AMI:** Amikacina. #: Número de muestras estudiadas, %: Porcentaje de sensibilidad, NE: No Estudiado

Tabla 3. Sensibilidad de las bacterias según antibiótico.

Reportamos una sensibilidad baja para imipenem y ampicilina/sulbactam, lo cual no concuerda a las recomendaciones que indican a estos como de elección. El principal mecanismo de resistencia fue la producción de carbapenemasa y el nivel de resistencia fue "MDR" superior a lo reportado^{27,28}.

Pseudomona Auriginosa

Causa entre 1.8% a 8.3% de neumonías comunitarias graves que ocasionan ingreso a "UCI" cuya letalidad va del 50% al 100%. Son la tercera causa de infecciones urinarias adquiridas asociadas a catéter. La sensibilidad para cefepime, ceftazidima, aztreonam, imipenem, meropenem, ciprofloxacina y gentamicina fueron muy bajas contrario a otros reportes. La sensibilidad para colistina fue elevada, lo cual nos permitirá aun un efectivo tratamiento contra cepas "MDR" y "XDR". Los mecanismos de resistencia más frecuentes fueron: "BLEE", productores de carbapenemasas y betalactamasas tipo AMPc^{10,11,29,30}.

Controlamos el sesgo de selección utilizando un "método de precisión" con criterios de inclusión basados en definiciones internacionales, para el perfil bacteriano se usaron las defini-

ciones de la "SEIMC" y para el shock séptico los parámetros The Third International Consensus Definitions for Sepsis and Septic Shock, lo cual aseguro la representatividad de nuestra muestra. El uso de un muestreo no probabilístico de casos consecutivos nos aseguró una adecuada proporción de exámenes para el análisis final. Para evitar los sesgos de medición, debido a datos retrospectivos, usamos un "método de estandarización". Los datos fueron recolectados por médicos especialistas con experiencia en la evaluación de cultivos y antibiogramas que unificaron su metodología. Sin embargo, no se pudo evitar las limitaciones por el menor número de muestras procesadas el 2014 por disminución en el abastecimiento de reactivos. Tampoco pudimos determinar los genes de resistencia en gérmenes "MDR", "XDR", "VISA" y productores de carbapenemasas, ni tuvimos control sobre la toma de cultivos. Para evitar los sesgos de confusión se utilizaron criterios de exclusión basado en un "método de restricción" que evitó el ingreso de muestras repetidas o de mala calidad. Los resultados del presente estudio descriptivo trasversal podrían extrapolarse a otras "UCIs" de los andes ubicados por encima de los 3,000 "msnm" en pacientes residentes de la altitud que cursan con shock séptico

Bacteria	Total, de Muestras	NIT	TET	TS	TOB	LEV	CIP	CEP	AZT	CEF	AS
		#	#	#	#	#	#	#	#	#	#
		S%									
<i>Staphylococcus aureus</i>	269	269	269	269	72	269	269	245	0	81	245
<i>Escherichia Coli</i>	224	204	221	220	208	222	223	224	156	222	221
<i>Acinetobacter baumannii</i>	150	118	11	141	119	148	150	141	144	133	141
<i>Klebsiella pneumoniae</i>	134	107	7	132	110	128	133	133	134	130	133
<i>Pseudomonas aeruginosa</i>	109	78	48	98	80	106	106	100	14	85	108
<i>Staphylococcus epidermidis</i>	85	85	85	85	16	85	85	71	0	23	71
<i>Enterococcus faecium</i>	36	36	36	0	9	36	36	29	0	9	30
<i>Staphylococcus hominis</i>	32	32	32	32	4	32	32	27	0	6	27
<i>Enterococcus faecalis</i>	100	44	53	0	25	9	7	NE	0	7	28
<i>Staphylococcus haemolyticus</i>	31	31	31	0	11	31	31	28	0	11	28
	68	0	NE	0	19	0	0	NE	0	50	
	29	29	29	29	8	29	29	25	0	10	25
	100	24	34	0	14	7	8	NE	0	8	

NIT: Nitrofurantoina, TET: Tetraciclina, TS: Trimetoprim/Sulfametoazol, TOB: Tobramicina, LEV: Levofloxacino, CIP: Ciprofloxacino, CEP: Cefepima, AZT: Aztreonam, CEF: Ceftazidima, AS: Ampicilina/Subactam. #: Número de muestras estudiadas, S%: Porcentaje de sensibilidad, NE: No Estudiado

Tabla 4. Sensibilidad de las bacterias según antibiótico.

ya que se realizó durante un periodo de 07 años en un hospital ubicado a 3,250 "msnm". Entre nuestras fortalezas destacamos que describimos la positividad de los cultivos asociados a un análisis bacteriano del mecanismo de resistencia, dato no observado en otros estudios. La relevancia de nuestro estudio se sustenta en la descripción de la realidad bacteriológica de una "UCI" ubicado por encima de los 3,000 "msnm" con una muestra grande que no distan de reportes mundiales que pone en evidencia una común problemática, el uso inadecuado de antibióticos generadores de multirresistencia.

Conclusiones

Encontramos: a) Un mayor porcentaje de bacterias gramnegativas. *Escherichia coli* fue la bacteria gramnegativa más frecuente y *Staphylococcus aureus* el grampositivo b) El cultivo positivo más frecuente estuvo relacionado a muestras del tracto respiratorio inferior. c) Dentro de las bacterias gramnegativas, *Pseudomonas aeruginosa* mostró elevada sensibilidad solo para colistina, el resto también para tigecilina. El mecanismo de resistencia más frecuente fue betalactamasa de espectro extendido. Las bacterias grampositivas tienen una elevada sensibilidad para tigecilina, linezolid y vancomicina. Su mecanismo de resistencia estuvo relacionado a una elevada resistencia para la ampicilina/sulbactam. d) No encontramos

diferencias de los perfiles bacterianos informados en la altitud y los del nivel del mar. Conocer el diagnóstico bacteriológico, la sensibilidad y la resistencia bacteriana a los antibióticos en el shock séptico nos dará un parámetro para la elección del inicio de la terapia antibiótica empírica dentro de la "UCI" y colaborar en la disminución de la mortalidad de estos pacientes críticos. Este estudio inicial será el primer paso para generar estudios prospectivos posteriores. Recomendamos a) Tomar en cuenta estos resultados para elegir empíricamente los antibióticos frente a un paciente que ingrese a "UCI" con shock séptico y b) Confirmar nuestros resultados de una elevada sensibilidad "in vitro" de Tigecilina con otros estudios analíticos.

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	Ampicilina Sulbactam (#/%)		Meticilina (*) (#/%)		Vancomicina (#/%)			Linezolid (#/%)	
	S	R	S	R	S	I	R	S	R
Enterococcus									
<i>Enterococcus faecium</i>	1 (3%)	29 (97%)	0 (0%)	9 (100%)	13 (36%)	0 (0%)	23 (64%)	32 (100%)	0 (0%)
<i>Enterococcus faecalis</i>	14 (50%)	14 (50%)	0 (0%)	11 (100%)	18 (58%)	0 (0%)	13 (42%)	25 (100%)	0 (0%)
			MSSA	MRSA		VISA	VRSA		
Staphylococcus Coagulasa Positivo									
<i>Staphylococcus aureus</i>	30 (12%)	215 (88%)	35 (13%)	234 (87%)	267 (99%)	2 (1%)	0 (0%)	257 (100%)	0 (0%)
<i>Staphylococcus Coagulasa Negativo</i>									
<i>Staphylococcus epidermidis</i>	2 (3%)	69 (97%)	0 (0%)	23 (100%)	80 (94%)	2 (2%)	3 (4%)	83 (100%)	0 (0%)
<i>Staphylococcus hominis</i>	2 (7%)	25 (93%)	0 (0%)	4 (100%)	32 (100%)	0 (0%)	0 (0%)	31 (100%)	0 (0%)
<i>Staphylococcus haemolyticus</i>	2 (8%)	23 (92%)	0 (0%)	8 (100%)	29 (100%)	0 (0%)	0 (0%)	28 (100%)	0 (0%)
(*) Resistente a Cefoxitina, R: Resistente, S: Sensible, I: Intermedio, MSSA: <i>Staphylococcus aureus</i> meticilino sensible. MRSA: <i>Staphylococcus aureus</i> meticilino resistente VISA: <i>Staphylococcus aureus</i> intermedio a vancomicina VRSA: <i>Staphylococcus aureus</i> resistente a vancomicina.									

Tabla 5. Mecanismos de Resistencia de las Grampositivas.

	MECANISMOS DE RESISTENCIA				NIVELES DE RESISTENCIA	
	BETALACTAMASA		CARBAPENEMASA	CIERRE DE PORINAS	XDR	MDR
	BLEE	AMPc				
<i>Escherichia Coli</i>	172 (77%)		12 (5%)			
<i>Klebsiella pneumoniae</i>	56 (42%)				9 (7%)	
<i>Acinetobacter baumannii</i>			116 (77 %)		3 (2%)	132 (88%)
<i>Pseudomonas aeruginosa</i>	99 (91%)	24 (22%)	79 (72%)	4 (4%)		
BLEE: Betalactamasa de espectro extendido. AMPc: betalactamasas tipo. AMPc. XDR: Resistencia extendida. MDR: Multirresistente.						

Tabla 6. Mecanismos de Resistencia de los Bacilos Gramnegativas.

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RESEARCH / INVESTIGACIÓN

Análisis estadístico de las enfermedades asociadas a la mortalidad por COVID-19 en un Hospital de Ecuador durante el año 2020

Statistical analysis of diseases associated with mortality due to COVID-19 in a Hospital in Ecuador during the year 2020

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2242

Resumen: En el presente estudio se implementó un modelo de regresión logística para analizar algunos factores asociados con el riesgo de muerte debido al COVID-19 en un hospital de Ecuador. Los factores y variables consideradas son la edad, el diagnóstico presuntivo y la asignación a una unidad de contingencia viral de pacientes en un hospital de Ecuador. También se realizó un análisis exploratorio que incluyó una visualización de series temporales para determinar el comportamiento de las muertes por sintomatología durante el año 2020. Se analizaron un total de 58536 pacientes fallecidos, de los cuales 319 pacientes murieron en área COVID-19 del hospital. El estudio concluye que hay un grupo particular de enfermedades categorizadas por la Clasificación Estadística Internacional de Enfermedades y Problemas de Salud Conexos (ICD) que están más relacionadas con el riesgo de muerte debido al COVID-19, junto con la asignación de dependencia, así como determinada edad y sexo del paciente.

Palabras clave: Regresión logística, riesgo de muerte, COVID-19, diagnóstico presuntivo, Clasificación Internacional de Enfermedades.

Abstract: In this study, a logistic regression model was implemented to analyze the associated factors with the death risk because of COVID-19, such as sex, age, presumptive diagnosis, and the viral contingency assignment to a viral contingency unit of patients a hospital in Ecuador. An exploratory analysis including a time series visualization was also made to determine the frequency of the deaths due to symptoms during the year 2020 in this hospital. For this study, 58536 deceased patients were analyzed, of which 319 patients died in the COVID-19 area of the hospital. The study concludes that a particular group of diseases categorized by the International Statistical Classification of Diseases and Related Health Problems (ICD) are more related to the death risk because of COVID-19, together with the dependency assignment, age, and sex of the patient.

Key words: Logistic Regression, death risk, COVID-19, presumptive diagnosis, International Classification of Diseases.

Introducción

El SARS-CoV-2 es parte de la familia de los coronavirus, los cuales no suelen afectar a los humanos¹; sin embargo, en los últimos años esta variante del virus ha infectado de forma severa a la humanidad, a través de la enfermedad denominada COVID-19². Tanto este virus, sus signos, así como los síntomas que provoca, eran poco conocidos antes de que estallara el brote en Wuhan (China) en diciembre de 2019. Desde el 11 de marzo del 2020 la Organización Mundial de la Salud OMS decretó al COVID-19 como pandemia a nivel mundial³, esto debido al alto número de muertes a escala global³, generadas por la propia enfermedad, así como por otras enfermedades catastróficas que al no ser tratadas a tiempo ocasionaron la muerte de quienes la padecían, enfermedades tales como hipertensión, problemas cardiovasculares, diabetes mellitus, cáncer y otras⁴.

Existen varias investigaciones acerca del incremento en la mortalidad por COVID-19 cuando se padece de enfermedades preexistentes⁴⁻⁷. Según un estudio la tasa de letalidad en China se incrementó en aquellos pacientes con enfermedades preexistentes: 10.5% para enfermedad cardiovascular, 7.3% para diabetes, 6.3% para enfermedad respiratoria crónica, 6.0% para hipertensión y 5.6% para cáncer⁸. Otro estudio rea-

lizado en Italia por el Instituto Superior de Sanidad de ese país en el cual se analizaron 355 muertes y de ellos sólo un 0.8% no tenían enfermedades preexistentes, mientras que el 99% tenía alguna enfermedad crónica preexistente (hipertensión arterial 76%, diabetes 36%, cardiopatías 33%)⁹.

Por otra parte, Ecuador se enfrenta frecuentemente con enfermedades propias del subdesarrollo como la desnutrición y las infecciones gastrointestinales; además, presenta enfermedades propias de países desarrollados tales como hipertensión, cáncer, obesidad y otras. Según los datos oficiales del Ministerio de Salud Pública del Ecuador, al 3 de agosto del 2021 se registran 487702 contagiados de COVID-19, de los cuales 31644 fallecieron. Además, según datos presentados por el Instituto Nacional de Estadísticas y Censos del Ecuador, las enfermedades crónicas como cáncer, del sistema circulatorio y del sistema digestivo ocupan los primero cinco lugares de morbilidad a partir de los 40 años, sin dejar de tener importancia en edades menores^{10,11}.

Con estos antecedentes, el presente estudio tiene como objetivo analizar los registros de las muertes que ocurrieron en un hospital de segundo nivel en Quevedo, Ecuador durante el año 2020 con el fin de identificar la relación entre la causa de

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muerte con el diagnóstico de COVID-19 así como de los síntomas que presentaron de acuerdo con la clasificación Internacional de Enfermedades¹².

Este estudio es de tipo descriptivo correlacional y explicativo, pues permite describir, relacionar e indagar acerca de las enfermedades asociadas a la mortalidad por COVID-19. Para este fin se aplicará el modelo de regresión logística ya que tiene múltiples aplicaciones, ya sea en el campo financiero, psicológico, educacional, en salud, entre otras¹³⁻¹⁵. En el área de salud hay varias investigaciones realizadas, entre ellas, hay una en particular donde se usa regresión logística para indagar si diversos factores socioeconómicos y culturales inciden en la probabilidad de que una mujer embarazada contraiga la enfermedad de Chagas¹⁶.

Métodos

Esta investigación analiza la base de datos de defunciones del año 2020 proporcionada por un hospital de Quevedo - Ecuador. Esta base de datos contiene las siguientes variables: Fallecido en área COVID, Sexo, Edad*, Enfermedades (DiagPre1**) y Contingencia Viral (ContViral***).

*Edad: para incorporar esta variable al estudio, se agruparon las edades considerando la clasificación etaria de la pro-

ducción estadística y epidemiológica de la unidad médica, la misma que es regulada por el Ministerio de Salud Pública del Ecuador (MSP). Los grupos de edad del estudio son: Menor a un mes, de uno a doce meses, de 1 a 14 años, de 15 a 40 años, de 41 a 60 años y más de 61 años.

**DiagPre1 representa el diagnóstico presuntivo 1, es decir hace referencia a un diagnóstico médico basado en una suposición razonable. Es el diagnóstico dado como primer paso por el médico tras la entrevista clínica, en donde se registran los síntomas que declara el paciente y los signos que el médico percibe durante la auscultación médica. Además, es un diagnóstico hipotético sobre las posibles causas del malestar o enfermedad del paciente. Luego, se confirma o descarta este diagnóstico con análisis adicionales realizados al paciente, con lo que se convierte en un diagnóstico de certeza o diagnóstico definitivo.

***ContViral: Contingencia Viral es una dependencia del área de emergencia creada a raíz del primer caso identificado de COVID-19 en la unidad médica para la diferenciación y tratamiento de los casos respiratorios de las demás emergencias hospitalarias como medida de prevención contra la contaminación cruzada.

La tabla 1 presenta la clasificación de enfermedades según la clasificación Internacional de Enfermedades¹²:

GRUPO	ENFERMEDAD DIAGPRE1	DESCRIPCIÓN
G1	"A" "B"	Ciertas enfermedades infecciosas y parasitarias
G2	"I"	Enfermedades del sistema circulatorio
G3	"J"	Enfermedades del sistema respiratorio
G4	"M"	Enfermedades del sistema osteomuscular y del tejido conjuntivo
G5	"R"	Síntomas, signos y hallazgos anormales clínicos y de laboratorio, no clasificados en otra parte
G6	"U"	Códigos para propósitos especiales, categoría asignada a pacientes por Covid-19.
G7	"C" "D" "E" "F" "G" "H" "I" "K" "L" "N" "O" "P" "Q" "S-T" "V-W-X-Y" "Z"	Tumores [neoplasias] Enfermedades de la sangre y de los órganos hematopoyéticos, y ciertos trastornos que afectan el mecanismo de la inmunidad Enfermedades endocrinas, nutricionales y metabólicas Trastornos mentales y del comportamiento Enfermedades del sistema nervioso Enfermedades del ojo y sus anexos y Enfermedades del oído y de la apófisis mastoides Enfermedades del sistema circulatorio Enfermedades del sistema digestivo Enfermedades de la piel y del tejido subcutáneo Enfermedades del sistema genitourinario Embarazo, parto y puerperio Ciertas afecciones originadas en el período perinatal Malformaciones congénitas, deformidades y anomalías Cromosómicas Traumatismos, envenenamientos y algunas otras consecuencias de causas externas Causas externas de morbilidad y de mortalidad Factores que influyen en el estado de salud y contacto con los servicios de salud

Tabla 1. Grupos y Clasificación de Enfermedades.

Análisis estadístico

Inicialmente se procedió con el pretratamiento de datos, eliminando datos duplicados y faltantes seguidamente se analizaron de forma descriptiva las variables para identificar el comportamiento de los fallecimientos. Luego se obtuvo un modelo de regresión logística, el cual permitió evaluar la relación de cada una de las enfermedades categorizadas de acuerdo con la Clasificación Internacional de enfermedades (ver tabla 1) y la muerte de un paciente en área COVID.

Este modelo también permitió obtener estimaciones de la probabilidad de un suceso, identificar los factores de riesgo que determinan dichas probabilidades, así como la influencia o peso relativo que éstos tienen sobre las mismas¹³. En general, en este estudio se aplica la regresión logística porque se trabaja con un resultado binario del cual sabemos que existen una gran cantidad de variables que pueden incidir sobre él.

En este modelo la variable dependiente de tipo dummy es "murió en área COVID", y se busca analizar la influencia de las variables predictivas sobre ella.

El modelo logit, es expresado como sigue:

$$\ln\left(\frac{p_j}{1-p_j}\right) = \vec{X}_j^t \vec{\beta} + \epsilon. \quad (1)$$

Básicamente, el modelo indica el comportamiento de los momios (relación entre la probabilidad de éxito y fracaso,

$$\frac{p_j}{1-p_j} \text{ en relación con los valores de } \vec{X}_j$$

En este estudio, a fin de medir la bondad del modelo, se graficaron las autocorrelaciones parciales de los residuos deviance, además se hizo el contraste de hipótesis:

H_0 : El modelo está cerca a los datos
vs

H_1 : El modelo está lejano a los datos (2)

El estadístico de prueba utilizado para (2) es el residual deviance, al cual lo denotamos como D, que tiene una distribución ji cuadrada (χ^2) con $n-p$ grados de libertad, donde n es el número de observaciones y p es el número de coeficientes estimados del modelo¹³.

La diferencia de los residual deviance entre modelos fue usada también para comparar las verosimilitudes de un modelo corto versus otro con más variables. En esta situación el contraste de hipótesis esta dado por:

H_0 : El modelo corto con q variables explicativas
vs
 H_1 : El modelo largo con p variables explicativas (3)

Siendo $q < p$ y el estadístico de prueba la diferencia entre los residual deviance de los modelos bajo H_0 y $H_1(D_0-D_1)$ que tiene una distribución ji cuadrada (χ^2) con $p-q$ grados de libertad.

Otra prueba de bondad de ajuste utilizada es la prueba de Hosmer-Lemeshow¹³, la cual permite contrastar si la modelación en regresión logística se ajusta a los datos; de manera general, permite evaluar si la tasa de eventos observados coincide o no con las tasas de eventos esperados en subgrupos de la población. El contraste de hipótesis es:

H_0 : El modelo es adecuado
vs
 H_1 : El modelo no es adecuado (4)

Y utiliza un estadístico ji cuadrada (χ^2) con $G-2$ grados de Libertad (5)

$$\chi^2 = \sum_{i=1}^G \frac{(O_i - E_i)^2}{E_i} \quad (5)$$

donde G es el número de subintervalos en los que se ha dividido el intervalo [0,1], representan las frecuencias observadas y esperadas dentro de cada subintervalo.

Para la obtención del modelo se utilizó el 70% de los datos, los cuales fueron seleccionados de manera aleatoria; el 30% restante se utilizó para la validación del modelo seleccionado. Para todas las pruebas se utilizó un nivel de significancia del 5%. El análisis se realizó con el software estadístico RStudio¹⁷.

Resultados

Luego de la respectiva depuración de la base de datos, esta quedó con la siguiente información: 58536 muertes, de los cuales 319 pacientes murieron en área COVID-19 del hospital; 115 mujeres y 204 hombres.

Análisis descriptivo

En el siguiente análisis descriptivo se presentan gráficos con la frecuencia absoluta del número de fallecidos por tipos de enfermedad (ver tabla 1), meses, sexo y grupo etario, así como un gráfico de cajas y bigotes para observar la frecuencia relativa de los fallecidos en área COVID-19, según determinadas características.

A continuación, en la figura 1 se muestra el gráfico del número de muertes por mes por grupo de enfermedad.

De los datos analizados, se identifica que, los meses abril, mayo, junio, julio, del año 2020 presentan un mayor número de muertes considerando un diagnóstico presuntivo de tipo de enfermedad de los grupos G6, G5 y G3, los cuales, tal como se describen en la tabla 1, son: G6: Propósitos especiales, categoría asignada a pacientes por COVID-19, G5: Síntomas, signos y hallazgos anormales clínicos y de laboratorio, no clasificados en otra parte y G3: Enfermedades del sistema respiratorio, respectivamente. Se aprecia un incremento en las muertes a partir de los meses abril y julio durante la primera ola de la pandemia.

Al llevarse a cabo la comparación entre ambos sexos de la población analizada se observa que, los hombres son los que han fallecido en mayor número (ver figura 2).

Los resultados en cuanto a la muerte por el tipo de enfermedades son similares a las de la figura 1.

Para analizar por edad al grupo de fallecidos, se utilizó la clasificación etaria propuesta por el MSP del Ecuador, descrita en la sección Metodología de este documento. Los resultados obtenidos se presentan en la figura 3.

Luego del análisis observado se identifica fallecimientos en la población de estudio a partir del año, estando la concentración de datos en la población mayor a 61 años, con tipo de enfermedad de los grupos G6, G5 y G3.

En general para el análisis presentado en las figuras 1, 2 y 3, coinciden proporcionalmente los tres grupos de enfermedades.

En la figura 4, se puede apreciar a través de los gráficos de cajas y bigotes, la distribución de los fallecidos en área COVID-19 diferenciados por edad, sexo y grupo de enfermedades preexistentes.

Regresión Logística

Se estimaron diversos modelos de regresión logística para lo cual se seleccionó de manera aleatoria el 70 por ciento de los datos para entrenamiento, es decir 40974 registros. A continuación, se muestran los modelos analizados:

El primero considera como variables predictivas: la edad, el género o sexo (1: Mujer, 0: Hombre), el grupo de enfermedad

Fallecidos en un Hospital de Quevedo

2020

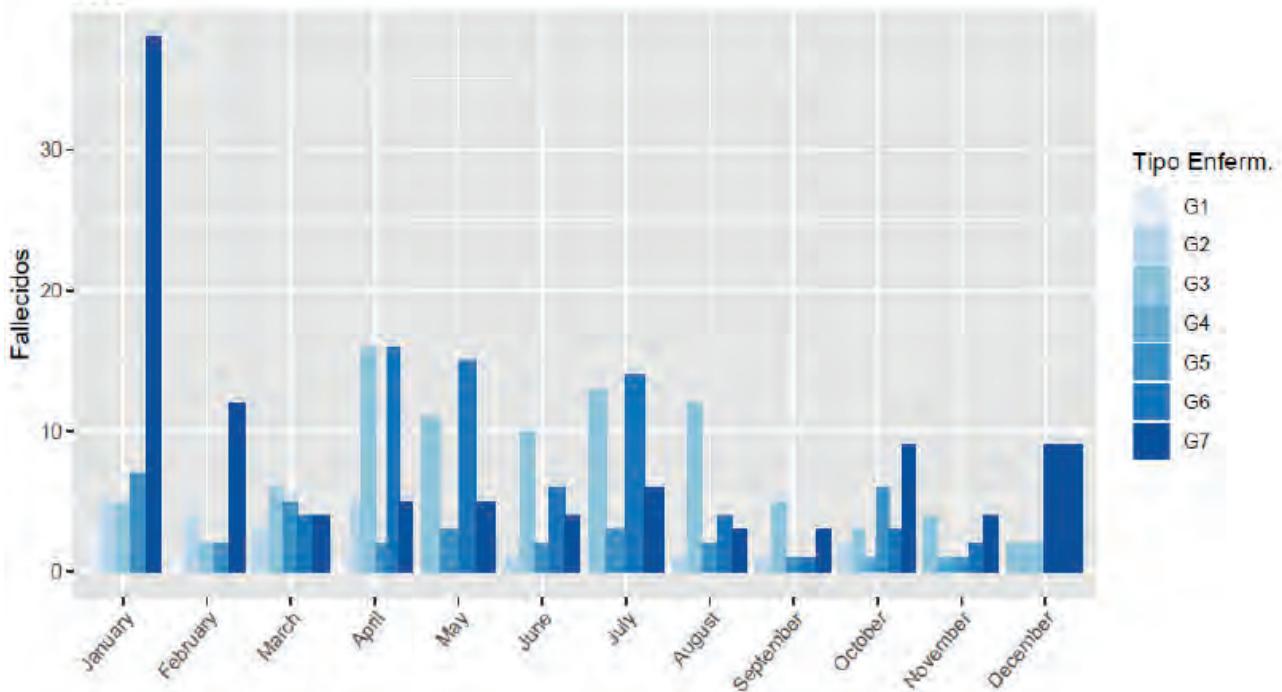


Figura 1. Número de fallecidos mensual por grupo de enfermedad.

Fallecidos en un Hospital de Quevedo por Genero

2020

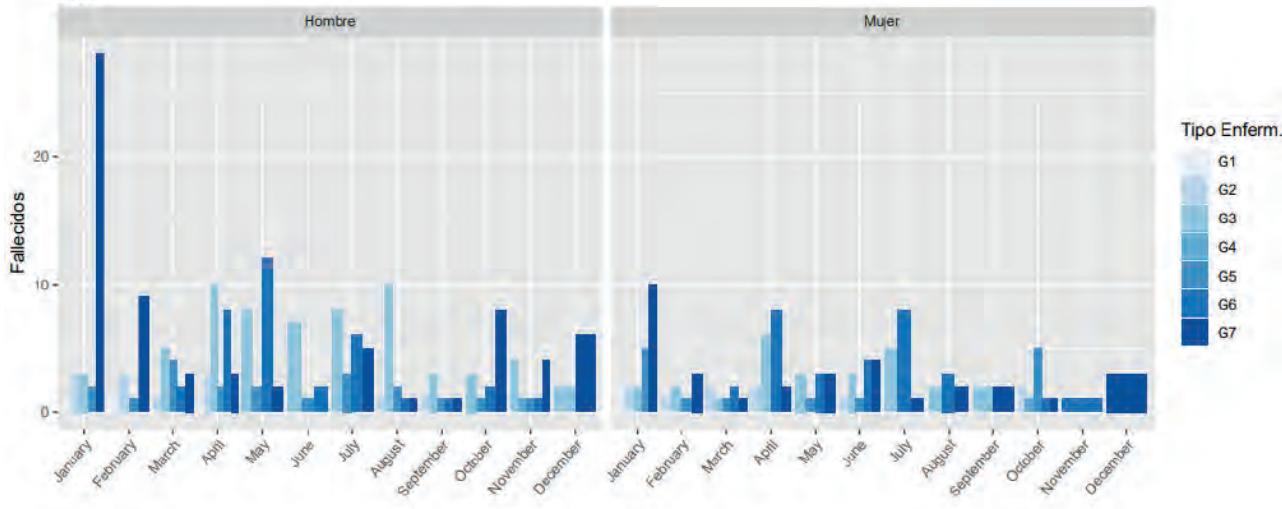


Figura 2. Número de fallecidos mensual por grupo de enfermedad y sexo.

al que puede incluirse al paciente como primer diagnóstico, y una variable binaria (Contviral) que asigna 1 si fue asignado a una dependencia de contingencia viral; mientras que la variable respuesta se llama Fallecido que toma el valor de 1 si el paciente falleció en área COVID-19 del hospital o no, es decir:

M1: Fallecido~Sexo+Edad+Contviral+Grupo

El segundo modelo considera las variables del modelo 1 más la interacción entre sexo y la edad, es decir:

M2: Fallecido~Sexo+Edad+Contviral+Grupo+ Sexo*Edad

El tercer modelo considera las variables del modelo 1 más la interacción entre sexo y Contingencia viral, es decir:

M3: Fallecido~Sexo+Edad+Contviral+Grupo+ Sexo*Contviral

Finalmente, un cuarto modelo que considera la interacción del Sexo y Grupo:

M4: Fallecido~Sexo+Edad+Contviral+Grupo+ Sexo*Grupo

La tabla 2 presenta la comparación de los logaritmos de las verosimilitudes de cada modelo y los valores p de las comparaciones de los modelos M2, M3 y M4 en relación con el modelo M1, es decir se usa el contraste (3). Al ser estos valores p mayores al nivel de significancia (0.05), indican que no existe evidencia estadística para rechazar el modelo más corto, esto también se puede apreciar en las verosimilitudes de cada modelo, las cuales son muy similares a pesar de que los modelos M2, M3 y M4 incorporan más variables.

Los resultados de la modelización de M1 se presentan en la tabla 3.

De las tablas 3 y 4 se puede apreciar que edad es una variable muy significativa, y al ver la razón de momios de esa

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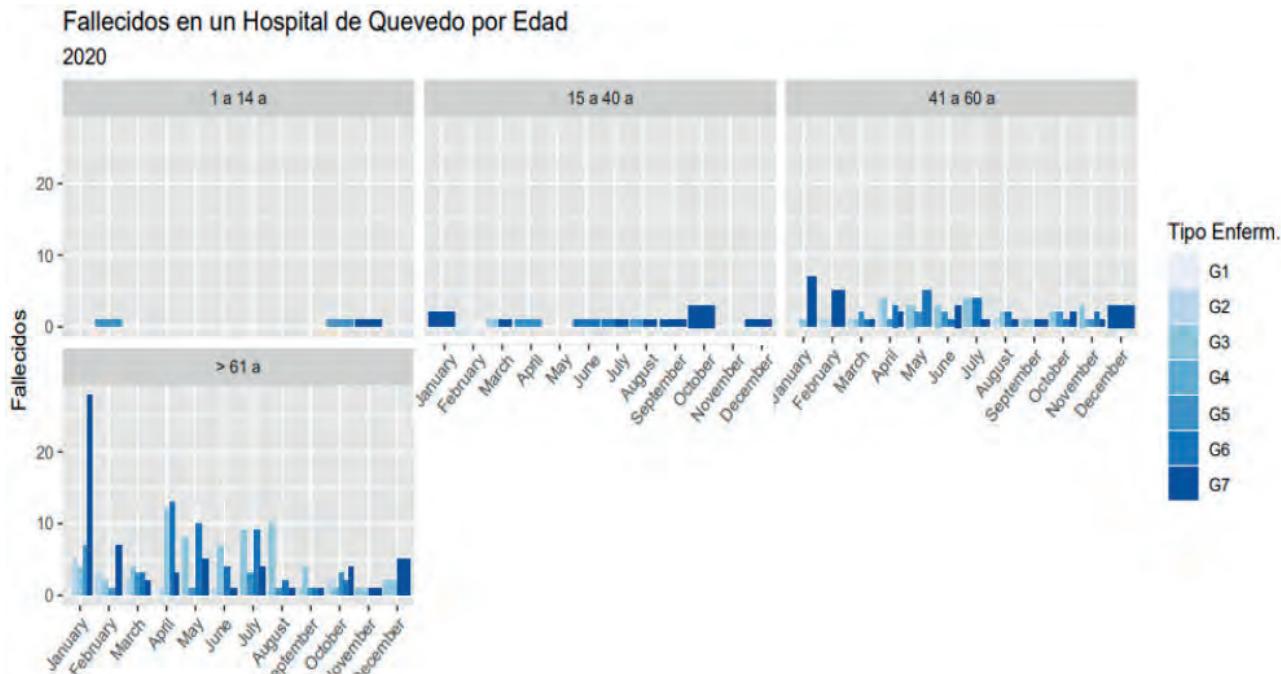


Figura 3. Número de fallecidos mensual por grupo de enfermedad y edad.

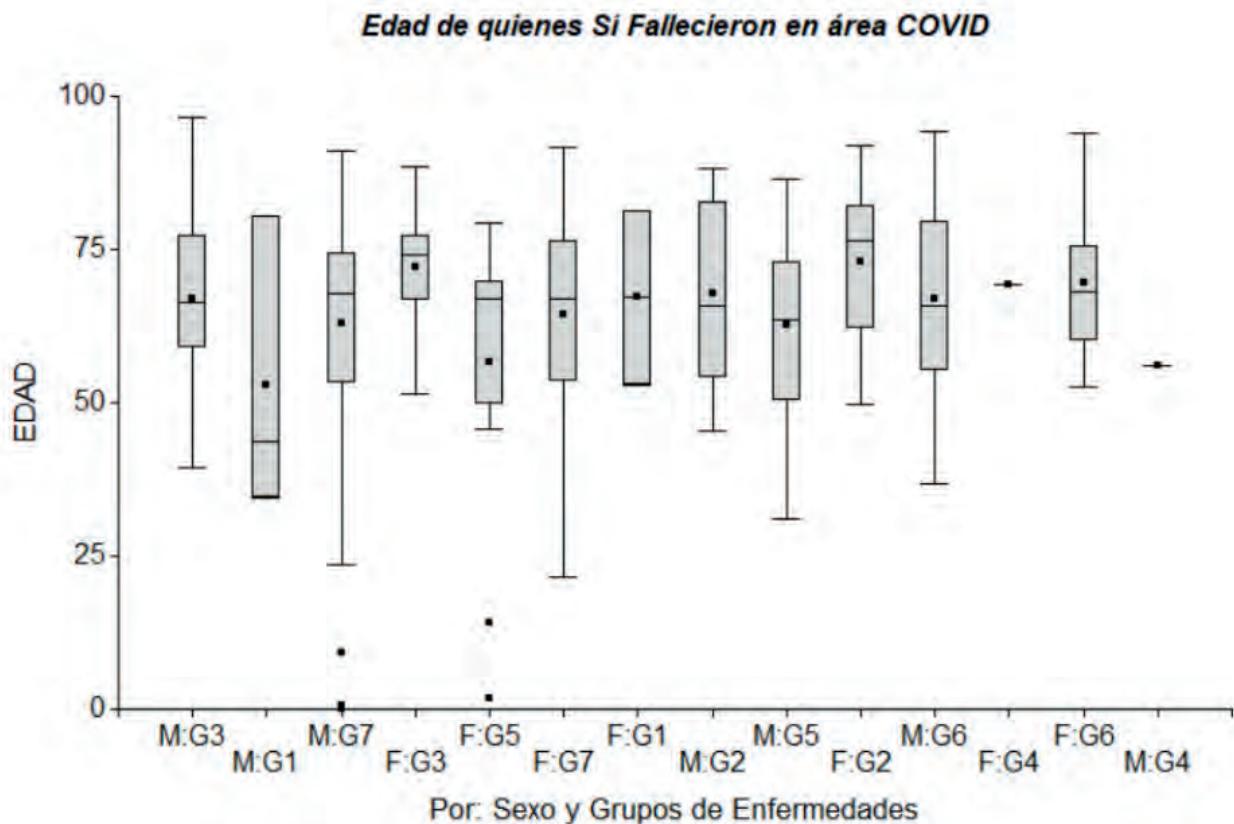


Figura 4. Distribución por grupo de enfermedad y Sexo de quienes fallecieron en área COVID.

variable, se puede interpretar que por cada año en que se incrementa la edad la posibilidad de muerte por COVID-19 se incrementa en un 7%.

Se sabe que los registros de muertes por COVID-19 en los países es inferior al presentado oficialmente por ellos, debido a que a veces la enfermedad no es detectada por falta de pruebas o en ocasiones la enfermedad genera por ejemplo un paro cardiaco y la muerte es encasillada en otro grupo de en-

fermedad, por ello se aprecia también la alta significancia de la variable contingencia viral y un alto valor de razón de momio, pues si el paciente ingresa por derivación de COVID-19 es muy fácil que la muerte sea englobada en el grupo G6^{6,18}.

Se puede apreciar que el padecer síntomas iniciales o que el primer diagnóstico del paciente esté englobado en los grupos: G1 (Enfermedades infecciosas y parasitarias), G3 (Enfermedad del sistema respiratorio), G4 (Enfermedades del sis-

Modelos	Logaritmo de verosimilitud	Valores p
M1	-974,46	
M2	-974,32	0,5935
M3	-973,45	0,1543
M4	-970,51	0,2444

Tabla 2. Comparación de los modelos.

	Estimación coeficientes	Error estándar	Estad. z	Pr(> z)	Sign.
Intercepto	-8,306130	0,381586	-21,767	<2e-16	***
Sexo	-0,323615	0,144203	-2,244	0,02482	*
Edad	0,068458	0,003913	17,494	<2e-16	***
ContViral	2,184720	0,228033	9,581	<2e-16	***
GrupoG1	-0,457665	0,526439	-0,869	0,38465	.
GrupoG2	0,340246	0,369376	0,921	0,35698	
GrupoG3	-0,232058	0,215908	-1,075	0,28246	
GrupoG4	-2,210573	0,755420	-2,926	0,00343	**
GrupoG5	-0,330204	0,320542	-1,030	0,30294	.
GrupoG7	-1,073571	0,288695	-3,719	0,00020	**

Tabla 3. Coeficientes estimados para el modelo M1

Sexo	Edad	ContViral	G1	G2
0,72	1,07	8,88	0,63	1,41
G3	G4	G5	G7	
0,79	0,11	0,718	0,34	
AIC	2069,9		Residual	2049,9
			Deviance	

Tabla 4. Razón de momios del Modelo M1.

tema osteomuscular y del tejido conjuntivo), G5 (síntomas no clasificados), G7 (otras), reducen la probabilidad de muerte en área COVID-19 en relación al grupo de referencia que es el G6 (Paciente ingresado con síntomas de COVID-19). Por otra parte, las personas que murieron y cuyo diagnóstico presuntivo inicial corresponden al grupo G2 (enfermedades del sistema circulatorio) incrementan la probabilidad de muerte en área COVID-19 en relación al grupo G6. Adicionalmente, el hecho de ser mujer reduce la probabilidad de muerte en área COVID-19 con relación a un hombre.

Considerando un paciente de sexo mujer, con edad de 50 años, asignada a una dependencia de contingencia viral y con un diagnóstico del Grupo G3, asociado a enfermedades respiratorias y aplicando el modelo logístico, el valor de probabilidad estimada es 3.7 % de fallecer en el área para COVID-19 del hospital. Sin embargo, considerando los mismos datos para un paciente de sexo hombre, se obtiene una probabilidad estimada de 4.8% de fallecer.

Con un paciente de 70 años, sexo hombre, y asignado al grupo G5 asociado a enfermedades de síntomas desconocidos, y con contingencia viral 1, sus probabilidades estimadas de fallecer son de 15%, mientras que una mujer bajo las mismas condiciones es del 12%. Por otro lado, si consideramos un paciente de 70 años, hombre, que tiene diagnóstico G6 (COVID-19) y asignado al área de dependencia viral, la probabilidad estimada de fallecimiento en área COVID-19 es 21%, y para una mujer bajo las mismas condiciones es de 17%.

A mayor edad y considerando una enfermedad dentro del grupo de enfermedades significantes para el modelo, el paciente tiene mayores probabilidades de fallecer.

Además, se realizó la prueba de bondad de ajuste a partir del test Hosmer y Lemeshow, obteniendo:

$$\text{X-squared} = 1.9029, \text{ df} = 2, \text{ p-value} = 0.3862$$

Este resultado indica que el modelo se ajusta bien a los datos. Otro indicador de que el modelo es adecuado es el valor p del contraste (2), el cual fue de 1 considerando que el residual deviance (2049,9) es un estadístico χ^2 cuadrado con 40964 grados de libertad. El gráfico de autocorrelaciones parciales también indica que los residuos deviance son aleatorios (figura 5).

Con el 30% de datos que no se consideraron para la construcción del modelo, se calcularon las tasas de verdaderos positivos y verdaderos negativos, así como la tasa de clasificación correcta, las cuales fueron respectivamente 60%, 96% y 97%.

Considerando los resultados de la tabla 3, en las que se muestra que los grupos G1, G2, G3, G5 y G6 tienen influencia estadísticamente similar en la probabilidad de morir en área COVID-19, tal vez debido a que la infección por SARS-CoV-2 presenta síntomas similares a cada uno de los grupos G1, G2, G3 y G5. Por otra parte, los grupos G4 y G7 tienen una influencia estadísticamente diferente a los otros grupos de la clasificación presentada en la tabla 1. Por esta razón se construyó un modelo en los que se hacía una reclasificación de las categorías de la tabla 1 y se consideró solo tres grupos: El grupo G4, el grupo G7 y la unión de los grupos G1, G2, G3, G5 y G6. Los resultados se presentan en la tabla 5:

Este modelo con menos variables es estadísticamente similar al mostrado en la tabla 2, con un valor p para el contraste (3) de 0.2122, por lo que se prefiere el modelo de la tabla 5 con la nueva reclasificación.

Se puede apreciar que los resultados de la razón de mo-

mios presentadas en las tablas 4 y 6 apenas varía. Con el 30% de datos que no se consideraron para la construcción del modelo, se volvieron a calcular las tasas de verdaderos positivos y verdaderos negativos, además la tasa de clasificación correcta, considerando esta vez el nuevo modelo, las cuales fueron respectivamente 64.28%, 96.4% y 96.23%; es decir, hubo una pequeña mejora.

Discusión

El COVID-19 ha afectado al Ecuador incrementando la tasa de mortalidad, especialmente en los primeros siete meses del año 2020, debido a que, al ser un virus poco estudiado, no existía un tratamiento farmacológico terapéutico efectivo conocido o una vacuna que prevenga y evite la propagación de este mortal virus.

Pese a que mayoritariamente se entendía que en un 80% de los casos solo producía síntomas leves respiratorios, al preexistir una comorbilidad aumentaba la probabilidad de que el cuadro se complicara generando insuficiencia respiratoria y posterior muerte.

El hecho que la unidad médica habilitara una área específica denominada contingencia viral, y que estableciera protocolos de atención para la población del cantón Quevedo, evitó la propagación del virus; esto puede visualizarse en las gráficas de los meses posteriores donde se observa un decremento progresivo de los casos, esto también se lo puede asociar con las medidas que se tomaron en el país para salvaguardar las vidas; estas medidas fueron difundidas por el COE (Comité de operaciones de emergencia) Nacional, el cual es un componente del Sistema Nacional para Emergencias y Desastres, responsable de promover, planear y mantener la coordinación y operaciones conjunta, entre diferentes niveles, jurisdicciones y funciones.

En el presente estudio, los factores que influyen en la probabilidad de fallecimiento por COVID-19 son la edad y sexo del paciente en conjunto con el grupo en el cual está clasificada la enfermedad diagnosticada en el hospital. En relación con el sexo, el ser hombre incrementa la muerte por COVID-19, este resultado también ha sido obtenido en otros estudios en los que se indica que las mujeres tienen un sistema inmunitario más fuerte que el de los hombres para combatir infecciones,

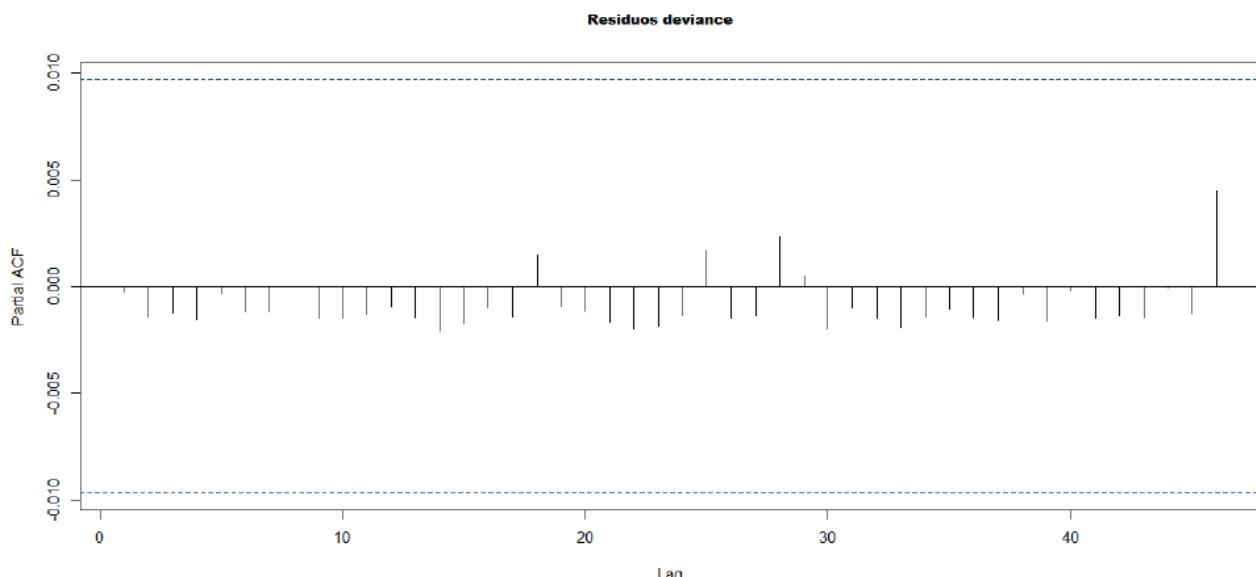


Figure 5. Autocorrelaciones parciales de los residuos.

	Estimación coeficientes	Error estándar	Estad. z	Pr(> z)	Sign.
(Intercepto)	-8,435841	0,282413	-29,871	<2e-16	***
Sexo	-0,276878	0,143037	-1,936	0,05290	*
Edad	0,067901	0,003779	17,967	<2e-16	***
ContViral	2,140644	0,169109	12,658	<2e-16	***
GrupoG4	-2,050675	0,720274	-2,847	0,00441	.**
GrupoG7	-0,859887	0,173207	-4,965	6,89-07	***

Table 5. Coeficientes estimados para el modelo con reclasificación de grupos.

Sexo	Edad	ContViral	G4	G7
0,76	1,07	8,50	0,13	0,42
AIC	2107		Residual 2095,2	

	Deviance

además que los hombres tienden a tener niveles más alto de consumo de tabaco y alcohol que las mujeres, lo que los pone en una situación menos favorable¹⁹.

Según la CDC (Centers for Disease Control), si se toma como referencia la edad de 18 a 29 años, la tasa de muerte en las personas de más de 30 años y menos de 40 años es 4 veces mayor; mientras que, en los mayores de 85 años la tasa de muertes es 600 veces mayor. Este resultado coincide con nuestro estudio, en la que la variable edad resultó muy significativa, incrementándose la probabilidad de morir por cada año adicional del paciente²⁰. Los hombres mayores a 61 años se han visto mayormente afectados por el virus de acuerdo con las estadísticas registradas de mortalidad registradas por el COE NACIONAL.

En este estudio se observó, que los síntomas que han sido categorizados en alguno de los Grupos de la tabla 1, dependiendo de la agrupación dada en la clasificación, incrementa la probabilidad de muerte por COVID-19 de los pacientes. Por ejemplo, si los síntomas son englobados como enfermedad respiratoria o cardiovascular ya sea porque es una enfermedad preexistente o porque recién presentan síntomas, incrementan la probabilidad de muerte por COVID-19. Otros estudios muestran que la hipertensión, las enfermedades cardiovasculares, la diabetes mellitus, el tabaquismo, la enfermedad pulmonar obstructiva crónica (EPOC), la malignidad y la enfermedad renal crónica se encontraban entre las enfermedades subya-

centes más prevalentes entre los pacientes hospitalizados de COVID-19, respectivamente.²¹ Este resultado muestra y confirma que esta enfermedad no solo se relaciona con problemas respiratorios sino también con problemas cardiovasculares.

Conclusiones

Con los resultados obtenidos en este estudio, se puede corroborar que la mortalidad por COVID-19 en Ecuador está también influenciada por variables como el género o edad. Adicionalmente, el hecho de que los pacientes sean ingresados al hospital bajo un diagnóstico presuntivo de enfermedad cardiovascular o respiratoria, ya sea porque sus síntomas son esos o porque tiene una enfermedad previa relacionada, puede incrementar la muerte por corona virus. Estos resultados muestran semejanza con estudios hechos en otros países.

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RESEARCH / INVESTIGACIÓN

Phylogenetic and molecular evolutionary analysis of SENV DNA isolated from Iraqi Hepatitis Patients

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Abstract: A recent group of transmissible, hepatotropic, single-stranded, circular, non-enveloped DNA viruses (SENV) has been discovered that is distantly linked to the widely distributed Torque Teno virus (TTV) family. This research aimed to use nucleotide sequencing to identify SEN-V's genetic alterations and investigate the similarities between isolates. Seven SENV isolates have been selected from the previous study, some associated with post-transfusion hepatitis, and used for determining the nucleotide sequence of SENV DNA by the chain termination method. According to the current analysis results, specific primer pairs were used to detect SEN viral DNA sequences; however, those specific primers can also detect closely related TT viral sequences instead.

Key words: SEN-V, Sequencing, Phylogenetic tree.

Introduction

In addition to well-known A to E hepatitis viruses, there is growing evidence that newer hepatitis viruses may exist and have a role in this disease¹. The SEN virus (SENV) is a member of a newly discovered family of hepatotropic, non-enveloped, single-stranded (ss) DNA of negative polarity viruses that was reported as non-A to E hepatitis virus in 1999². SEN-V is a circular 3,900 nucleotides, possibly belonging to the Anelloviridae family, including Torque Teno virus (TTV), TTV-like minivirus, SANBAN virus and SAN YONBAN virus. Among nine detected genotypes of SEN-V; genotypes D and H are more frequent in hepatitis cases³⁻⁵. SENV and TTV have related genomic structures; SENV's nucleotide sequence found two dominant, partially overlapping open reading frames (ORF1 and ORF2)⁶⁻⁹.

About 20% of hepatic infections are not associated with hepatitis viruses (A–E), which may be interpreted as other viruses' involvement. Wide ranges of SENV infections are reported in individuals with liver disease. SENV is ubiquitous with distinct geographic variations. Epidemiological studies have evidenced wide ranges of SENV in other pathological conditions, such as autoimmune diseases, respiratory conditions, cancer, thalassemia patients and patients on maintenance hemodialysis. In spite of several studies, the pathogenetic role of SENV has not yet been clarified¹⁰⁻¹³. The current study aims to detect the genetic changes in SEN-V and/or similarities between isolates in hepatitis patients.

Methods

Selection of samples

Seven (7) DNA samples of SENV, which were previously extracted from the blood of post-transfusion hepatitis, those DNA samples were used to identify the genetic alterations of SENV by Sanger nucleotide sequencing. Until used, these specimens were held in a deep freeze (-44°C).

Detection of SENV DNA

The samples were taken out of the freezer and thawed. For the isolation and purification of DNA from specimens, the

Viral Nucleic Acid Extraction Kit II (Cat. #VR00, Geneaid, Taiwan) was used. The operation was carried out following the manufacturer's instructions. According to Karimi-Rastehkenari *et al.*², SENV DNA was determined using nested polymerase chain reaction (PCR) with SENV-specific primers. For amplification, PCR fragment was chosen, which was expected to cover 119 bp of the SENV. SENV was amplified for the first round with forward primer AI-1F (5'-TWCYCM AAC GACCAG CTA GAC CT-3'; W = A or T, Y = C or T, M = A or C) and reverse primer AI-1R (5'-GTT TGT GGTGAG CAG AAC GGA-3'), while for the second-round PCR amplification with specific forward and reverse primers H-1020F (5'-TTT GGC TGC ACC TTC TGG TT-3') and H-1138R (5'-AGA AAT GAT GGG TGA GTG TTA GGG-3'), respectively.

Genomic amplification

According to Khudair *et al.*¹¹, DNA amplification reactions were carried out using nested traditional PCR. For the first and second round reactions, the cycling conditions were similar as follows: initial denaturation at 95°C for 5 minutes for 1 cycle, followed by DNA amplification by sequentially heating for denaturation of DNA template at 95°C for 30 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 45 seconds for 35 cycles, then final extension 72°C for 5 minutes for 1 cycle. The amplicons were electrophoresed in a 1 percent agarose gel for visualization. The band size was determined using an ultraviolet (UV) transilluminator and a direct comparison to a 100 bp DNA marker. One band of 119 bp DNA was found in SENV DNA positive samples, as shown in figure (1). The amplified SENV DNA samples have a code number of (1, 5, 68, 100, 122, 123, and 136). These seven samples were sent to Macrogen Company in South Korea and their corresponding primers for sequencing.

Analysis of sequences

The nucleotide sequence similarities between isolates and the documented reference sequence were determined in this study using the BLAST program (<http://www.ncbi.nih.gov>) and the BioEdit program. Nucleotide sequences were translated to a protein sequence using the online Expasy translate server. Using the NCBI-BLAST server, a specific comprehensive viral tree was

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built, in which the observed viral variant was compared to their neighbor homologous viral sequences¹⁴. Then, using Clustal Omega for making multiple sequence alignments. In addition, FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) a program was used to graphically view phylogenetic trees, including the observed variant, as a polar cladogram. In the comprehensive tree, the viral sequences of each classified phylogenetic species group were colored accordingly.

Results

2252

Sequencing of SENV local isolates

According to the sequencing company's instructions, seven SENV isolates' 119-bp PCR second round amplicons were commercially sequenced from both ends. Only specific chromatographs from ABI sequence files were studied further, ensuring that the annotation and differences were not due to PCR or sequencing errors. The exact location and other aspects of the retrieved PCR fragment were established by comparing the observed DNA sequences of both local specimens with the retrieved DNA sequences of SENV (GenBank acc.GQ452051.1).

The SENV sequences were highlighted in table (1); the grey-colored regions correspond to forward and reverse primers, respectively. The forward primer is positioned in the positive strand, while the position of the reverse primer was in the negative counterpart. The alignment results of all sequenced samples revealed the presence of seven mutations, in which substitution was observed, as shown in table (2). The sequencing chromatogram of the observed point mutation was documented in these amplicons, as shown in figure (2). The exact positions of observed mutation were listed in the NCBI reference sequences figure (3) to summarize all of the results

obtained from the sequenced 119 bp fragment. Interestingly, The NCBI Blast server disclosed that there were noticeable differences between the previously sequenced samples (Var. Nos. 1, 5, 100, 123, and 136) and the (Var. No. 68 and 122), as shown in figure (4).

Discussion

Viral hepatitis is a worldwide disease that presents a severe threat to human health. To date, five hepatitis viruses have been discovered, ranging from A to E, accounting for 80% to 90% of hepatitis cases. However, none of the hepatitis viruses were found in the remaining 10% to 20% of cases with classic viral hepatitis symptoms, indicating that other viruses, such as SENV, are likely to play a role in the pathogenesis of non-A-E hepatitis^{3,13}.

In this study, the total number of aligned nucleic acid sequences in the comprehensive phylogenetic tree figure (4), regardless of viral variants, was 123 sequences including seven different local SENV isolates from viral hepatitis patients. in addition to both SENV and TTV sequences, this viral-specific phylogenetic tree consists of several viral species, namely TTV-like viruses, Spodoptera Litura Nucleopolyhedroviruses, enterobacter phages, Gallid herpes viruses, Canine distemper viruses, as well as uncultured viruses^{15,16}. The comprehensive phylogenetic tree was found that observed viral variants encompassed two-variable phylogenetic positions; the first one is located in the SENV referring sequencing group, while the other group is located in the TTV referring sequencing counterpart. The current study-based phylogenetic tree indicated that two main categories are originated from the presence of specific differences between the sequences of both viral groups.

The first viral group is just derivatives of the main SENV referring sequences. This sort of difference belongs to the ob-

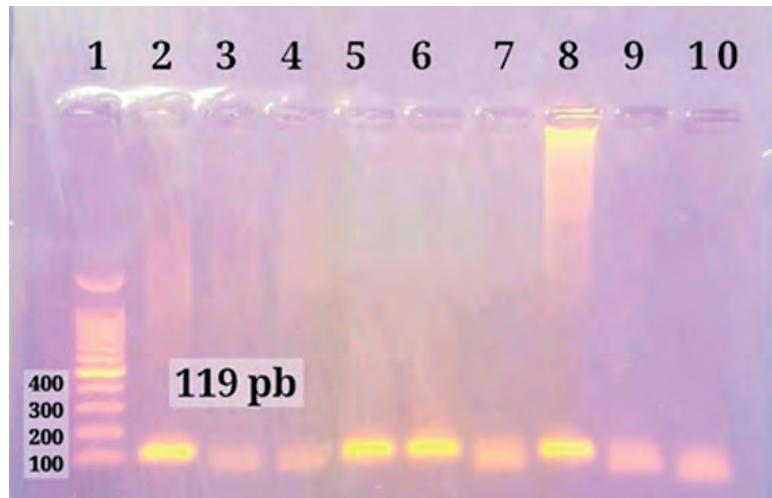


Figure 1. Agarose gel electrophoresis of PCR products second round. Line 1: 100 bp ladder. Lines 2,5,6 and 8 SENV-H positive 119 bp. Lines 3,4,7,9 and 10 negative samples.

Amplicon	Referring locus sequences (5' - 3')	length
SENV	TTGGGCTGCACCTTCTGGTTCTACAGACACCCAGAAAGTGGAC TTGTAGCTCAGTTGACAACGTTCCCCCATGAAAATGGAC GAGAACACAGCCCCTAACACTCACCCATCATTCT	119bp

Table 1. The position and length of the PCR amplicon used to amplify SENV virus. The amplified sequence was extended from 1 into 119 of the NCBI reference DNA sequence (GenBank acc. no. GQ452051.1). The grey-colored regions refer to forward and reverse primers, respectively.

No.	Nitrogen bases	Changes in Nitrogen bases	Samples No.	Position (Subject)	Position (reference)	Amino acid change	SNP
1	C	T	Var. No. 5 & 136	21	21	-	Not registered
2	C	T	Var. No. 100	31	31	p.11P>S	Not registered
3	A	G	Var. No. 5 & 136	36	36	-	Not registered
4	G	A	Var. No. 5 & 136	54	54	-	Not registered
5	T	C	Var. No. 1	66	66	-	Not registered
6	C	A	Var. No. 5 & 136	72	72	-	Not registered
7	G	A	Var. No. 5 & 136	87	87	-	Not registered

Table 2. The observed single-nucleotide polymorphism (SNP) pattern in comparison to the NCBI referring sequences of the SEN virus (GenBank acc. no.GQ452051.1).

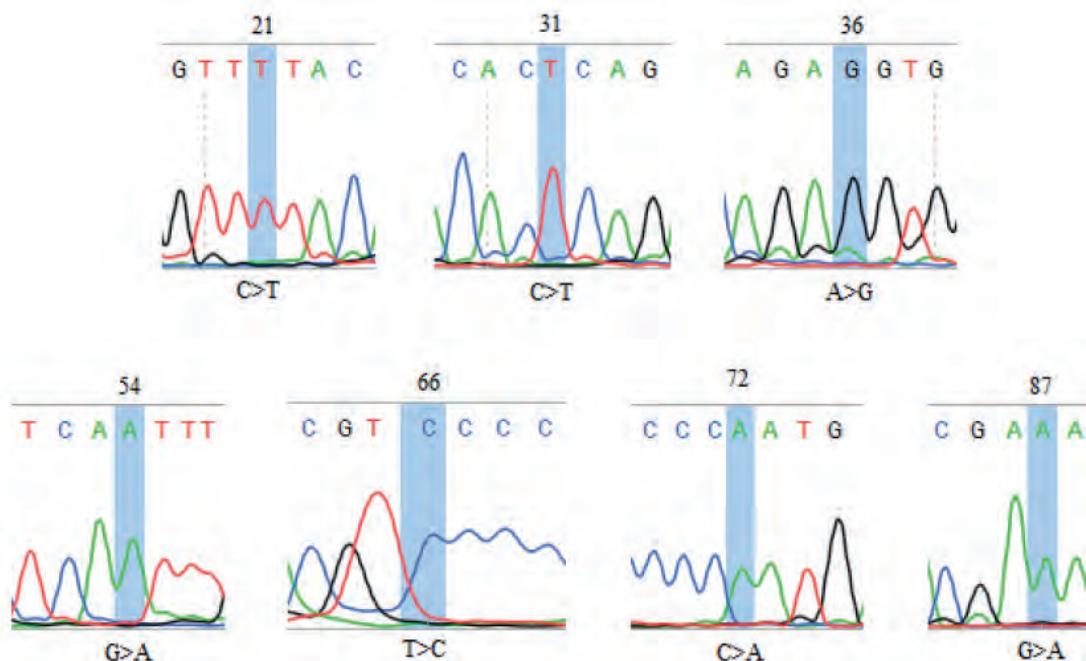


Figure 2. The DNA chromatogram sequence of local isolates of SEN virus. The locations of the observed substitution mutations were highlighted. The symbol ">" denotes a substitution mutation.

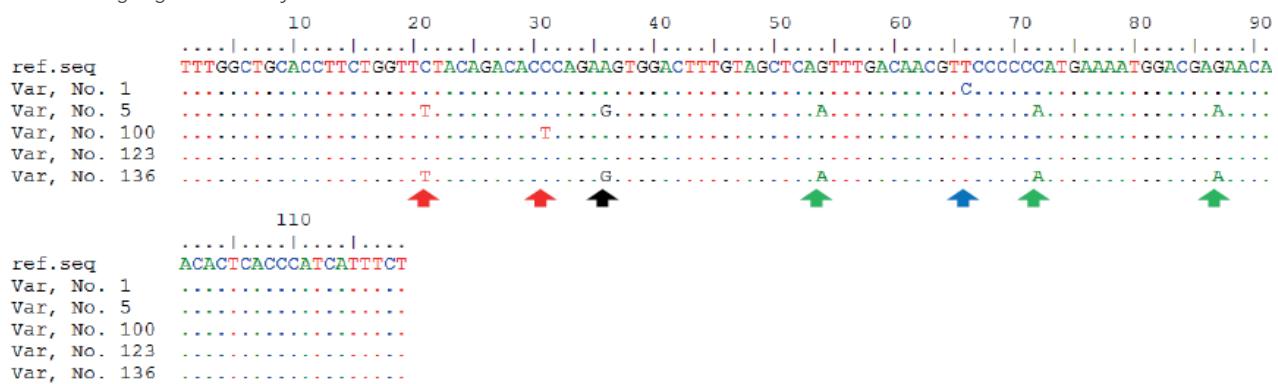
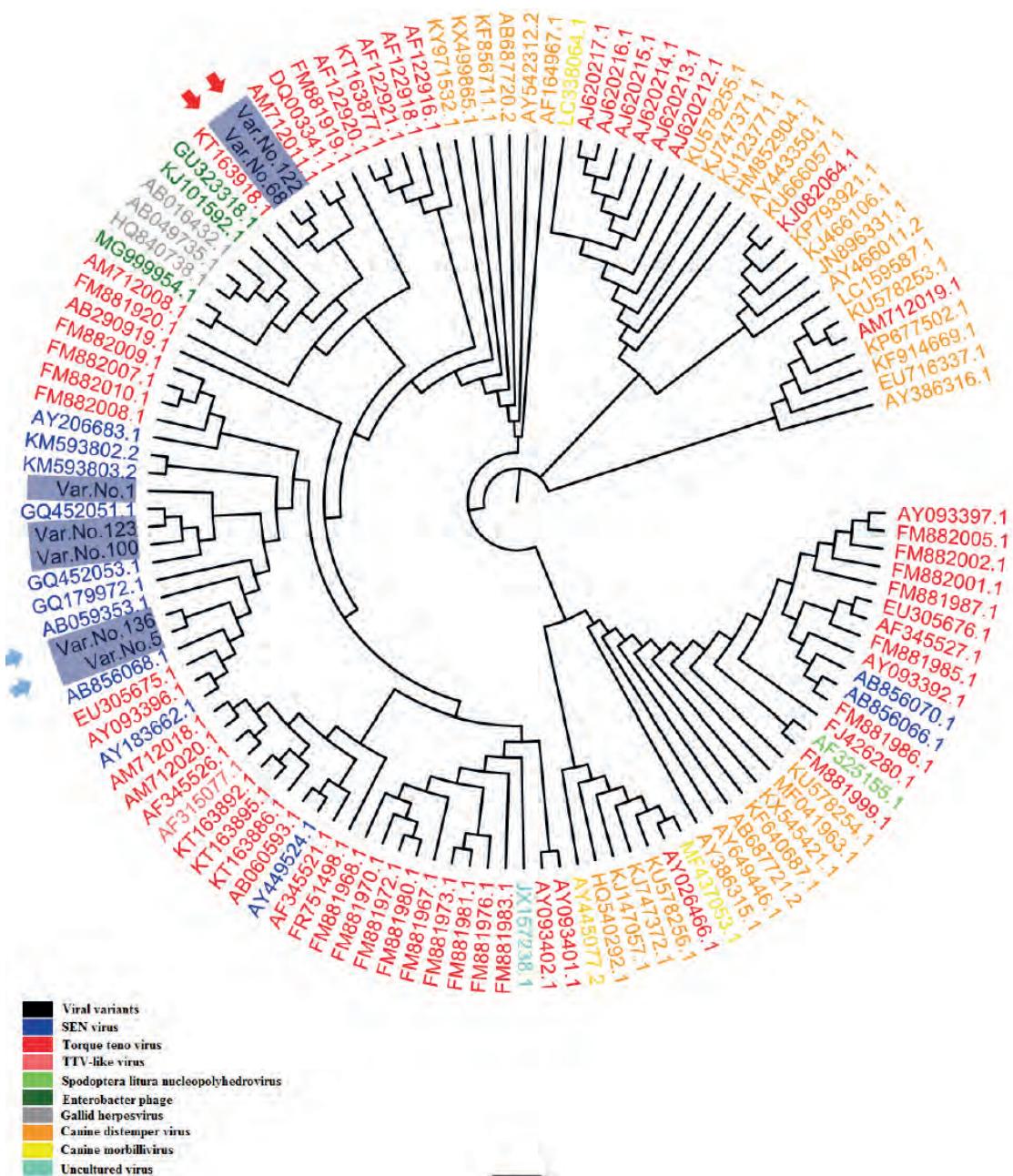


Figure 3. The DNA sequences of the observed local viral isolates were aligned with the corresponding reference sequences of the SEN virus. The substitution mutations were highlighted in the PCR products according to their positions. The NCBI referring sequence is denoted by the symbol "ref.seq." The colored arrows indicate substitution mutations that have been found.



3.0

Figure 4. The comprehensive phylogenetic tree of local viral isolates from SENV (Nos. 1, 5, 100, 123, 136, 68 and 122.) The black color denotes viral forms that have been sequenced, while the other colors denote viral species that have been deposited with the NCBI. The SENV positions of Var. Nos. 1, 5, 100, 123, and 136 are indicated by blue colored arrows, while the Var. Nos. Red-colored arrows indicate 68 and 122. All of the numbers mentioned above referred to each referencing species' Genbank accession number. The number "3.0" at the bottom of the tree denotes the degree of scale range among the species classified by the comprehensive tree.

served DNA variations that were revealed in five sequenced viral samples. The Var. No. 1 was exerted only one variation from the SENV referring sequence (g.66T>C). In addition, the situation is the same in Var. No.100 that showed transition replacement of pyrimidine base with another pyrimidine (g.31C>T) which led to replacement of Proline (P) at position 11 with Serine (S). Whereas Var. Nos. 5 and 136 have exerted more variations (g.21C>T, g.36A>G, g.54G>A, g.72C>A, and g.87G>A), as shown in table (2). Thus, both Var. No. 5 and Var. No. 136 were occupied a distinctive position in the tree. Such position was however located within SENV phylogenetic regions, as shown in figure (4).

The both local Var. Nos. 68 and 122 were distant from the SENV phylogenetic position. In contrast they showed similarity to Torque Teno virus (acc. no. TK163918.1). The current finding was that two samples No. 68 and 122 deviated from the expected SENV sequenced samples into the unexpected Torque Teno viruses. This is perhaps due to several reasons; SEN and TTV have many in common sequences as they share many homologies. The genomic sequences of SENV are relatively similar to TTV, and both are classified within the Anelloviridae family³. Add to that, both SENV and TTV are single-stranded non-enveloped DNA viruses of 3,900 nucleotides^{2,17-20}. The extraordinary diversity data obtained from the present study fo-

llows other reports that notified such a particular interconnection between SENV and TTV^{7,21}. In addition, The high genomic homology seen between these two SENV isolates and several TTV isolates could point to SENV's evolutionary history concerning TTV1. According to one study, some of the TTV-related isolates can be pathogenic¹⁵. Through specifically designed primers pairs were utilized in this study to detect SENV DNA sequences, it's probably that such specific primers may detect the closely related TTV sequences instead²².

Conclusions

This study showed that several silent point mutations had been found in SENV ORF1 region. However, only one mutation in isolate No.100 led to the replacement of Proline with Serine. The present phylogenetic tree provided a clue about the identity of these local isolates, and a more extensive scale screening study is required for more information about the pattern of relatedness between SENV sequences and their TTV counterparts in different diseases in our region.

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Conflict of Interest

There are no potential conflicts of interest for the writers to disclose.

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RESEARCH / INVESTIGACIÓN

Traditional practices in post-partum care among Indonesian and Filipino mothers: a comparative study

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Abstract: This study was conducted to assess the traditional practices in post-partum care among Indonesian and Filipino mothers to propose a program to improve maternal and child health. The study utilized a descriptive research design for Indonesian mother respondents ($n=110$) and Filipino mother-respondents ($n=119$) conveniently selected. Traditional practices on post-partum care focused on hygienic care, behavioral precautions, breastfeeding, baby care; dietary modifications; and physical activities. Descriptive statistics (frequency and percentage), weighted mean, and independent t-test were used to describe and analyze quantitative information. Four dimensions, including hygienic care (p -value 0.038); breastfeeding and baby care (p -value 0.000); dietary modifications (p -value 0.000); and physical activities (p -value 0.000), showed a statistically significant difference in the assessment of mother respondents on their traditional practices in post-partum care. Meanwhile, the dimension on behavioral precautions (p -value 0.250) yielded statistically no significant difference on the assessment of mother respondents on their traditional practices in post-partum care. Four dimensions, including hygienic care (p -value 0.038); breastfeeding and baby care (p -value 0.000); dietary modifications (p -value 0.000); and physical activities (p -value 0.000), showed a statistically significant difference in the assessment of mother respondents on their traditional practices in post-partum care. Meanwhile, the dimension on behavioral precautions (p -value 0.250) yielded statistically no significant difference on the assessment of mother respondents on their traditional practices in post-partum care.

Key words: Traditional, Practices, Postnatal Care, Cultural Diversity, Indonesia, Philippines.

Introduction

There are many sources of variability in post-partum practices, both within and between cultural groups. Some traditional post-partum methods are based upon what we would consider supernatural or religious beliefs. Medical anthropology has long described health and illness belief frameworks in diverse cultures that include different ideas, such as religious, magical, or mystical beliefs¹. Furthermore, the various health beliefs and explanatory models may vary depending on the observation level among the different social spheres of culture. Designated professional healers or medical practitioners may differ in their thoughts from folk healers, such as midwives, who may also differ from lay popular beliefs at large².

Traditional post-partum healing beliefs in South Asia are centered on the notion that a woman's body is drained of all its energy after birth. The mother must have complete rest and receive good nutrition to help restore vitality³. For instance, many women from Indonesia and the Philippines continue to practice a wide range of traditional beliefs and practices during the post-partum period. Healing methods that have survived for centuries are still common practice and focus much attention on the recovery of new mothers⁴. By recognizing and appreciating common local beliefs, care providers, primarily nurse-midwives, can better provide culturally competent care. Instead of reducing the choices available to women during the post-partum experience, providers should understand, respect, and integrate cultural interpretations of childbirth and women and their families⁵.

Indonesian women's post-partum beliefs are grounded in religion and long-held health practices. Although the recommended reduction strategies are in place, the country is challenged with increasing maternal and neonatal mortality

rates⁶. Meanwhile, in the Philippines, post-partum recovery is also surrounded by a wide variety of beliefs, traditional practices, and rituals that involve both mother and infant. Cultural beliefs may be considered implementing maternal care and other health programs that fit their cultural practices⁷.

In many societies in the Southeast Asian region, traditional beliefs and practices are believed to be vital to maternal and child health⁸. Deep cultural and social meanings are attached to practices related to behaviors, activities, foods, hygiene, and infant care with variance by regions⁹. There are pretty diverse interpretations of the traditional post-partum beliefs and practices, even in the urbanized communities¹⁰. For example, in Indonesia and the Philippines, comparative post-partum mothers' beliefs and practices have not been well documented.

The Philippines is full of superstitions and beliefs regarding childbearing, mainly because Filipinos believe that there is nothing to lose if they abide with these beliefs derived from their traditions, customs, and culture. They emphasized that when a woman is pregnant, one foot is confined to a hospital while the other is bound 'six feet below the ground'¹¹. Similarly, in the Philippines, a low healthcare services utilization in post-partum women contributes to significant maternal deaths during the post-partum period¹².

The problem of morbidity or mortality in post-partum mothers is related to socio-cultural and environmental factors in the community. Many cultural practices harm public health behavior, resulting in a greater risk of infection¹³. In some cultures, abstinence from eating in pregnant women can affect nutritional intake¹⁴. The low level of community knowledge significantly affects maternal health¹⁵. In Nigeria, people with low knowledge will surrender to the gishiri incision, a vaginal

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surgery performed by traditional birth attendants in cases of obstructed labor¹⁶. Public perception of maternal mortality is largely colored by non-medical causes such as religion, beliefs, and supernatural factors¹⁷.

The purpose of this study is to compare the significant traditional maternal health beliefs and practices carried out by women during a post-partum stage in Indonesia and the Philippines with emphasis on influence on maternal health utilization.

Methods

2258

Design

This study utilized a descriptive research design that permitted an understanding of phenomena, meanings, and personal values in evaluating the traditional practices in post-partum care among Indonesian and Filipino mothers. Structured survey questionnaires were used to gather quantitative data. This approach afforded the collection of quantitative data about real-life experiences of Indonesian and Filipino mothers who have observed traditional post-partum practices passed on from one generation to another. Quantitative design generates a more rounded and objective understanding of phenomena (Hayes, Bonner, and Douglas, 2013). A descriptive approach was used for quantitative analysis, which will provide a clear interpretation of the traditional practices of post-partum care in which a cultural-sensitive care program may be developed to enhance maternal and child health.

Population, Sample, and Sampling Technique

The study population included Indonesian mothers from various localities of North Tapanuli, Indonesia, and Filipino mothers from different suburban districts of Manila. In determining the study sample, the researcher used convenience sampling to choose readily available subjects that the researcher believes are typical or representative of the accessible population. While the computation of sample size for this study was not required, a minimum of 100 Indonesian mothers and 100 Filipino mothers met the inclusion criteria such as conforming to traditional beliefs and practices in post-partum care and conveniently available in the selected research locales. In contrast, post-partum mothers with complicated pregnancies and over the post-partum stage are excluded from this study. Non-probability sampling was focused on a sampling technique where the selection of respondents was based on the judgment of the researcher based on the following inclusion criteria as Indonesian mother who conforms to traditional beliefs and practices in post-partum care, Filipino mother who conforms to traditional beliefs and practices in post-partum care, willing to participate voluntarily in the study.

Research Instruments

The research study referred to an open-access and validated tools on Postpartum Practices Survey by Shouman and colleagues of the Mansoura University of Egypt, designed to compare practices related to post-partum care and be modified to align with Indonesian and Filipino mother respondents.

The adapted self-administered survey questionnaire consisted of three parts: Profile Characteristics; Traditional Practices on Postpartum Care. Profile variables included age; marital status; education; religion, employment, and the number of children. Traditional practices on post-partum care focused on hygienic care, behavioral precautions, breastfeeding, baby

care, dietary modifications, and physical activities. A four-point Likert scale was used to measure the responses. To identify the respondents' perspectives of the practices on post-partum care in two cultural settings, the modified instruments needed to be translated from English to Indonesian and back to the English language to ensure items in the questionnaire are properly adequately aligned with respondents originating from Indonesia and the Philippines. The content of the survey questionnaire in this study needed to be subjected to validation by some experts in traditional post-partum practices. All necessary recommendations by the experts were integrated into the internal organization of the research instrument to ensure all the items apply Indonesian and Philippine cultural background; a pilot study was done before conducting the main study to assess the reliability or validity of the data.

Furthermore, conducting a pilot enabled the participant's understanding of the questions and the timing required to complete the survey. The pilot study included 10 Indonesian and 10 Filipino mother-respondents who were not part of the actual survey. Internal consistency reliability was tested for each domain not just to examine the reliability and validity of the instrument but also to assess congruence with Leininger's Cultural Care Diversity and Universality Model that forms the theoretical framework of this study. The research instrument yielded an impressive overall 0.94 Cronbach alpha. An alpha value higher than 0.9, the internal consistency is excellent, and if it is at least higher than 0.7, the internal consistency is acceptable. (Richardson and Yu, 2015).

Data Collection Procedure

The researcher had initially secured approval from the Graduate School to conduct the study. The researcher further sought administrative approval from the local authorities in selected research locales included in the study. The researcher considered qualified Indonesian and Filipino mother respondents who were deemed principal participants in the study. After all approvals and permission had been secured, the researcher started screening eligible participants based on the inclusion criteria. The purpose of the study was carefully explained to all participants. Voluntary participation was clarified among the qualified respondents and written informed consent was obtained. Confidentiality of all gathered data was assured. Privacy and anonymity of the study respondents were maintained by eliminating all potential identifiers. The researcher had personally facilitated the distribution and collection of completed survey questionnaires and was around to answer any clarification from the respondents. Staff nurse respondents took approximately ten (10) minutes to complete the questionnaire. Completed self-administered questionnaires were immediately collected and checked for completeness by the researcher for analysis. Descriptive statistics (frequency and percentage), weighted mean, independent t-test were used to describe and analyze quantitative information. Data collection was carried out between August 2019 and March 2020.

Ethical Considerations

The research study ensured the voluntary participation of the participants. Anonymity, confidentiality, and privacy of all gathered information from this study were maintained until the completion of the study. Moreover, the study, in general, adhered to all ethical standards conducted in the study. A cover letter was attached to the questionnaire to explain the details of the study. Written informed consent was obtained, and it was clarified to all participants that they were under no obli-

gation to accomplish the survey questionnaire. All potential identifiers were eliminated in the questionnaire. The study's research protocol was subjected to the Ethics Review Board of the Trinity University of Asia.

Statistical Treatment of Data

In realizing the purpose of this study, the numerical data were treated utilizing the Statistical Package for Social Science (SPSS) software. Weighted mean was utilized for the assessment of Indonesian and Filipino mother-respondents on traditional beliefs and practices. Descriptive statistics (frequency and percentage), weighted mean, and independent t-test were used to describe and analyze gathered information.

Results

Results were extrapolated from questionnaires accomplished by the Indonesian mother-respondents ($n=110$) and Filipino mother-respondents ($n=119$) in this study. Data collection was carried out between August 2019 and March 2020.

Based on the age characteristic, more Filipino mother respondents are 20 years old and younger (13.4%) than the Indonesian mother respondents (9.1%). However, more Indonesian mother respondents are between 31 and 40 years old (34.5%) than Filipino mothers (25.2%). According to their marital status, the more significant proportion of the mother respondents from both countries was married (Indonesian mothers, 96.4% and Filipino mothers, 63.9%). More Filipino mother respondents are single (21.8%) than the Indonesian mother respondents (3.6%). However, more Filipino mother respondents are separated from their spouse (14.3%) compared to Indonesian mother respondents (0%). More Filipino mother respondents finished college (37.0%) than the Indonesian mother respondents (16.4%). However, a minimal percentage of Indonesian and Filipino mother respondents have only reached elementary education with 2.7% and 13.4%, respectively. More Filipino mother respondents are unemployed (36.1%) than the Indonesian mother respondents (15.5%). Interestingly, about a quarter of the Indonesian and Filipino mother respondents are self-employed, with 29.1% and 25.2%, respectively. Moreover, more Filipino mother respondents had five children or more (10.1%) than the Indonesian mother respondents (3.6%).

Table 2 summarizes the assessment of traditional practices in post-partum care on the dimensions of hygienic care, behavioral precautions; breastfeeding and baby care; dietary modifications; and physical activities. With responses of mother respondents to the individual dimension ranging from agreeing to agreeing strongly, Indonesian mother respondents presented an Overall mean of 3.09 verbally interpreted as agreeing. In contrast, Filipino mother respondents depicted an overall mean of 2.99 verbally interpreted as agreeing as well.

For the majority of Indonesian mother respondents, the most notable dimensions of traditional practices in post-partum care pertain to breastfeeding and baby Care ($x=3.37$), behavioral precautions ($x= 3.15$), and physical activities ($x= 3.11$). However, the least notable dimensions refer to dietary modifications ($x= 2.96$); and hygienic care ($x= 2.88$). Meanwhile, for the majority of Filipino mother respondents, the most notable dimensions of traditional practices in post-partum care pertain to physical activities ($x=3.43$), behavioral precautions ($x= 3.22$), and breastfeeding and baby Care ($x=3.01$). However, the least notable dimensions refer to hygienic care ($x=2.73$); and dietary modifications ($x=2.55$).

Table 3 supports the analysis in determining significant

differences in the assessment of Indonesian and Filipino mother respondents on their traditional practices in post-partum care. An independent t-test was used to compare the five dimensions of traditional practices in post-partum care at a 5% significance level. Four dimensions, including hygienic care (p -value 0.038); breastfeeding and baby care (p -value 0.000); dietary modifications (p -value 0.000); and physical activities (p -value 0.000), showed a statistically significant difference in the assessment of mother respondents on their traditional practices in post-partum care. Meanwhile, the dimension on behavioral precautions (p -value 0.0250) yielded statistically no significant difference on the assessment of mother respondents on their traditional practices in post-partum care.

2259

Discussion

Hygienic Care

Assessment of two groups of respondents showed a congruent point of view about hygienic care in which they both strongly agree on regular cleansing of the breast to provide safe and most nourishing milk to the baby. Moreover, both cultures maintain cleanliness in the environment to avoid infection and avoid taking a cold bath after giving birth not to get chill but refrain from taking a bath with added herbs with medicinal properties to relieve aches and pains.

Traditional practices are standard in various cultures around the world, including Indonesia. Out of 1,331 ethnic groups currently living in Indonesia, around ethnicities still practice their local traditions¹⁸. As early as 1961, anthropological studies described how a wide range of cultural ceremonies and traditions were performed by Javanese families connected with weddings, pregnancy, and childbirth. In the Timor communities of Indonesia, one common post-partum tradition is the Sei or smoke tradition, in which new mothers and their newborn babies sit or lie above embers from biomass fuel (e.g. wood and agricultural crop residue) for up to 40 days¹⁹.

As regards hygienic care, Chinese women are advised to restrict bathing and hair washing during puerperium to prevent possible headaches and body pain in later years (You. 2015). Some women do not take showers during puerperium because they are fearful of cold. Many women add herbs to bathwater to smell aromatic as they believe that herbs promote wound healing¹². A similar study in Fujian Province, China, found that most rural mothers seemed to adapt to the tradition by bathing with boiled water with wine or motherwort herb (a familiar herb with medicinal properties) to prevent the problems of absorption through the skin. Wine and motherwort are both thought to have disinfecting properties and will therefore prevent infection. They believed that as they were with the baby, they needed to be clean to protect them from illness. It also made them feel comfortable and happy³. Women also rub their bodies with herbs because they believe that rubbing helps uterine involution²⁰.

The use of an abdominal corset is every day among women to prevent pendulous abdomen¹². Most women perform perineal care by using water mixed with salt to prevent vaginal infection, promote wound healing, and eliminate unpleasant odours. In addition, slightly less than two-thirds of women used murr in painting their perineal wound and episiotomy sutures to promote healing. Some mothers in rural and urban areas wash their perineal area with boiled water and use iodine or alcohol to clean incisions or tears. Among the traditional practices among women is to take sitting baths, by putting on

Characteristics	Post Partum Mothers			
	Indonesians		Filipino	
	n	%	n	%
Age				
20 y.o	10	9.1	16	13.4
21 – 30 y.o	56	50.9	64	53.8
31 – 40 y.o	38	34.5	30	25.2
≥ 41 y.o	6	5.5	9	7.6
Marital Status				
Single	4	3.6	26	21.8
Married	106	96.4	76	63.9
Separated	0	0	17	14.3
Educational Attainment				
Elementary	3	2.7	16	13.4
High School	89	80.9	59	49.6
College	18	16.4	44	37.0
Religion				
Moslem	101	91.8	3	2.5
Christian	9	8.2	116	97.5
Employment Status				
Employed	61	55.5	46	38.7
Self-Employed	32	29.1	30	25.2
Unemployed	17	15.5	43	36.1
Number of Children				
1 Child	26	23.6	8	6.7
2 Children	42	38.2	19	16.0
3 Children	28	25.5	55	46.2
4 Children	10	9.1	25	21.0
5 Children or more	4	3.6	12	10.1

the floor of the container filled with boiling water and herbs are added to the water so that the genital area absorbs the plants' vapors, and after that, they sit in the water which is used to prevent vaginal infection²¹.

Behavioral Precautions

Assessment of two groups of respondents showed commonalities in traditional practices regarding behavioral precautions where both cultures strongly agree on seeking help and support from their husbands, friends, family, and other

relatives during the post-partum stage. Moreover, Indonesian and Filipino mothers limit reading, watching, or browsing on a computer or mobile phone to prevent eye strain; seek advice from parents, elderly and religious workers inspirationally; and focus on positive thoughts to keep them motivated during post-partum recovery. While Indonesian mothers stay inside the house and rest entirely within a month after delivery, Filipino mothers also adhere to this habit as a behavioral precaution.

In common sense, the protection period gains an essen-

Table 1. Frequency Distribution of Respondent Characteristics.

Variables	Indonesian Mothers		Filipino Mothers	
	Mean	Verbal interpretation	Mean	Verbal Interpretation
Hygienic care	2.88	Agree/True	2.73	Agree/True
Behavioral precautions	3.15	Agree/True	3.22	Agree/True
Breastfeeding and baby care	3.37	Strongly Agree/ Very True	3.01	Agree/True
Dietary Modifications	2.96	Agree/True	2.55	Agree/True
Physical activities	3.11	Agree/True	3.43	Strongly Agree/ Very True

Table 2. The mean and verbal interpretation on the traditional Practices.

Variables	df	t Value	Critical t Value	P Value
Hygienic care	227	2.087	± 1.97	.038
Behavioral precautions	227	-1.154	± 1.97	.250
Breastfeeding and baby care	227	-5.307	± 1.97	.000
Dietary Modifications	227	6.162	± 1.97	.000
Physical activities	227	-4.960	± 1.97	.000

Table 3. Difference between the Assessment of the Indonesian and Filipino Mother Respondents on Their Traditional Practices in Postpartum Care.

tial meaning for women who respect the culturally learned standards and rules to avoid relapse due to complications resulting from inappropriate self-care. This action refers to the care human beings take of themselves through favorable practices to preserve their health. Numerous sources can influence women's preparation for adequate self-care during this period, including the health team, the media, and advice from mothers, grandmothers, and non-professional friends. Nevertheless, lack of orientation on the need for puerperal consultations upon discharge from hospital and professionals' lack of knowledge on the practices used in the home context can contribute to women's adoption of unhealthy conducts²².

It should be reminded that it is in the domestic sphere that knowledge, decisions, and practices operate, which are sometimes conflicting with maternal health care needs. Therefore,

it is fundamental for health professionals to get to know popular practices, encourage health promotion practices, and problematize harmful conduct that puts the well-being of the mother and child at risk. Studies reveal that puerperal women's major doubts relate to diet, bodily hygiene, physical exercise, and sexual intercourse. The belief in hypokalaemia emphasizes foods considered lactogenic, including canjica, milk, and rice pudding. Daily baths continue according to each woman's customs, but washing the head is prohibited during the protection period. Surrounded by meaning, the worst consequence of this practice is presented as death²³.

Breastfeeding and Baby Care

Assessment of two respondents showed opposing views regarding placing religious articles on a baby's clothing to pro-

tect them from evil spirits. With the notion of protection from evil spirits, most Indonesian mothers embrace putting religious articles on their baby's clothing. On the contrary, most Filipino mothers do not follow the same practice 136 anymore. Nevertheless, both cultures showed commonalities in traditional practices regarding breastfeeding and baby care, particularly on breastfeeding their baby to provide the most nourishing milk; and protecting their newborn from anything that might harm them. Although Filipino mothers also apply baby oil or powder to their baby after giving a bath to provide warmth and comfort; and do not expose their baby outside late in the afternoon or when the mother feels the weather is cold, it is apparent that this kind of traditional practice is being performed by more Indonesian mothers in which they strongly agree.

Indonesia is one of many developing countries that continue struggling to improve children's and mothers' health outcomes. In Indonesia, infant mortality rates are highest among children whose mothers gave birth at age 40 or older, had high parity (3 or higher), and became pregnant after a short birth interval/less than 24 months. The rate is also highest for children living in rural areas, mothers with no education, and children in the lowest wealth index. According to the 2012 Indonesia Demographic Health Survey (IDHS), only one-third of Indonesian mothers follow WHO-UNICEF (2003) recommendation to provide breast milk only (exclusively breastfeeding practice) for the first six months of an infant's life. However, infants who take breastfed are generally breastfed until well into their second year or beyond, and the median duration of any breastfeeding is 21 months²⁴.

However, some women restrict water intake during the puerperium because of fear of water retention²⁵. In a study carried out in China, consuming a particular type of herbs during puerperium like Almjelb, Anise, hella is believed to facilitate lochial drainage, improve milk production, and expels cold the body, and mothers report that they consume more food than usual. The meals number ranged from five to eight in a day, starting at 5 am finishing with the meal before sleeping at night²⁶. There is a belief that post-partum women should eat much food because women are weak, and food will help rebuild their strength, promote recovery, and improve breastfeeding^{3,27}. Women consume food more than usual because they always feel hungry and to compensate for blood loss. Colostrum has been called mother's gold liquid, a thick, yellow substance produced toward the end of a female's pregnancy and is emitted by her mammary glands during the first 48 hours after giving birth²⁸.

Sadly, some women do not give colostrum to their babies, as they consider it insufficient and has no benefits for giving it to the baby. The women discarded the colostrum because it is dirty, "like pus," and potentially harmful to the baby²⁹. Women who know colostrum give it to their babies. In addition, more women have no intention to breastfeed their babies³⁰. Some women believe that they have a problem in milk production and breastfeeding increases breast size and body weight. The identified obstacles to breastfeeding are perceived milk insufficiency, maternal employment, breast and nipple problems, and pressure from family³¹.

Dietary Modifications

Two groups of respondents showed opposing views on taking more hot soup with traditional vegetables to increase breastmilk production and eating more nutritious food to regain strength. Indonesian mothers both integrate taking hot soups and eating more nutritious food on their diets to increase

breastmilk production and regain more strength. On the contrary, Filipino mothers do not apply the practices mentioned earlier in their dietary practice. Nevertheless, both cultures showed commonalities in refraining from eating foods that are thought not good for wound healing but follow recommended traditional diets advised by older female family members and avoid cold drinks while on post-partum recovery to prevent chill.

Mothers who have just given birth need good nutrition to support their healing and recovery. Furthermore, for mothers breastfeeding, their diet also directly impacts their baby's health and growth³². Pregnancy takes an enormous toll on a women's body, and recovering from birth is a delicate and slow process that takes intention and support. Post-partum wellness has been misinterpreted as weight loss, but in actuality, a woman's body needs careful attention for recovery and healing in the form of nourishing foods, rest, and support^{33,34}.

In line with Garner's research, people in China believe that post-partum women are weak and lose energy and blood during delivery. For this, they should eat a lot of "warm" food full of proteins as this will help her regain strength, promote recovery, improve breastfeeding, enrich the blood, enhance recovery of the mother, facilitate discharge of lochia, and stimulate the production of breast milk.

Physical Activities

Assessment of two respondents showed commonalities in the traditional practice except that one group illustrates a higher degree of agreement over the other. More Indonesian mothers strongly agree with babysitting, bathing, and walking around the house, while Filipino mothers would also do the same on a much lesser consideration. Similarly, Indonesian practice avoids sexual activity while on post-partum recovery to avoid stress and infection, while Filipino practice also observes the same practice even more. Both groups of mother-respondents also avoid sexual activity while on post-partum recovery to avoid stress and infection; entertain occasional visits from friends, family, and other relatives; and get the body massage with aromatic or therapeutic oil or wear an abdominal corset to relieve pains and bleeding. Thus, both Indonesian and Filipino mothers traditionally adhere to physical activities that are essential to post-partum care.

In line with Garner's research³ claim that all families believed that when the mother goes outside, wind will enter her body and cause illnesses not only arthritis and rheumatism later in life but also headache, poor appetite, and colds. They added that having adequate rest in the post-partum period helps the weak mother regain her strength and health to care for the new baby and resume normal activities. They regarded housework as a predisposing factor to the mother's exposure to either water or wind, causing arthritis and chronic aches.

Practices on post-partum and infant care are actions done by women and are not explained scientifically, but they continue to perform and are believed to be favorable to maintain their well-being since their mothers, mothers-in-law, and neighbors have practiced it and have guaranteed their health³⁵. For example, mother roasting 'can involve lying beside a stove for up to 30 days, squatting over a burning clay stove, sitting on a chair over a heated stone or a pot with steaming water, or bathing in smoke from smoldering leaves. These practices may be replaced by hot water bottles in Australia and placing a post-partum woman close to a heater. Post-partum women may be massaged with coconut oil to restore their lost health, expelling blood clots from the uterus, returning the uterus into

a normal position, and promoting lactation. Some women perform various practices to dry out 'the womb'³⁶.

Limitations

Current research has not characterized all post-partum cultures in all corners of the country compared with the culture of post-partum mothers in the Philippines.

Conclusions

Four dimensions, including hygienic care (*p*-value 0.038), breastfeeding and baby care (*p*-value 0.000); dietary modifications (*p*-value 0.000); and physical activities (*p*-value 0.000), showed a statistically significant difference in the assessment of mother respondents on their traditional practices in post-partum care. Meanwhile, the dimension on behavioral precautions (*p*-value 0.250) yielded statistically no significant difference on the assessment of mother respondents on their traditional practices in post-partum care.

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Conflict of Interest

The author(s) declares no conflict of interest.

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RESEARCH / INVESTIGACIÓN

Clinical Characteristics and Outcome of SARS-CoV-2 Patients. An Experience from Anbar province - West of Iraq

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Abstract: The SARS-CoV2 infection emerged in Iraq in February 2020. In this study, we describe the clinical characteristics and outcomes of the initial SARS-CoV2 patients. A total of 529 patients were included in this study from April to August 2020 in Anbar province. Patients were confirmed to be infected in nasal swabs by real-time RT-PCR or chest CT scan findings. The gathered data included the demographic variables (age, sex, residency), presence of comorbidity (hypertension, diabetes mellitus, respiratory illness, coronary heart disease, chronic kidney disease, obesity), and history of contact with a known case of SARS-CoV2. The results showed that 64% of the patients were males and 36% were female, 48% of the patients lied in the age category 40-59 years, 74% had exposure history, 95% did not have a history of smoking, 46% were overweight, 60% had no comorbidity, 78% presented with mild/moderate disease, 70% had typical chest CT scan finding (CO-RAD 5), and 76% of patients showed positive PCR. The fatality rate is 16%. Most of the patients had a history of exposure to a confirmed case of SARS-CoV2 before the illness. The severity and outcome were correlated with risk factors and comorbidity. Combining chest computed tomography images with the qPCR analysis of nasal swab samples can improve the accuracy of SARS-CoV2 diagnosis.

Key words: SARS-CoV-2, Clinical Characteristics, Outcome, West of Iraq.

Introduction

SARS-CoV2 is a novel virus causing a global pandemic with significant morbidity and mortality. It is a coronavirus from Coronaviridae's family and was first discovered in Wuhan, China, in December 2019; it is also named Covid-19 (Corona Virus Disease 2019)¹. The discovery of the virus was made after having an increasing number of patients with acute respiratory tract infection not attributed to known viral infections like the influenza virus, avian flu, and severe acute respiratory syndrome coronavirus (SARS-CoV). Lockdown of the area where the disease appeared first with other precautionary measures has failed to contain the virus, and it spread rapidly to other countries in Asia (South Korea, Iran, India, Pakistan, and many others) and then globally^{2,3}. In its update in March 2020, the World Health Organization (WHO) declared the disease a pandemic in a press announcement. This is not the first outbreak caused by this kind of viruses in the last 2 decades, since it had been described in 2002 (Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and 2012 (the Middle East Respiratory Syndrome Coronavirus (MERS-CoV)). The mortality rate of SARS-CoV was 10%, while that of MERS-CoV was 37%^{4,5}. The spread of the virus varied enormously among different countries and within the same country or region. However, many studies have confirmed that the number of affected cases tremendously overshoots reported cases confirmed by PCR testing⁶.

The average incubation period of the disease is 6 days⁷, but it could be as long as 14 days⁸. Most patients develop only mild symptoms of fever, mild cough, loss of taste and smell sensations, and muscle cramps. Nonetheless, a minority might present with severe pneumonia, acute respiratory distress syndrome, and multi-organ failure^{9,10}. Radiologically, bilateral pulmonary parenchymal ground-glass and consolidative pulmonary opacities are the most frequent CT; however, occasionally, rounded morphology and peripheral lung distribution might be evident^{11,12}. Iraq reported the first case of covid-19 in

late February 2020 in Al-Najaf province. The disease then gradually involved all governorates and provinces. The first confirmed case in Anbar Province (West of Iraq) was on 25th March 2020, and the first death was on 31st May 2020¹³. Knowledge of the natural course of the disease and its virulence is essential in organizing proper plans and procedures to face this illness effectively. Studies have shown that Covid-19 has a wide range of disease severity and mortality¹⁴, representing a fundamental challenge facing health care providers. Several studies were carried out in different parts of Iraq targeting these objectives, and this study represents another one to describe the clinical characteristic and outcomes of the disease in the West of Iraq.

Materials and methods

Depending on the local protocol, patients who tested positive to SARS-CoV2 using Real-Time Polymerase Chain Reaction (RT-PCR) and patients with classical clinical and radiological features and history of contact with Covid-19 patients (even with negative RT-PCR) were enrolled in this cross-sectional study. We used a CT-based system (CO-RADS) to assess the suspicion of pulmonary involvement in SARS-CoV2¹⁵. Patients were either admitted to Ramadi Teaching Hospital or treated at home based on their clinical status. Those who were treated at home were followed by medical personnel regularly. The management line is based on the Iraqi Ministry of Health's national guidelines for clinical care and treatment of SARS-CoV2, updated regularly.

A total of 529 patients were included in this study during the period from April to August 2020. The gathered data included the demographic variables (age, sex, place of living), presence of comorbidity (Hypertension, diabetes mellitus, respiratory illness, coronary heart disease, chronic kidney disease,

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se), and history of contact with a known case of SARS-CoV2. Patients were categorized into 4 categories. Mild disease is when a patient complains of one or more of the following: Low-grade fever, mild cough, runny nose, and sore throat. The moderate disease is when a patient shows clinical and radiological features of pneumonia without hypoxia: Fever, cough, chest tightness, clinical and radiological evidence of consolidation(s). Severe disease is when a patient shows clinical and radiological features of pneumonia with hypoxia which requires assisted ventilation. Acute disease is when a patient suffers from clinical shock or organ(s) failure¹⁶. The Human Research and Ethics Committee, Anbar University (Ministry of Higher Education and Scientific Research- Iraq) approved this study (Notification letter No. 69/2020).

Statistical analysis

It was carried out using SPSS, version 22. Since all variables were of categorical types, the Chi-Square test with a 5% significance level was used to examine the investigated relationships between patients' characteristics and survival status. If the chi-square test's assumption (expected count) was violated, either Fisher's exact test or likelihood ratio Chi-square test was used as suitable.

Results

During the study period, 529 patients with SARS COV-2 were either admitted in Ramadi hospital or followed at home. Of them, 64% were males, 48% of age 40-59 years, 74% with exposure history, 95% without a history of smoking, 46% overweight, 60% without comorbidity, 78% with mild/moderate covid, 70% having CO-RAD 5 chest CT scan finding and 76% with positive PCR. Out of 529 covid-19 patients, 84% survived, while the remaining 16% could not survive (case mortality rate is 16%).

Table (1) shows the characteristics of SARS-CoV2 patients by survival status. There was no significant difference in the distribution of survival status by gender. The percentage of non-survival increased with age, and the association was significant ($p=0.001$). The majority of non-survivors had no smoking history. The highest percentage of non-survivors was among overweight patients (35%), and the difference was statistically significant ($p=0.001$). Almost all non-survivors (99%) had at least one comorbidity (hypertension, diabetes, coronary heart disease, chronic obstructive lung disease, chronic kidney disease) compared to 29% of survivors, and the association was significant ($p=0.001$).

Demographic	Survival Status				p-value
	Survivor (n=444)	Not Survivor (n=85)	Total (n=529)		
sex	Male	282 64%	56 66%	338	0.677
	Female	162 36%	29 34%	191	
Age group	<25 years	22 5%	1 1%	23	0.001
	25-39 years	106 24%	11 13%	117	
	40-59	229 52%	25 29%	254	
	≥60 years	87 20%	48 56%	135	

Table 1. Survival status of SARS-CoV2 patients by demographic characteristics.

Table (2) survivors reported less exposure history (72%) compared to non-survivors (86%), and the difference was statistically significant ($p=0.008$). All non-survivors (100%) were diagnosed with severe/critical covid-19 status compared to only 8% of survivors, and the association was significant ($p=0.001$).

The percentage of non-survivors increased with disease severity, and CORAD level as shown by CT scan as 85% of non-survivors had CO-RAD 5 ($p=0.001$) (Table 3).

Discussion

SARS-CoV2 pandemic continues spreading despite all measures exerted by health authorities to face it. Understanding patients' characteristics about the survival rates of the disease is valuable in this confrontation. We are still gaining knowledge in identifying clinical characteristics of severe disease and mortality. This study summarizes the clinical characteristics of 529 cases with SARS-CoV2 infection concerning the outcome, i.e., survivors vs. non-survivors. Knowing these characteristics will have a significant impact on improving survival. The reported overall case fatality in different Iraq provinces is as follows: Southern 9.4%, Northern 2.1%, Eastern 16.7%, and the Capital 8.7%¹⁷. This study estimated 16% as a case fatality rate in Anbar province – west of Iraq.

There is a consensus among researchers and clinicians that the increasing age of the patients is significantly linked to a higher non-survival rate in SARS-CoV2 patients^{18,19}. Likewise, the current results showed that elderly patients were prone to more severe disease and higher mortality. The possible cause is not well understood; however, viable hypotheses emerge, including changes to the immune cell repertoire, epigenome, nicotinamide adenine dinucleotide (NAD+) levels, and inflammasome activity biological clocks, and covalent modifications of human and viral proteins²⁰.

Several studies confirmed that SARS-CoV2 infection is more common in patients with comorbidities^{1,10,18}. This is consistent with the current study. However, the results could not find any dominance of particular comorbidity over the others in terms of outcome. Factors that could explain this include the increased expression of the angiotensin-converting enzyme-2 (ACE2), cytokine storm, and drug interactions in patients with comorbidities²¹.

Many studies have mentioned that the male gender is one of the risk factors for increased severity and mortality independent of age²²⁻²⁴. However, this study showed no significant difference in terms of survival status and gender. Similar fin-

Risk Factors		Survival Status				p-value	
		Survivor (n=444)		Not Survivor (n=85)			
Smoking	Yes	24	5%	4	5%	28	0.522
	No	420	95%	81	95%	501	
Body weight/ BMI	Normal (18.5-24.9)	178	40%	26	31%	204	0.001
	Over weight (25-29.9)	215	48%	30	35%	245	
	Obesity (30-34.9)	36	8%	20	24%	56	
	Morbid Obesity (+40)	15	3%	9	11%	24	
Comorbidity	Yes	127	29%	84	99%	211	0.001
	No	317	71%	1	1%	318	
Comorbidity	Hypertension	31	7%	45	53%	76	0.001
	Diabetes	21	5%	55	65%	76	0.001
	Coronary heart disease	14	3%	12	14%	26	0.001
	Chronic obstructive lung disease	8	2%	11	13%	19	0.001
	Chronic kidney disease	4	1%	2	2%	6	0.248
	Others(Stroke, Chronic liver diseases, Rheumatoid Arthritis and Malignancies)	6	1%	2	2%	8	0.621

Table 2. Survival status of SARS-CoV2 patients by risk factors assessment.

COVID Status		Survival Status				p-value	
		Survivor (n=444)		Not Survivor (n=85)			
Exposure history	Yes	321	72%	73	86%	394	0.008
	No	123	28%	12	14%	135	
Disease severity	Mild/Moderate	410	92%	0	0%	410	0.001
	Severe/Critical	34	8%	85	100%	119	
Chest CT-Scan	CORAD3	21	6%	0	0%	21	0.001
	CORAD4	88	27%	12	16%	100	
	CORAD 5	217	67%	65	84%	282	
PCR	POSTIVE	327	75%	70	82%	397	0.129
	NEGATIVE	111	25%	15	18%	126	

Table 3. Survival status of SARS-CoV2 patients by disease exposure and severity.

dings have been seen in other studies²⁵⁻²⁷. A possible explanation would be that in certain countries, like Iraq, women are at higher risks due to demographic factors or local health characteristics which has been shown in other studies conducted in other parts of the country^{17,28}. The history of smoking was considered as a nasty prognostic factor in numerous studies^{1,29-32}. This is in contrast with the current findings, as the majority of non-survivors were a non-smoker. This may be related to the low numbers of smokers in this study compared to non-smokers or incomplete records. In addition to this, severe SARS-CoV2 targets the older population (>65 years), in whom smoking rates are approximately 3-5 fold lower than that in the general population³³. Obese patients are at higher risk of developing severe and critical illness than non-obese patients³⁴⁻³⁶. A similar finding was shown in this study. This is likely because obese patients are known to have a defective immune system in addition to underlying comorbidities^{37,38}. Obesity changes the role of immunity by altering the response of cytokines, resulting in a decrease in the cytotoxic cell response of immuno-competent cells that have a key anti-viral role and disturb the balance of endocrine hormones, like leptin, that affect the interaction between metabolic and immune systems^{39,40}. Chest CT scan is a valuable tool in the diagnostic process of viral pneumonia cases associated with SARS-CoV2. The sensitivity and specificity of the chest CT in diagnosing SARS-CoV2 and the radiation exposure have to be judged together. Arguments exist regarding the value of chest CT scan for SARS-CoV2 diagnosis, particularly those patients who exhibit typical clinical symptoms and have negative RT-PCR results in highly infected regions⁴¹.

Conclusions

The SARS-CoV2 infection caused severe respiratory illness with significant morbidity and mortality. Understanding these factors can enhance defining patients at higher risk and allow a more targeted approach to prevent those deaths. Using chest CT scan images with nasal swab sample qPCR analysis may improve the accuracy of SARS-CoV2 diagnosis.

Conflict of interest

Authors have no conflict of interest.

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RESEARCH / INVESTIGACIÓN

The inhibitory effect of aqueous and alcoholic extract of red pepper on some isolated pathogenic bacteria from different areas of human body

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2270

Abstract: In most underdeveloped nations, traditional medicine, including herbal treatment, is still widely used. Due to the growth of antibiotic resistance, this study aims to use pepper as an anti-bacterial as alternative to antibiotics. Pepper is one of the most important plants used as a medicine for a long time in various countries and civilizations. This study aims to use pepper as an anti-bacterial in alternative to antibiotics. The current study included the inhibitory efficacy of aqueous and alcoholic red pepper extract on seven bacterial isolates: *Staphylococcus aureus*, *Streptococcus spp*, *Escherichia coli*, *Klebsiella spp*, *Salmonella spp*, *Pseudomonas aeruginosa*, *Proteus spp*. These isolates were isolated from different pathologies and regions, and they were diagnosed according to the site of infection. Several antibiotics were also used as a standard control sample for germs. This study shows that the highest inhibitory Effect against *E. coli* bacterium, as the average inhibition diameter was about 16.5 mm, and it is an excellent inhibitory when compared to the antagonist's gentamicin and nitrofurantoin as it showed good inhibitory efficacy against the bacteria such as *S. aureus*, *P. aeruginosa*, and *Salmonella spp*. While the effect on Klebsiella was equal, on the other hand, the aqueous extract did not show any efficacy against *Proteus spp*, as was shown in the results. The results also showed that *Staph. Aureus* bacteria were the most affected by the alcohol extract of the red pepper as it showed a high inhibition zone compared with the control sample tetracycline and nitrofurantoin. The plant's aqueous and alcoholic red pepper extracts were effective against the tested bacterial isolates. The plant's aqueous and alcoholic red pepper extract has good inhibitory efficacy against the studied bacterial isolates.

Key words: Red Pepper Alcoholic extract, Aqueous extract, antagonism, Inhibition activity.

Introduction

Traditional medicine, including herbal medicine, is still widely practiced in most developing countries. Red pepper is the dried, ripened fruit pod from the *Capsicum* species' pungent (hot) varieties. In addition to Cayenne, red pepper also goes by other names, including chili pepper, chile pepper, hot pepper, and red chillies¹. Pepper is one of the most important plants that have been used as medicine for a long time in different countries and civilizations². Because modern medicines such as antibiotics that previously enjoyed almost comprehensive adequacy against pathogens weaken their effectiveness³.

The spread of resistant pathogens, manufactured medicines are one of the most severe problems that impede the success of treatments for diseases caused by germs. Since ancient times plant extracts and volatile oils have drawn attention as a source of natural products as they have shown their therapeutic power as an alternative for treating many infectious diseases⁴. The effect of medicinal plants on the growth of microorganisms may lie in one of its components⁵; it contains active substances, including (volatile oils, glycosides, saponins, tannins, alkaloids, lipids, carbohydrates, resins, and sterolylates⁶. Pepper has antimicrobial properties that are important for the human health of plants⁷ and antioxidant properties^{8,23}. Studies have shown that they have anti-bacterial, antifungal, and antiviral properties⁹. Research in the use of plant extracts and their active components as anti-bacterial agents, as the inhibitory effect of the active substances in these plants, is dilute and easy. Bodies can interact with them; in addition to that, they contain many active substances that work together to treat the disease, so it was used for many pathological symptoms⁹. The World Health Organization (WHO) indicated that a large proportion of the world's population primarily uses folk

medicine in health care and medicine¹⁰. It is worth noting that the world is now more inclined than before to treat herbs and medicinal plants after the results of the research have shown the effectiveness of treatment with them, and there has become a near-global race to register the most significant possible number of patents for the results of the research that is being conducted.

The present study aimed to isolate and diagnose different multiresistant Gram-positive and gram harmful bacteria and to study the effect of red pepper extracts on the isolated bacteria—the Comparison effect of watery and alcoholic pepper extract with several commonly used antibiotics.

Materials and methods

Sixty samples were collected from patients with various infections and pathological injuries from different areas of the body from Tikrit Teaching Hospital were placed directly after taking them in test tubes containing the sterile medium (Brain heart infusion broth), after which the tubes were transferred to the laboratory, incubated at 37 ° C for 24 hours in blood base agar and MacConkey media.

Bacterial isolates

Seven bacterial species were isolated from different parts of human bodies, and they confirmed that they are pathogenic bacteria. Nutrient Agar base and MacConkey Agar were incubated at 37 ° C for 24 hours

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Preparing plant extracts

The aqueous extract was prepared by mixing 40 grams of dry red pepper with 160 milliliters of sterile distilled water. While the alcoholic extract, mix 40 grams of the dry plant model with 160 milliliters of ethyl alcohol at a concentration of 95% to prepare the alcoholic extract, stir the mixture with a shaker device, and leave the mixture in the refrigerator for 24 hours. For soaking. Filter using filter paper to get rid of unpowdered particles and fibers. After filtration, the extract is placed in the incubator at 40 ° C until all liquids are evaporated and the extract remains in Baker's base^{6,11}.

Sterilization of the alcoholic extract

It was prepared by dissolving 1 g of the plant extract in 5 cm³, sterilizing the mixture at a temperature of 62°C for 15 minutes, thus obtaining the standard center of the alcoholic extract used to prepare the subsequent dilution^{10,12}.

Diffusion test method

The methods included drilling a nutrient agar medium of equal dimensions with a diameter of 6 mm using the cork borer. 0.1 ml of the bacterial leeches is spread on the surface of the medium, and then the pits are filled with the extractor solution, and the plates are then incubated at 37 ° C for 18 hours, then the extract's effectiveness is evaluated. Inhibition on bacteria by measuring the diameter of the inhibition circuit

Antibiotic sensitivity test

The method (Kirby Bauer)¹³ was followed to test the susceptibility of germs to the types of antibodies used prepared by the company (Bioanalyse) where (5-4) pure colonies of these germs were transferred to the medium of the nutritious broth and the bacterial cultures were immunized to a degree. 37 C for a period of (14 - 16) hours, then dilute the bacterial suspension with the physiological solution compared to the standard control tube, which is equivalent to 10 cells / cm³, then transfer 0.1 cm of the bacterial suspension to the medium of neutron agar and by using a cotton swab spread on the surface of the plate using a cotton swab left the dishes at a degree. The room temperature was set for 30 minutes, then the antibiotic tablets were fixed and using sterile forceps, the dishes were preserved

for (14-16) hours, then the area of growth inhibition was measured by one millimeter¹⁴, and then the results were recorded.

In this study, samples were taken from patients suffering from pathological injuries and different body areas according to the area of injury shown in Table (1).

Diagnosis and identification

The bacterial colonies growing on media (McConkey agar and nutrient agar media) were identified through microscopy, culture characteristics, and biochemical tests, which included tests (catalase, oxidase, indole, red methyl, vox-Proskauer, Simmons citrate, motility, urease, Klingler iron agar test).

As for the tests for bacteria of the genus *Staphylococcus* included (catalase test, clotting test, growth on mannitol salt agar, oxidase and urease).

Results and discussion

The alcoholic extract showed higher inhibitory efficacy than aqueous on the growth of isolates of multiple antibiotic-resistant bacteria.

The aqueous extract of the red pepper plant showed good inhibitory efficacy against the studied microbial species, as it had the highest inhibitory action against *E. coli*, with the average diameter of the inhibition circle diameter 16.5 mm, and it is an excellent inhibitory when compared with the antibiotics Gentamicin and Nitrofurantoin as it showed good inhibitory efficacy against because this pepper contains a large number of fatty acids esters, amides, monoterpenes, diterpenes¹⁵, triterpenes, sesquiterpenes, and phytosterols were shown in Sharma *et al.*¹⁶. It also exhibited significant antimicrobial activity against pathogenic microorganisms¹⁷. The bacteria *S. aureus*, *P. aeruginosa*, and *Salmonella. Sp.* The results were identical to the results of Araujo *et al.*¹⁸. The results in the current study disagree with Soetarno *et al.*¹⁹ how tested the antimicrobial property of pepper and found ethanol extracted pepper was more effective than aqueous extracted.

The extract did not show any inhibitory efficacy against the bacterium *Proteus. sp*, while the alcohol extract showed the inhibitory activity against the bacterium itself. From the rates of inhibition diameters, the bacterium was found most affected by the alcohol extract of the plant, which showed High

Bacteria Type	isolation area	Type of infection
<i>S. aureus</i>	Throat	Laryngitis
<i>Streptococcus spp</i>	Gum	Gingivitis
<i>Salmonella spp</i>	Intestinal	Food poisoning
<i>E. coli</i>	Intestinal	Intensive inflammation
<i>Proteus spp</i>	Urinary tract	Urinary tract inflammation
<i>P.aeruginosa</i>	Intestinal	Intestinal diarrhea
<i>Klebsiella</i>	Intestinal	Intestinal diarrhea

Table 1. Shows the areas of bacterial isolation and the disease state isolated from them.

Extract concentration Mg/mm	<i>E.coli</i>	<i>Staph aureus</i>	<i>Streptococcus spp</i>	<i>Ps. aeruginosa</i>	<i>Kl. pneumonia</i>	<i>Proteus spp</i>	<i>Salmonella spp</i>
Aqueous extract							
200	16.5	12.5	11.5	12	9.2	0	11
100	12	11	10.3	10.5	8.5	0	9.5
50	9	9	8.7	9	0	0	7.5
25	0	0	0	0	0	0	0
Alcoholic extract							
200	11	15	12	13.8	9.3	11	10.5
100	8.5	12.5	10.3	10.3	7.2	9.5	7
50	0	11	8	0	0	8	0
25	0	9	6.4	0	0	0	0

Table 2. The inhibitory efficacy of chilli pepper plants aqueous and alcoholic extract in bacterial species compared with the antibiotics used as a control sample (Diameter of the damping circuit measured in mm).

Antibiotic	Results Antibiotic Sensitivity						
Gentamicin	14	15	10	30	20	23	20
Ampicillin	0	0	0	0	0	0	14
Ciprofloxacin	0	0	0	0	0	0	17
Tetracycline	13	8	7	0	15	10	8
Nitrofurantoin	12	12	6	20	0	15	20

Table 3. Antibiotics used to sensitivity test.

sensitivity inhibition when compared with the control sample tetracycline and nitrofurantoin as the results shown in the same Table showed that the alcoholic extract of the plant has inhibitory activity affecting the bacteria *E.coli*, *P. aeruginosa*, and *Streptococcus sp*. These results are similar to the results obtained by (20) Antimicrobial activity of ethanol extracts from different Capsicum against *S. aureus*, *Saccharomyces* was investigated by (14 and 21) and anti-bacterial

The activity of chili against *Bacillus subtilis* and *E. coli* was reported by De et al.²². The alcoholic extract of the red pepper plant was found to have inhibitory efficacy against many types of germs, and the *Klebsiella sp* bacterium was cleansed. The bacterial species was least affected by the aqueous and alcoholic extract of the plant; as the average diameter of the inhibition, the circuit was about 9.2 mm at a concentration of 200 mg/cm³ bitter, which the substance's inability can explain. The effective dissolved in water and alcohol to penetrate the biological envelope of the germ, as the reason for this may be attributed to its possession of medicine. The mucosal time (reduced) may give it an essential defensive factor that protects it from the inhibitory action of the active components of the extract²³. Many researchers demonstrated the effect of plant extracts on bacterial inhibition^{1,2,24}. The use of various compounds to inhibit pathogenic bacteria has been discussed in previous studies, such as the direct influence of Ag and TiO₂ nanoparticles on pathogenic bacteria such as *P. mirabilis* and *Proteus vulgaris* in a previous study²⁵. However, several studies have discovered that applying physical forces therapy to pathogenic bacteria, such as Audible Sounds and Magnetic Fields, can help to reduce the resistance of *S. aureus* to infection²⁶.

Conclusions

The plant's aqueous and alcoholic red pepper extracts were effective against the tested bacterial isolates. The highest in-

hibitory action of the plant's aqueous red pepper extract was against *E.coli*, while there was no inhibitory effect against the bacterium *Proteus sp*.

Conflict of interest

The author declares that there is no conflict of interest for this study.

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RESEARCH / INVESTIGACIÓN

Role of Leptin with hypothyroidism in Iraqi diabetic type 2 patients

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2274

Abstract: In thyroid subjects, leptin hormone and thyroid-stimulating hormone levels connect; both are emphatically corresponding with adiposity. "Leptin hormone was essentially raised in the hypothyroid topic," to levels like those seen in corpulent thyroid topic. This study aimed to determine leptin hormone levels, T3, T4, TSH, HbA1c %, FBG, lipid profile in diabetic and diabetic with hypothyroidism patients and compare the outcome with the healthy group. 90 samples were registered in this study with their ages ranging between (40 - 65) years that dole out into 3 groups as follows: thirty healthy groups (G1), thirty patients with diabetes (G2), and category three (G3) include diabetic patients and hypothyroidism as an associated disease. This study revealed a slightly significant elevated leptin in G3, contrasting to G2 and G1. Meantime no significant elevated spotted between G2 and G1. The conclusion could be from this investigation that leptin levels were changed in patients' gatherings that might be utilized in checking and early analysis of thyroid dysfunction in these patients relying upon the significant connection for leptin chemical with T3, T4, and TSH.

Key words: Leptin hormone, hypothyroidism, and diabetic type2.

Introduction

Diabetes mellitus (DM) and thyroid disorders are two endocrine problems that influence each other in various reactions. Thyroid chemicals add to the guideline of carbohydrates digestion and pancreatic capacity^{1,2}. Sensible components for progression of T2DM in thyroid issue patients might be identified with the upset hereditary articulation of qualities related to physiological distortions prompting hindered glucose utilization by the expanded hepatic glucose yield, muscles, and glucose absorption rise from digestive system³. Leptin is a (146) amino acid protein chemical secreted by adipocytes in light of an increment in fat mass. It is by all accounts a vital atom in the input circle that directs energy balance. Leptin has a double activity: it diminishes hunger and builds energy utilization, making more fat scorched⁴. Leptin gives data to the sensory system on the measure of energy put away in the fat tissue. Serum leptin levels profoundly correspond with muscle to fat ratio mass in grown-ups, youngsters, and babies. Obese individuals have altogether higher flowing leptin than typical. In obesity, a diminished affectability to leptin happens (like insulin opposition in type 2 diabetes), bringing about powerlessness to recognize satiety despite high energy stores and high levels of leptin⁵. Thyroid hormones and leptin are two chemicals that manage power balance via central signaling mechanisms⁶. Aggravation of thyroid capacity is related to noticeable changes in energy consumption and body weight; furthermore, it has accordingly been the subject of much exploration to contemplate the shared jobs of thyroid hormones and leptin in this regard⁷. In thyroid subjects, leptin hormone and thyroid-stimulating hormone levels connect; both are emphatically corresponding with adiposity. "Leptin hormone was essentially raised in the hypothyroid topic" to levels like those seen in corpulent thyroid topic. "The information is steady with the speculation that leptin hormone and the pituitary-thyroid pivot interface in the thyroid state, and that hypothyroidism reversibly increments leptin focuses"⁸⁻¹¹.

Materials and methods

Ninety individuals with ages ranging between (40-65) years were joined up with this examination.

They separated into three gatherings as follows:-

1. Gathering (G1) that comprises 30 sound people as control bunch.
2. Gathering (G2) that consists of 30 diabetic patients.
3. Gathering (G3) that includes diabetic patients and hypothyroidism as an associated disease.

Blood samples were gathered from all gatherings after a time of fasting 12-14 hours. The study was conducted between December 2020 – April 2021 in the diabetic & endocrinology center in Al-Yarmouk Teaching Hospital / Iraq.

Estimation of Leptin levels by Competitive Elisa Reveals¹². The T3, T4, and TSH were determined by Enzyme-linked Fluorescent Immunoassay (ELFA) competition method with a final detection¹³. Whole blood was used in the determination of HbA1c. The HPLC method¹⁴ determined the HbA1c. Serum glucose was measured using kits from (Randox Company, United Kingdom) based on the PAP enzymatic determination of glucose¹⁵. TC¹⁶, TG¹⁷, and high-density lipoprotein¹⁸ were estimated using the enzymatic method (Human Gesellschaft für Biochemical and Diagnostica mbH, Germany). The levels of LDL and VLDL were analyzed by using Friedewald equation¹⁹.

Statistically study outcomes were communicated as mean ± SD. T-test was used for comparison among the three studied groups. The P-vales (< 0.05) no significant, (> 0.05) significant, (> 0.01) highly significant were considered statically.

Results and discussion

Descriptive was introduced in Table (1), which shows the levels of HbA1c%, F.S.G, T3, T4, TSH, and leptin for every considered gathering. Table (1) showed a highly significant increase in patients' gatherings (G2, G3) contrasting with control bunch in HbA1c% and FSG levels. Likewise, a significant rise in G3 contrasted with G2 was found.

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Results uncovered a significant decrease in levels of T4 and T3 in G3 when contrasted with group one and group two. Furthermore, there is a highly significant increase in TSH level in group three contrasted with groups one and two.

Diabetic patients appear to impact thyroid capacity in the hypothalamic control of thyroid-stimulating hormone discharge and at peripheral tissue by changing T4 to T3. High glucose levels lead to decreased deiodinase enzyme concentration in the liver, decreased T3, rising levels of opposite T3, and low, typical, or undeniable levels of T4²⁰.

Results in Table 1 uncovered a significant rise in listening levels in G3, contrasting with G1 and G2. These outcomes show that serum leptin is somewhat raised in subjects with moderate hypothyroidism, potentially because of the immediate activity of thyroid hormones.

Leptin is another hormone assuming a significant part in the guideline of power balance by tweaking food consumption, thermogenesis, just as lipid and glucose digestion. Concerning interactions between leptin and thyroid hormones, the impact of thyroid capacity and hypothyroidism, specifically on flowing leptin hormone levels, gave clashing information. Subsequently, diminishes, increments, or non-change in leptinaemia were accounted for in hypothyroid patients. This is the way might clarify disparate outcomes that leptin hormone levels fundamentally invert changes in lipid cluster, this boundary differing as indicated by sex, age, and span of hypothyroidism²¹.

Table (2) shows the levels of lipid profile for G1, G2, and G3. Results revealed a significant rise in lipid profile levels without HDL in groups two and three, contrasting to G1. Results likewise showed a significant rise in lipid profile without HDL in G3 contrasting to G2; Results also showed a significant decline in HDL levels in group two and group three contrasting with

G1, while no significance was found in group two-three contrasting with group two in HDL levels.

Conclusions

The conclusion could be from this investigation that leptin levels were changed in patients' gatherings that might be utilized in checking and early analysis of thyroid dysfunction in these patients relying upon the significant connection for leptin chemical with T3, T4, and TSH.

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Parameters	Group 1	Group 2	Group 3	T-Test	T-Test	T-Test
				G1 vs. G2	G2 vs. G3	G1 vs. G3
HbA1c%	5.42±0.40	8.23±1.057	10.98±1.39	HS	S	HS
F.B.G (mmol/L)	4.356±0.76	10.607±2.53	11.091±2.935	HS	NS	HS
T3 (nmol/L)	1.67±0.385	2.77±0.173	1.08±0.064	S	S	S
T4 (nmol/L)	82.5±9.550	115.22±27.4	47.91±15.506	S	HS	HS
TSH (nmol/L)	2.23±0.416	3.44±0.07	21.44±4.82	S	HS	HS
Leptin (ng/mL)	3.7 ± 2.1	3.79 ± 0.8	5.07 ± 1.06	NS	S	S

* P-vales (> 0.05= NS, < 0.05= S, < 0.01= HS)

Table 1. HbA1c%, F.S.G, T3, T4, TSH, and leptin hormone levels for groups 1, 2, 3.

Parameters (mg/dL)	Group one	Group two	Group three	T - Test G1 vs G2	T - Test G2 vs G3	T - Test G1 vs G3
TC	178.4±7.2	191.4±5.7	262.44±5.6	NS	S	S
Triglyceride	93.04±18.22	175.0±48.8	238.5±7.8	S	S	S
HDL-c	43.166±4.5	34.177±5.32	35.0±3.20	S	NS	S
LDL-c	86.1±12.23	115.9±33.47	201.05±4.1	S	S	S
VLDL	18.608±3.644	35.0±9.76	47.7±1.56	S	S	S

* P-vales (> 0.05= NS, < 0.05= S, < 0.01= HS)

Table 2. Lipid profile levels for groups 1,2,3.

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RESEARCH / INVESTIGACIÓN

Prevalence of antibodies in Iraqi Urinary Tract Infection patients using radial immunodiffusion (RID) assay

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Abstract: Candida has different types that could cause bloodstream infections. A total number of 150 samples were collected from candidemia patients and examined. The *Candida spp.* Species isolated from blood samples were analysed. These were identified by culturing the species using different media, namely the chromogenic agar test. Then, the virulence factors of all samples were tested. The *Candida glabrata* isolates were tested with six commercial antifungal drugs. *C. glabrata* 67 (44.6%), *C. albicans* 34 (22.6%), *C. krusei* 18 (12%), *C. tropicalis* 17 (11.3%), and *C. parasilosis* 14 (9.3%). the production of phospholipase ranged between 0.63-0.99 mm. It was found that 96% of the species showed phospholipase activity in aerobic conditions. The protease activities of *Candida spp.* Isolates were experimentally tested by area of inhibition around the colonies, where 59.3% had the double (++) protease activity, 31.4% with (+) grade, and 9.3% had (-) grade or clear zone around the colony. The hemolytic capacity ranged from 0.69-0.89 in the optimum aerobic environments. Finally, 38.33% of the isolated *Candida spp.* were positive and 61.67% negative for biofilm formation. Out of the total positive *Candida spp.* for biofilm formation, 21.73% were strong biofilm producers, and 78.27% were weak. Minimum fungicidal concentration (MFC) of Fluconazole for *C. glabrata* isolates was not appropriate (NA) due to the occurrence of low inhibition tested for species. Micafungin exhibited the lowest fungicidal activity against *C. glabrata* ranging from 0.03 - 0.125, while Fluconazole showed the highest.

Key words: Candidemia, chromogenic agar, *Candida glabrata*, Antifungal.

Introduction

Urinary tract infections (UTIs) are common bacterial infections, with nearly more than one hundred and fifty million individuals infected yearly¹. In the United States, estimated 10.5 million clinic cases with UTI symptoms and²⁻³ million cases visit the emergency department²⁻⁴.

The urinary tract includes kidneys, ureters, bladder, and urethra. Several natural factors are secret with urine from the urinary tract for protecting against all infection agents; also, the anatomical and mechanical barriers have a significant role, such as the glycoprotein plaque uroplakins⁵ and a layer of hydrated mucus⁶. Furthermore, the urinary tract lining is made by immune cells and epithelial cells, which have an important role in protecting against bacterial infection and preventing bacteria from getting into the urinary tract⁶. UTIs are most common in females more than males, and the incidence rate has increased during the last (30) years⁶.

UTIs increase in the elderly due to the immune-compromised individuals, and the urinary catheter has an essential role in increasing the percentage of infection⁷.

Immunity has two types innate immunity and adaptive immunity⁸⁻¹⁰. The individual with innate immunity has resistance against the infection. Innate immunity includes many chemical and cellular components. The infection gets in and spreads, but the person with high immunity has resistance against the bacteria¹¹. Adaptive immunity has two types of humoral immunity, and cellular immunity includes all the naturally acquired immunity due to infection vaccination¹²⁻¹⁵.

Features likely to contribute to disease of the human immune system involve the responses to antigens (B- and T-cell features, PMNs), the efficiency of bacterial destruction (e.g., lysozyme, complement), and types of antimicrobial substances produced (e.g., immunoglobulins, Cytokines)¹⁶. Immunoglobulins are a group of serum proteins with a crucial antimicrobial activity; IgM represents an indicator of recent infection, IgG levels increased in chronic infections¹⁷.

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Materials and methods

This study was conducted around UTI Iraqi patients during the period from November 2019 to April 2020. This study included 40 patients and 20 healthy controls.

Samples collection

Serum samples were collected from the patient in Al-Khalis General Hospital and National Laboratories in Al-Khalis District / Diyala Governorate (Iraq) from November 2019 to April 2020. After examining their urine samples by the microscope, they were divided into two groups first is patients with UTI, and the other group is healthy who does not suffer from UTI; this group is considered the control for comparison with the first group.

Estimation of antibodies

Using radial immunodiffusion test to detect levels of antibodies in serum is done by using (LTA, Italia). Normal values of antibodies were IgG (800-1800 mg/dl) and IgM (60-280 mg/dl).

Statistical Analysis

The Statistical Analysis System (SPSS) program was used to detect the effect of different factors in study parameters least significant difference, using T-test for comparing between the means value and the using of the Chi-Square test for comparing between the percentage.

Results and discussion

The patient group involved 40 patients, 8 males 20% and 32 females 80%, with a mean age of 40.60 ± 2.295 years. The healthy control group consisted of 20 subjects, 11 males (55%) and 9 females (45%), with a mean age of 36.80 ± 3.441 years, as seen in tables 1 and 2 explain the distribution of the two study groups according to gender and age.

Results in Tables 1 and 2 show UTI infections occur in females more than males; the mean age is 40.60 ± 2.295 years. The higher incidence of UTI in females than males might be due to a variety of factors, such as the proximity of the female urethral meatus to the anus¹⁸. Also, most school girls are avoiding urinal in school, which is accompanied by urinary infection¹⁹. Alternatively, vaginal flora has a vital role vaginal infection with coliforms which leads to urinary tract infection²⁰. The female anatomic feature also contributes to higher prevalence among the female subject. This finding is agreed with earlier studies²¹⁻²².

There was a significant increase in the mean concentration of IgG in sera of patients (854.82 ± 138.79) mg/dl compared with the mean concentration of IgG in sera of the control group (616.4 ± 67.65) mg/dl. These results show high significance between two groups UTI patients and control group according to IgG while non-significant to IgM show table 3. This finding is agreed with AlSaadawi and Alkhaled (2015)²³. Elevation in serum concentration of IgG in patients suffering from UTI may

be the reason for the significant increases in the concentration of complement components in patients suffering from UTI²⁴. Higher values IgG undergo increases to protect and combat infection²⁴.

Table 4 shows the non-significant difference in gender distribution between two groups according to IgG and IgM. IgG and IgM prevalence in UTI patients was (838.82 ± 121.49) mg/dl and (70.23 ± 8.48) mg/dl for male IgG and IgM respectively, while in females was (858.82 ± 171.63) mg/dl and (77.28 ± 7.62) mg/dl for IgG and IgM respectively, this similar with El Mashad *et al.* 2017²⁵.

Non-significant between two groups (with RBC and without RBC) according to IgG and IgM. With RBC group was (823.85 ± 152.75) mg/dl and (82.01 ± 15.78) mg/dl for IgG and IgM respectively, while in without RBC group was (871.49 ± 199.17) mg/dl and (72.57 ± 4.93) mg/dl for IgG and IgM respectively show table 5.

Group	Patient		Control	
	Number	%	Number	%
Male	8	20	11	55
Female	32	80	9	45
Total	40	100 %	20	100 %
Chi-Square (χ^2)			7.548**	
Sig.			.006	
			(P<0. 05).	

Table 1. Distribution of the two-studied group according to gender.

Study group	N	Minimum	Maximum	Mean	Std. Error
Patient	40	4	75	40.60	2.295
Control	20	3	67	36.80	3.441
Total	60	3	75	39.33	1.909
T			0. 937*		
Sig.			0.981		
			(P<0. 05).		

Table 2. Distribution of the two-studied group according to age.

Groups	N	%	IgG		IgM	
			Mean	Std. Error	Mean	Std. Error
Patients	40	33.3	854.82	138.79	75.87	6.30
Control	20	66.7	616.4	67.65	87.94	15.15
T			1.176**		-.868	
Sig.			.069		.029 NS	
			(P<0. 05).			

Table 3. APrevalence of IgG and IgM among UTI patients and control group.

Groups	N	%	IgG		IgM	
			Mean	Std. Error	Mean	Std. Error
Male	8	20	838.82	121.49	70.23	8.48
Female	32	80	858.82	171.63	77.28	7.62
T			-0.057		-0.443	
Sig.			0.210		0.365	
			(P<0. 05).			

Table 4. Effect of gender on IgG and IgM prevalence in UTI patients.

Groups	N	%	IgG		IgM	
			Mean	Std. Error	Mean	Std. Error
With RBC	14	35	823.85	152.75	82.01	15.78
Without RBC	26	65	871.49	199.17	72.57	4.93
T			-0.162		0.710	
Sig.			0.623		0.015 NS	
(P<0.05).						

Table 5. Effect of present RBC on IgG and IgM prevalence in UTI patients.

Conclusions

We conclude that UTI infection occurs in females more than males; the mean age is 40 years. In all patients with UTI compared to control, the IgG serum level increases and IgM serum level decreases. Serum IgG and IgM showed high significance between two groups of UTI patients and the control group according to IgG while IgM showed non-significant and serum IgG and IgM showed no significant difference in UTI patients and RBC groups.

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RESEARCH / INVESTIGACIÓN

Effect of cinnamon on blood sugar and anthropometric measurement in type two diabetes patients

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2280

Abstract: There is some emerging evidence that suggests certain supplements help lower blood sugar levels. One of these is cinnamon, which exhibits characteristics that mimic insulin, such as the activity of biologically active substances to activate insulin receptor kinase, increasing glucose uptake, autophosphorylation of the insulin receptor, and glycogen synthase activity. To assess the effect of the cinnamon substance on lowering fasting blood sugar, modifying body weight, body mass index (B.M.I.), and waist circumference in type two diabetics. A randomized controlled intervention clinical trial. The study was conducted on 60 patients with type II diabetes mellitus; the study followed both gender patients for four weeks. The contributors were allocated randomly into a group that receives 2 g/d cinnamon substance (intervention group), and a group without cinnamon substance is given (control group). Fasting plasma glucose (F.P.G.) and anthropometrics measurement at the beginning (beforehand cinnamon supplementation and 4 week at the end of the study duration). After 4 weeks of cinnamon taken, serum F.P.G. levels significantly improved ($P \leq 0.0001$). Anthropometric measurements (weight, height, B.M.I.) were reduced significantly (change mean $\leq P=0.001$) for all. The Present study disclosed that supplementation of 2 gm cinnamon improves F.P.G. and has a good role in anthropometric indices (weight, waist circumference, B.M.I.).

Key words: Cinnamon, T2DM, F.B.G., B.M.I.

Introduction

The diabetes epidemic is predictable to grow more threatening over the next several years. By the year 2050, it is projected that 1 out of 3 persons will have diabetes¹ T2DM is categorized by a mixture of insulin resistance (reduced tissue sensitivity or receptiveness to insulin) and beta-cell failure². Prediabetes is the subclinical changes in fasting plasma glucose, impaired glucose tolerance, or both. The degree of changes is among euglycemia, and the hyperglycemia of type T2DM³. Medical Nutrition Therapy delivered is also fruitful and crucial to preventing the development of prediabetes and obesity from typing T2DM⁴ cinnamon has a natural substance of attention because it has been theorized to deliver health benefits, such as the capability to lesser blood glucose and serum lipids. The insulinotropic properties of cinnamaldehyde have been preliminarily examined and are supposed to be accountable for indorsing insulin release, increasing insulin sensitivity, enhancing insulin disposal, and employing activity in insulin receptor kinase and the instruction of protein-tyrosine phosphatase 1B (PTP1B)⁵ Additional, methyl chacone polymer present in cinnamon augments the triacylglycerol lipase action that hydrolyzes fat molecules in diet, rises glycogen synthesis in the liver, augments glucose uptake and phosphorylation skeletal muscles and adipocytes insulin receptors⁶ Cinnamon act as a natural lipase inhibitor generally used in traditional medicine to reduce cholesterol and body weight⁷. A four-month management with a dietary addition encompassing cinnamon, chromium and carnosine reduced fasting blood sugar and augmented fat-free mass in obese or overweight pre-diabetic subjects⁸. Cinnamon consumption for short period is related to a notable decrease in both systolic B.P. and diastolic B.P., Though cinnamon demonstrates positive effects on potential lowering B.P., It would be early to indorse cinnamon for control B.P. as the restricted available studies⁹. Cinnamon is known to

have anti-diabetic properties, in addition to which, it is also perceived to have anti-oxidant, anti-inflammatory and anti-bacterial properties¹⁰ cinnamon is currently marketed as a remedy for obesity, glucose intolerance, diabetes mellitus and dyslipidemia^{10,11} At present cinnamon is sold as both a preventative and therapeutic supplement for many ailments including, metabolic syndrome, insulin resistance, type 2 diabetes, hyperlipidemia and arthritis¹⁰.

The study's objectives were to assess the effect of cinnamon on lowering fasting blood sugar, modifying body weight, body mass index (B.M.I.), and waist circumference in type two diabetics.

Materials and methods

Study design

A randomized clinical controlled trial intervention design (pre& post) which accompanied at the Al-wafaa Specialist Center for Diabetes and Endocrine Disease /Nenaveh Health Directorate in Iraq and was carried on T2DM patients (N=60) with uncontrolled diabetes mellitus patients, male and female patients aged (25 – 70) years.

Exclusion criteria

Exclusion criteria from taking the cinnamon supplement, acute concurrent illness, upper G.I.T. disease (GERD, gastritis, peptic ulcer), cancer, allergy to cinnamon, asthma & difficult breathing lactation, and pregnancy were excluded from the study. History was taken of Medical, surgical, drug, and usual diet history at the beginning of the trial. All contributors were taught to maintain their usual dietary behaviors and physical activity throughout the study.

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Participants

The study's two groups were randomly divided into the intervention group (n=30) receiving cinnamon substances and the control group(n=30) without cinnamon supplementation. Cinnamon capsules were already prepared and given as 2 gm as two capsules in two divided doses with lunch and dinner daily for 4 weeks. Compliance with taking cinnamon has been done by checking the capsule containers.

Biochemical analysis-anthropometric measurements

F.P.G. was measured and recorded at the beginning (before cinnamon substance supplementation) and the end of the study. Venous blood samples (1 cc) were collected afterward fast overnight for measuring fasting blood sugar. Blood sample for F.B.S. assay directly analyzed.

Measurement of weight was done by the standardized scale and for height by a standardized stadiometer. B.M.I. was calculated by dividing weight (Kg) by squared height (m²) at the beginning and the end of the study and categorized according to WHO criteria. The WHO procedure instruction for assessing waist circumference is that the measurement is prepared at the estimated midpoint among the lower margin of the last rib and the iliac crest top.

Anthropometrics measurements are a series of quantitative measurements of the muscle, bone, and adipose tissue used to assess the body's composition. The elements of anthropometry are height, weight, body mass index (B.M.I.), body circumferences (waist, hip, and limbs), and skinfold thickness.

Statistical analysis

Version 21 of IBM SPSS has been used for analytic statistics. Participant representative data are offered as mean, range & S.D. Paired sample t-test was used to compare the characteristics of the study group before and after their Intervention (P-value=0.05). T-test Independent samples were used to evaluate the difference between the intervention and control group in the mean change of study group characteris-

tics (P-value <0.05). The mean alteration characteristics of the study group (P-value<0.05) were considered significant.

Ethical permission

Permission to pilot the study was gained from Nenaveh Health Directorate in Iraq authorities. After elucidating the objects of the study for patient& relatives and earnings agreement for diabetes patients comprising in this study, privacy and confidentiality was considered. Also, allow the participant to leave the study at any time have been decided and provide them with complete information about cinnamon substance with its side effect and role in Type two D.M. also, I got signed consent from intervention and control groups participate in the research.

Results

Patients in this study had a mean age of 50.6 ± 9.3 years, cutting-edge within the range of 25-70 years. The male constituted 45%, and the female was 55%. Regarding residency, 56% of residence were in urban areas while 43 % lived in rural regions; As seen in the table (1). According to the mean duration of diabetes mellitus in participants was 6.6 ± 5.9 .

The Comparisons characteristics of the study group before and after the intervention (4 weeks after cinnamon intake) (table 2). The difference between the intervention and the control group in the mean change of characteristics study group (table.2).

The patients were monitored for four weeks, the mean level of F.P.S. for the intervention group at the beginning of the study was 176.93, and after follow-up, the mean level was 141.4; this difference was highly significant.

Anthropometric measurements (weight, waist circumference) decreased during the study period in the intervention group with a p-value <0.0001 for both. The B.M.I. mean level became better after supplementations of cinnamon with a p-value was <0.0001 in the intervention group (table2).

Demographic characters			
variant	groups	frequency	percent
gender	male	27	45.0
	female	33	55.0
	Total	60	100.0
residence	Urban	34	56
	Rural	26	43
	Total	60	100
employment	employ	16	26.7
	Not employ	44	73.3
	Total	60	100

Table 1. The demographic characters.

N=60	Intervention group			Control group		
	mean			mean		
	before	after	p-value	before	after	p-value
weight	85.75	84.85	<0.0001	86.30	87.08	<0.0001
Waist circumference	103.97	101.62	<0.0001	106.30	106.36	0.895
B.M.I.	31.11	30.40	<0.0001	32.27	32.55	<0.0001
FBS	176.93	141.4	<0.0001	183.36	192.40	0.035

*Significant P value < 0.05

2282

Table 2. Comparisons characteristics of the study group before and after the intervention.

N=60	Intervention group Mean SD	Control group Mean SD	P-Value
Weight change	-0.89 ± 0.79	0.79 ±0.88	<0.0001
Waist circumference changes	-2.35 ±1.16	0.07 ±2.75	<0.0001
BMI change	-0.71 ±0.37	0.28 ±0.88	<0.0001
FBS change	-35.07 ±32.34	9.03 ±22.36	<0.0001

* Significant P value < 0.05

Table 3. Shows the difference between the intervention and the control group in the mean change of the characteristics study group.

Discussion

Most patients with type two diabetes tend to ingest alternative medication to regulate their blood sugar; cinnamon is the most frequent. There are (4-18) weeks for cinnamon supplementation in patients with type two diabetes for their health status improvement with doses (150 mg/day-6 gm/day)².

This study aims to assess the effect of cinnamon supplementation of 2 gm daily dose for four weeks on F.B.S., B.M.I., weight, waist circumference; we found a significant statistical improvement while no significant statistical difference in HbA1c.

Effect on F.B.G

In a meta-analysis of ten randomized controlled trials (n = 543) patients, a cinnamon substance with doses of (120 mg/d - 6 g/d)for four to eighteen weeks, levels of fasting plasma glucose reduced with a mean difference (-24.59 mg/dL; 95% CI) with upper & lower limit(-40.52 to -8.67)mg/dL) consequently⁵. Another intervention study with a single cinnamon supplement (3g) during 16 weeks resulted in improvements in fasting blood sugar & all constituents of metabolic syndrome significant in a sample of Asian North Indian sample patients⁶. Based on the study designed as a triple-blind placebo-controlled randomized clinical trial in Iran findings was, cinnamon could improve FBS¹² the different findings of another intervention study that also done in Iran concluded that an 8-week of

3 grams per day intake of cinnamon supplementation had no significant effects on F.P.G¹³.

Effect on weight

A systematic review plus controlled clinical trials (meta-analysis) revealed that supplementation with cinnamon significantly decreases body weight. But we need long-term Randomized controlled trials that are recommended of high quality to approve these results¹⁴. Another study of Randomized controlled trials found that adding cinnamon, magnesium, and chromium supplements to honey of kanuka type was accompanied by weight reduction¹⁵. Though cinnamon supplementation caused alterations in the weight, the result changes were statistically insignificant of that systematic review and meta-analysis of clinical trials stud¹⁶. Another systematic review and meta-analysis of clinical trials found that cinnamon intake had no beneficial changes on anthropometric parameters¹⁷.

Effect on waist circumference

The significant reduction in waist circumference is in type two diabetes patients who took three months' cinnamon substance¹⁶. A controlled intervention randomized clinical trial (triple-blind) revealed that Cinnamon supplementation was directed to improve anthropometric measures (B.M.I., body fat, and visceral fat)¹².

A Randomization effects model with meta-analysis (con-

trolled clinical trials) found that Cinnamon supplementation affects insignificantly waist circumference¹⁴ Cinnamon Supplementation can reduce blood glucose without changes in anthropometric indices, including waist circumference¹⁷.

Effects on B.M.I

Our study result agree with R.C.T.s in which Cinnamon supplement steered to the improvement of B.M.I. and body fat were significantly more noticeable in patients with high B.M.I. diabetes patients (B.M.I. \geq 27)¹² Twenty-one R.C.T.s of 1,480 diabetes patients were comprised, The meta-analysis displayed a significant reduction of B.M.I. with cinnamon supplementation¹⁴. Inconsistent with our study, a systematic review & clinical trials meta-analysis for type 2 diabetes patients no significant effect of cinnamon supplementation on the B.M.I.¹⁶. Also another systematic review of clinical trials & meta-analysis Cinnamon supplementation cannot improve B.M.I. in type 2 diabetes patients¹⁷.

Limitation of study

The short study period was short because I was limited in period time and because of COVID-19 lockdown, lack of previous studies in the research area, need more research with a large sample size, and one participant was excluded from the intervention group because he complained of gastritis.

Conclusions

The results of this study revealed that a two-gram daily dose of cinnamon improves fasting blood glucose, anthropometric parameters (weight, waist circumference, B.M.I.). However, it has no significant consequence on HbA1C after four weeks of supplementation of type II diabetic patients. Hence cinnamon could have an essential role in postponing or avoiding the progression of Type II diabetes, and we need further research, especially in Iraq.

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RESEARCH / INVESTIGACIÓN

Determination of the prevalence of *blaOxa-like* gene and *ISAbal* elements among extensive-drug resistant (XDR) *Acinetobacter baumannii* isolates

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2284

Abstract: The capacity of Multi-drug resistant (MDR) *Acinetobacter baumannii* to survive in any state of affairs concerning the gaining of various gene types of virulence and antimicrobial agent resistance are the main anxiety in the hospital's environments. So, it is very crucial to determine the prevalence of insertion sequences in *A. baumannii* in the hospitals. Detecting the *blaOxa-51* gene through the polymerase chain reaction (PCR) was performed to confirm *Acinetobacter baumannii* and the search for *ISAbal* element. Between October 2020 and February 2021, 540 distinct clinical specimens were gathered from five hospitals in Baghdad. Thirty-eight *A. baumannii* isolates were obtained from various clinical specimens. The isolates were initially identified phenotypically using standard microbiological techniques and by the Vitek2 compact automated machine. Isolates of *A. baumannii* were identified genotypically by amplification of the *blaOxa-51-like* gene. Antimicrobials are studied by Kirby-Bauer (disc diffusion) technique on Muller-Hinton agar as specified by the recent clinical and laboratory standard institute (CLSI) guidelines (2020). The actual results of the current study indicated that from total isolated (38) *A.baumannii* isolates, 23 isolates (61%) were resistant to meropenem and 25 isolates (66%) were resistant to imipenem. The *blaOxa-51* gene was identified in all strains examined, *ISAbal* was also present in all *A. baumannii* isolates. *ISAbal* has a high predominance between drug-resistant *A. baumannii*. Identifying these parameters can assist in the control of infection and decreasing the microorganism's prevalence rate.

Key words: Insertion sequence, The *blaOxa-51-like* gene, *Acinetobacter baumannii*.

Introduction

An opportunistic infectious agent, *A.baumannii* encompasses a high rate of occurrence among immunocompromised people, significantly those that have experienced long (more than ninety days) hospital residence¹. Commonly related to aquatic environments². It's been recognized as a "red alert" human infectious agent in recent years, causing concern among medical professionals, owing to its extensive spectrum of antibiotic resistance³.

The development of multidrug-resistant (MDR) pathogens has more and more become a cause for profound importance concerning each healthcare facility and community-acquired infections⁴. According to the World Health Organization (WHO), Antimicrobial resistance has recently been recognized in concert as one of the 3 most vital issues facing human health⁵. The therapeutic selections are restricted, typically leading to unsuitable medical care and resulting negative consequences on patient⁶.

The primary antibiotic resistance mechanisms are enzymatic (production of β -lactamases and enzymatic modification of aminoglycosides) and non-enzymatic (changing membrane permeability, activating efflux pumps) and altering of the target site)⁷.

Insertion sequences (IS) are among the most basic mobile genetic elements (MGEs) and are found across the animal kingdom. To present, more than 4500 IS from 29 families have been discovered^{8,9}. In *Acinetobacter* spp., more than thirty different types of Insertion Sequences have been discovered, suggesting that ISs had a significant role in developing this species and contributing to the multidrug-resistant phenotype shown in this genus¹⁰.

Insertion elements have two distinct characteristics: short transposable elements (up to 2500bp) and only code for proteins involved in transposition¹¹.

In *A. baumannii*, *ISAbal* has been found in conjunction

with numerous antibiotic resistance genes¹². *ISAbal* has been shown to function in the expression of the *Bla ampC* gene, the antibiotic resistance gene of *A. baumannii*, which encodes the naturally occurring cephalosporins enzyme, and the *blaOXA-23* gene, which encodes a carbapenem-degrading oxacillinase. Nevertheless, it could also act in the case of other resistance genes^{13,14}. A composite transposon (defined as Tn2006) formed by two copies of *ISAbal* bracketing this β -lactamase gene, responsible for the movement of *blaOXA-23*¹⁵.

ISAbal has been found to regulate the expression of the *bla ampC* gene, which encodes the naturally occurring cephalosporins enzyme, and the *blaOXA-23* gene, which encodes a carbapenem-hydrolyzing oxacillinase enzyme. However, it may also play a role in the development of additional resistance genes. *ISAbal* has been demonstrated to produce carbapenem resistance in *A. baumannii* by causing overexpression of the naturally existing *blaOxa-51-like* gene¹⁶.

The expression of the *blaOXA-23* gene has been linked to *ISAbal* and *ISAbal4*^{15,17}. The expression of the *blaOXA-58* gene has been related to the insertion sequences *ISAbal*, *ISAbal2*, *ISAbal3*, and *IS18*¹⁸. Therefore, determining the prevalence of insertion sequences genes in *A. baumannii* in hospitals is very crucial.

Materials

Specimens' collection

Between October 2020 and February 2021, 540 distinct clinical specimens were collected from five hospitals in Baghdad, including The Burns Hospital, Gazi Al-Hariri Hospital, Baghdad Teaching Hospital, Welfare Teaching Hospital in the Medical City, and Al-Yarmouk teaching hospital. Collected

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specimens were sputum, blood, fluids such as (cerebrospinal, pleural, and peritoneal), urine, and swabs.

Isolation of Bacteria

In the laboratory (Teaching Laboratories/Medical City, Baghdad) under aseptic conditions, the collected specimens were cultured directly on MacConkey agar and blood agar, incubated for 24 hrs. at 37°C. The colonies were non-hemolytic opaque creamy on blood agar, while on MacConkey agar were non-lactose fermenting colonies. For obtaining pure, well-isolated colonies, they were subcultured on another MacConkey agar plate and incubated for another 24 hrs. at 37°C¹⁹.

Bacterial Identification

Microscopical examination

One isolated colony was transferred to a microscopic slide, fixed then stained with Gram stain (GS). Gram reaction, cell shape, and arrangement were recorded. The results were compared with Brooks *et al.*(2013)²⁰.

Biochemical tests

To phenotypically identify the isolates as *A. baumannii*, biochemical tests such as the ability to grow at 42°C, culture on selective medium, negative for oxidase test, absence of lactose fermentation, and others were utilized.

Identification of *Acinetobacter baumannii* using VITEK® 2 system.

As stated by the manufacturer's instructions, identification cards of Gram-Negative Bacteria (ID-GNB) were used on the VITEK® 2 system to recognize isolates at the species level. The bacterial isolates were inoculated at 37°C on MacConkey agar plates and, after incubated overnight, taken a single colony then suspended. In 0.45 % sodium chloride, the turbidity measurement for the bacterial suspension to meet the McFarland (0.5) standards, The (Gram-Negative Vitek 2 Identity card) then was manually placed into the Vitek-2 system, along with the bacterial suspension tub, the software also prepared

according to (BioMerieux, France) the manufacturer's instructions²¹.

Identification of *A.baumannii* by PCR

Using a primer specific for *bla OXA51-like* genes, PCR was utilized to amplify *bla OXA51-like* genes used for *A. baumannii* isolate recognition.

Test of antimicrobial susceptibility profile

According to the latest clinical and laboratory standard institute (CLSI) criteria (2020), the isolates were tested for antimicrobial susceptibility to 18 antimicrobial agents using the Kirby-Bauer disc diffusion technique on Muller-Hinton agar (Oxoid /England)²². The antibiotic discs (Mast Group /UK) used throughout the study for *A.baumannii* isolates are Piperacillin-Tazobactam (100/10 µg/disc), Ampicillin-sulbactam (13/10 µg/disc), Ticarcillin-Clavulanate (55/13 µg/disc), Cefepime(33 µg/disc), Cefotaxime (33 µg/disc), Ceftazidime (33 µg/disc), Ceftriaxone (33 µg/disc), Imipenem (13 µg/disc), Meropenem (13 µg/disc), Colistin sulphate (25 µg/disc), Tobramycin (13 µg/disc), Gentamicin (13 µg/disc), Amikacin (33 µg/disc), Doxycycline(30 µg/disc), Tetracycllin (30 µg/disc), Ciprofloxacin (5µg/disc), Levofloxacin (5µg/disc) and Trimethoprime-Sulphamethoxazole(1.25/ 23.55 µg/disc).

Genomic DNA extraction from bacterial isolates

Extraction of bacterial DNA from isolates under study using a commercial Extraction system (ZR Fungal/Bacterial DNA Miniprep Kit) designed to isolate DNA from Gram-negative bacteria according to the manufacturer's instructions. For each reaction, a totally of 4 µl of extracted DNA was used.

Molecular recognition of *BlaOXA-51-like* gene and Insertion sequence elements

To detect XDR *A.baumannii* isolates, PCR was used to detect the *BlaOXA-51-like* gene and *ISAbal* elements. Primer sequences for each gene reported above are listed in table (1). These primers (Macrogen, South Korea) were received in a lyophilized state, dissolved in sterile deionized distilled water

Primer	The sequence of primers (5' ---3')	Product size	Accession number
OXA51	CTTTTGGCTAAATGGAAGCG	434	CP081137.1
OXA51	CGGGTGTCTTAGTTATCCAAC		
ISAbal F	CACGAATGCAGAAGTTG	549	CP029569.1
ISAbal	CGACGAATACTATGACAC		

Table 1. Sequences of primers used throughout the study.

Steps	OXA-51	ISAbal	Repeats
Activation	94°C/5min	94°C/5min	1 cycle
Denaturation	94°C/45s	94°C/45s	
Annealing	56°C	50°C	40 cycles
Extension	72°C/45s	72°C/45s	
Final extension	72°C/7min	72°C/7min	-

Table 2. Programs were used in the PCR for *OXA-51-like* gene and *ISAbal* element.

to a final concentration of 100 picomole/μl, and kept in a deep freezer until use, as advised by the vendor.

The PCR amplification procedure for the genetic level to detecting genes under study by follows steps: Final volume for PCR mixture was 25 μl (12.5 of Green Master Mix 2x, 4 μl extracted template DNA, 1.5 μl from each forward and reverse primer, 5.5 μl nuclease-free water were added in 0.2 ml PCR Eppendorf tubes, mixed for a short time via vortex then been loaded to Veriti™ 96-Well (applied biosystems) Thermal Cycler. The program used for each monoplex PCR reaction was set according to each primer. The best annealing temperature was chosen after the gradient runs through the optimization process of each oligonucleotide primer.

For amplification of the *Bla OXA-51-like* gene, the DNA thermal cycler device Veriti™ 96-Well (applied biosystems) was programmed in the following amplification conditions: Following a 5-minute activation at 94°C, 40 cycles of 45 seconds at 94°C (denaturation), 56°C (annealing), and 45 seconds at 72°C (extension) were conducted. In contrary to other genes, the ISAbal annealing temperature was 50°C. The last cycle was followed by 7 minutes at 72°C (Table 2). Amplified PCR products were examined on 1.5% agarose gel at an electrical current of 7 volt/cm² in 1X TBE buffer with added Red safe dye (INTRON) has been exposed till the tincture had reached the other side of the gel. The SiZer™-1000 Plus DNA Marker and SiZer™-100 DNA Marker (Intron / Korea) were used as markers during PCR products electrophoresis. After that, the agarose gel was removed from the tank and visualized by a UV transilluminator documentation system (Cleaver scientific /UK) at 336 nm, then photographed using a digital camera.

Sequencing was carried out by Macrogen DNA Sequencing (Seoul, Korea) using 3730xl DNA Analyzer (Applied Biosystems™, Foster City, CA). Two samples from ISAbal PCR products with forwarding primer (17 pmol/ μl) for each gene were selected and sent to sequencing. Raw reads generated in this study were trimmed or filtered to remove low-quality sequences using (SnapGene software). Once sequencing reads had been obtained, the data analysis process was started, the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) was used to analyze DNA sequences and do similarity searches.

Results

540 clinical specimens were collected between October 2020 and February 2021 from some hospitals in Baghdad, including Baghdad medical city and Al-Yarmouk hospitals. Thirty-eight bacterial isolates were obtained, which differentiated to *A.baumannii*. Following conventional identification techniques, antimicrobial sensitivity testing was carried out by examining the findings in (Figure 1), revealing a high degree of resistance of *A.baumannii* clinical isolates to the majority of the antimicrobial agents under test.

In this study, antibiotic resistance profile of *Acinetobacter baumannii* for 18 antibiotics as following: Trimethoprim/sulphamethoxazole 32 (84%), Ciprofloxacin 27 (71%), Piperacillin/tazobactam 26(68%), Ticarcillin/clavulanate 26 (68%), Cefazidime 26(68%), Ceftriaxone26(68%), Cefotaxime 25 (66%), Imipenem 25(66%), Doxycycline 26(68%), Levofloxacin 25 (66%), Cefepime 24(63%), Tetracycline 24(63%), Meropenem 23(61%), Gentamicin 22 (58%), Tobramycin 15(39%), Amikacin 17(45%), Ampicillin/sulbactam 12 (32%), Colistin 0(0%). The exact and important results of current study indicated that from total isolated (38) *A.baumannii* isolates, 23 isolates (61%)

and 25 isolates (66%) were resistant to meropenem and imipenem respectively.

All the *A. baumannii* isolates were positive for *blaOxa-51-like* genes and ISAbal genes. Figures 1 and 2 demonstrate agarose gel electrophoresis of PCR products of blaOxa-51-like genes and ISAbal elements, respectively.

The sequence was analyzed by BLAST software at NCBI. Figure (4) shows the alignment result with USA: San Diego isolates (Accession number CP053098.1). The sequence was found to share 99% nucleotide homology with the reference isolates. Tables 3 and 4 show nucleotide changes and Features of ISAbal(Forward) from *Acinetobacter baumannii* (X9 isolate) with *Acinetobacter baumannii* ATCC 17978 chromosome from the USA: San Diego.

The sequence was analyzed by BLAST software at NCBI. Figure (4) shows the alignment result with USA: San Diego isolates (Accession number CP050388.1). The sequence was found to share 99% nucleotide homology with the reference isolates. Tables 5 and 6 show the nucleotide changes and features of ISAbal(Forward) from *Acinetobacter baumannii* (X10 isolate) with *Acinetobacter baumannii* strain VB473 chromosome from India.

Discussion

A.baumannii has developed as a well-established nosocomial pathogen with a high level of antibiotic resistance. Various medical facilities frequently report extensively drug-resistant, and pan drug-resistant isolates²³. By 2007, up to 70% of isolates in specific locations had evolved multidrug resistance, particularly resistance to carbapenems, which were formerly thought to be the gold standard for treating MDR *A. baumannii* infections²⁴.

The antibiotic of choice for treating *A. baumannii* is carbapenems. Due to rising resistance rates, *A. baumannii* infections are becoming increasingly ineffectual. Resistance to the newer antibiotic tigecycline is also quickly developing. Colistin, a previously abandoned antibiotic, is now used as a last option, yet resistance to this medication is increasing at an alarming pace throughout the world²³.

Aside from its proclivity for the critically ill in intensive care units, *A. baumannii* has lately been linked to a slew of infectious diseases among military troops injured in the Iraq and Afghanistan wars⁴.

Resistance of Carbapenem is frequently connected to the making of oxacillinase enzymes. Metallo β-lactamases (MBL), on the other hand, can cause carbapenem resistance in *A. baumannii*²⁵.

The actual results of the current study indicated that of 38 *A.baumannii* isolates, 23 isolates (61%) and 25 isolates (66%) were resistant to meropenem and imipenem, respectively. The antibiotic resistance results of imipenem and meropenem are less than that found by al Al-Saadi (2018)²⁶. From 162 *A.baumannii* isolates, 112 isolates (88.19%) and 107 (84.25%) were resistant to meropenem and imipenem, respectively. Prior investigators in Iran found that the resistance rates to imipenem and meropenem were (95.23%) and (98.09%) respectively²⁷, indicating that these bacteria have a wide range of resistance mechanisms. This would imply significant risks among hospitalized patients, mainly where this antibiotic class was previously considered the standard therapy for *A.baumannii* infections²⁸.

The recognition of the *Bla OXA-51-like* gene can be utilized to identify *A.baumannii* reliably and straightforwardly^{29,30}.

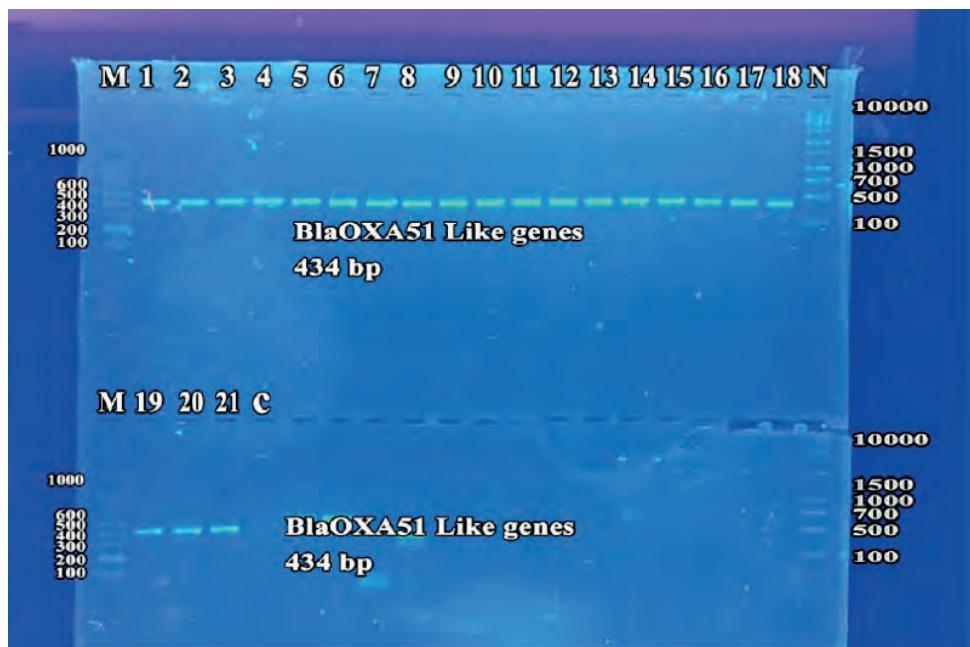


Figure 1. Detection of *blaOXA51* like gene by monoplex PCR for *A.baumannii* isolates. lanes 1-21, XDR *A.baumannii*; Lane C, Negative control. Lane M, 100 bp DNA marker. Lane N, 1000 bp plus DNA marker. Detection was done on agarose gel (1.5%) at 5 Volt/cm for 1.5 hours, stained by Red Safe dye, and imagined on a UV transilluminator documentation system.

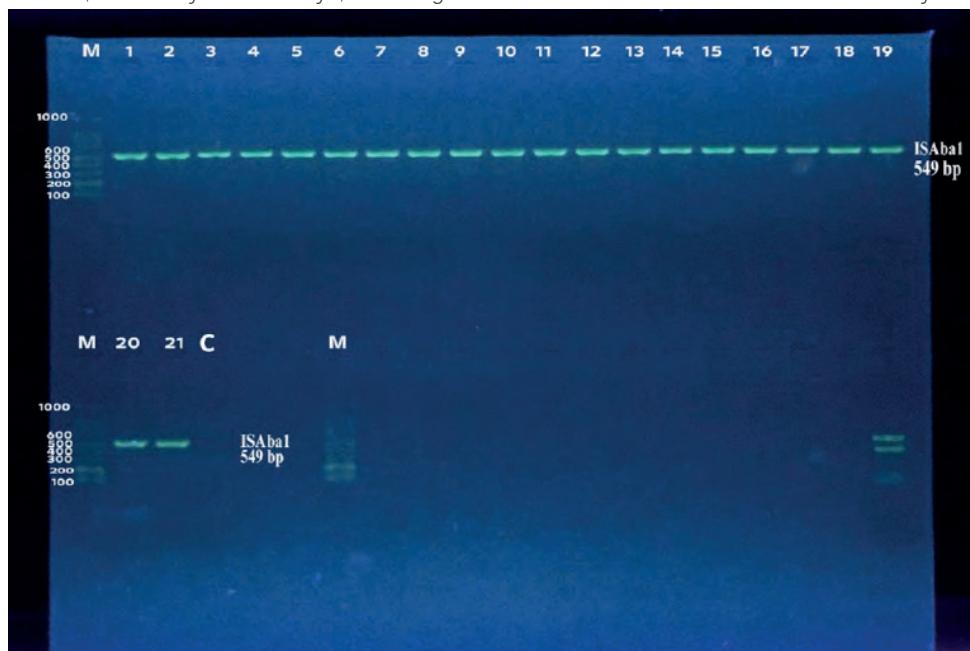


Figure 2. Detection of *ISAbal* gene by monoplex PCR of isolates. Lanes 1-21: XDR *A.baumannii* isolates. Lane C: Negative control. Lane M: 100 bp DNA marker. Detection was done on agarose gel (1.5%) at 5 Volt/cm for 1.5 hours, stained by Red Safe dye, and imagined on a UV transilluminator documentation system.

Furthermore, because this gene was controlled by insertion sequences such as *ISAbal*, the presence of intrinsic chromosomally placed genes of the *bla OXA-51-like* gene did not correlate with the amount of carbapenem resistance of *A.baumannii* isolates³¹.

Additionally, all isolates of *A.baumannii* carried the *Bla OXA-51-like* gene and attributed the imipenem resistance state to the presence of *ISAbal* upstream of the *blaOxa-51-like* gene serves as a promoter for gene expression as one of these isolates' resistance methods^{32,33}.

From 21 XDR *A. baumannii* isolates all have *blaOxa-51-like* genes. This corroborated those of previous local investigations^{26,34,35}.

A study in Egypt revealed that genes encoding *blaOxa-51* (belonging to class D carbapenemases) were found in 100% of the studied isolates³⁶. According to research by Bahador et al., all 62 CRAB isolates tested positive for *blaOxa-51-like* genes³⁷.

The present data could affirm that all *A.baumannii* isolates were positive for the *ISAbal* gene. It was found that the prevalence of *ISAbal* was 100%^{27,36}. *ISAbal* was the most common insertion element (90.6%)³⁸. The prevalence of *ISAbal* is equal to that seen in 59 Spanish isolates (93.2%)³⁹. While Taiwan (36%)⁴⁰ and India (33%)⁴¹ have lower prevalence rates than the rest of the world. The presence of various insertion sequences in *A. baumannii* makes it resistant to carbapenems⁴².

These insertion sequences are found near genes that code

Gene: ISAbal(Forward) from <i>A.baumannii</i> (X9 isolate)						
No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
1	Deletion	52	-/A	CP053098.1	<i>Acinetobacter baumannii</i>	99%

Table 3. Nucleotide changes of ISAbal(Forward) from *Acinetobacter baumannii* (X9 isolate) with *Acinetobacter baumannii* ATCC 17978 chromosome from the USA: San Diego.

Feature	Studied isolate	Reference isolate
Molecule Type	Genomic DNA	Genomic DNA
Isolation Source	Swab	Fatal meningitis
Host	Homo Sapiens	Homo Sapiens
Country	Iraq, Baghdad	USA: San Diego

Table 4. ISAbal(Forward) features from *Acinetobacter baumannii* (X9 isolate) with *Acinetobacter baumannii* ATCC 17978 chromosome from the USA: San Diego.

Gene: ISAbal(Forward) from <i>A.baumannii</i> (X10 isolate)						
No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
1	Deletion	83	-/A	CP050388.1	<i>Acinetobacter baumannii</i>	99%
	Deletion	85	-/A			

Table 5. Nucleotide changes of ISAbal(Forward) from *Acinetobacter baumannii* (X10 isolate) with *Acinetobacter baumannii* strain VB473 chromosome from India.

Feature	Studied Isolate	Reference Isolate
Molecule Type	Genomic DNA	Genomic DNA
Isolation Source	Swab	Sputum
Host	Homo Sapiens	Homo Sapiens
Country	Iraq, Baghdad	India

Table 6. Features of ISAbal(Forward) from *Acinetobacter baumannii* (X10 isolate) with *Acinetobacter baumannii* strain VB473 chromosome from India.

for several OXA-type carbapenemases and are implicated in their overexpression¹³.

Conclusions

In conclusion, *A. baumannii* is a significant pathogen in several nations. According to the findings of this study, it has a high resistance rate against most antibiotics, threatening in patients as a red alarm bacterium in hospitals, producing a high rate of death and morbidity due to its numerous mechanisms of resistance and the fact that it is not or only rarely treated with conventional antibiotics.

This bacterium can cause dangerous and long-term infections, especially in youngsters and people with immunological deficiencies. Our research focused on specific mobile components transported between species to change the antimicrobial pattern and enhance antimicrobial resistance. ISAbal has a high prevalence among extreme drug-resistant *A. baumannii* isolated from several Baghdad hospitals. Identifying these parameters can aid in controlling infection and reducing the microorganism's prevalence rate.

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Acinetobacter baumannii ATCC 17978 chromosome, complete genome Sequence
ID: CP053098.1 Length: 4005343 Number of Matches: 1
Range 1: 3737775 to 3738256

Score	Expect	Identities	Gaps	Strand
883 bits(478)	0.0	481/482(99%)	1/482(0%)	Plus/Minus

Query 2	CTCTGTCTGCGAACACATTACAATACGGTCTTACCAAAATGGCTATAA-GCGTTGAA	60
Sbjct 3738256	CTCTGTCTGCGAACACATTACAATACGGTCTTACCAAAATGGCTATAAAGCGTTGAA	3738197
Query 61	TCAAAGCAATACGCTTTCGTATCTGAATTCCACGTTATTAAGCAATGTCCAAGGA	120
Sbjct 3738196	TCAAAGCAATACGCTTTCGTATCTGAATTCCACGTTATTAAGCAATGTCCAAGGA	3738137
Query 121	TAGGTATCGTATTCCACGATAAACGATTGCGAGCATCAGGATATTAATATTCGTTTC	180
Sbjct 3738136	TAGGTATCGTATTCCACGATAAACGATTGCGAGCATCAGGATATTAATATTCGTTTC	3738077
Query 181	CCCATTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGGTCAATGAAAACATATTGA	240
Sbjct 3738076	CCCATTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGGTCAATGAAAACATATTGA	3738017
Query 241	AAATCAACTGAGAAATTGACGATAATCAAATACTGACCTGCAAAGAACGCCTGCATAC	300
Sbjct 3738016	AAATCAACTGAGAAATTGACGATAATCAAATACTGACCTGCAAAGAACGCCTGCATAC	3737957
Query 301	GTCGATAAAATGATTGTGGTAAGCAGTGTGATGGCAAGGCTTGTAGATGCAGAAGAAAGAT	360
Sbjct 3737956	GTCGATAAAATGATTGTGGTAAGCAGTGTGATGGCAAGGCTTGTAGATGCAGAAGAAAGAT	3737897
Query 361	TACATGTTGCTTTAAAATAATCACAAGCATGATGAGCGCAAAGCACTTTAAATGTGACT	420
Sbjct 3737896	TACATGTTGCTTTAAAATAATCACAAGCATGATGAGCGCAAAGCACTTTAAATGTGACT	3737837
Query 421	TGTTCCATTTAGATATTGTTAAGATAAGATAACTCATTGAGATGTGTCATAGTAT	480
Sbjct 3737836	TGTTCCATTTAGATATTGTTAAGATAAGATAACTCATTGAGATGTGTCATAGTAT	3737777
Query 481	TC 482	

Figure 3. Sequence alignment of ISAbal(Forward) of *A.baumannii*(X9 isolate) with *Acinetobacter baumannii* ATCC 17978 chromosome from USA: San Diego.

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Acinetobacter baumannii strain VB473 chromosome, complete genome
 Sequence ID: CP050388.1 Length: 3948250 Number of Matches: 27
 Range 1: 164726 to 165214

Score	Expect	Identities	Gaps	Strand
891 bits(482)	0.0	487/489(99%)	2/489(0%)	Plus/Plus
2290				
Query 2		GATAAACTCTGTCTGCGAACACATTACAATACGGTCTTACCAAAAATGGCTATAAA		61
Sbjct 164726		GATAAACTCTGTCTGCGAACACATTACAATACGGTCTTACCAAAAATGGCTATAAA		164785
Query 62		GCGTTGAATCAAAGCAATACGCTTTCGTATCTGAATTCCACGTTTATTAAGCAATGT		121
Sbjct 164786		GCGTTGAATCAAAGCAATACGCTTTCGTATCTGAATTCCACGTTTATTAAGCAATGT		164845
Query 122		CCAAAGGATAGGTATCGTATTCCACGATAAACGATTGCGAGCATCAGGATATTAATATT		181
Sbjct 164846		CCAAAGGATAGGTATCGTATTCCACGATAAACGATTGCGAGCATCAGGATATTAATATT		164905
Query 182		TCGTTTCCCCATTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGGTCGAATGAAAA		241
Sbjct 164906		TCGTTTCCCCATTCCAATTGGTTCTATCTAAAGTCAGTTGCACTTGGTCGAATGAAAA		164965
Query 242		CATATTGAAAATCAACTGAGAAATTGACGATAATCAAAATACTGACCTGCAAAGAAGCG		301
Sbjct 164966		CATATTGAAAATCAACTGAGAAATTGACGATAATCAAAATACTGACCTGCAAAGAAGCG		165025
Query 302		CTGCATACGTCGATAAAATGATTGGTAAGCAGTTGATGGCAAGGTTTAGATGCAGA		361
Sbjct 165026		CTGCATACGTCGATAAAATGATTGGTAAGCAGTTGATGGCAAGGTTTAGATGCAGA		165085
Query 362		AGAAAGATTACATGTTGCTTAAATAATCACAGCATGATGAGCGCAAAGCACTTAA		421
Sbjct 165086		AGAAAGATTACATGTTGCTTAAATAATCACAGCATGATGAGCGCAAAGCACTTAA		165145
Query 422		ATGTGACTTGTCCATTAGAGATTGTTAAGATAAGATAACTCATTGAGATGTGT		481
Sbjct 165146		ATGTGACTTGTCCATTAGAGATTGTTAAGATAAGATAACTCATTGAGATGTGT		165205
Query 482	C-T-GTATT 488			
Sbjct 165206	CATAGTATT 165214			

Figure 4. Sequence alignment of ISAbal(Forward) of *A.baumannii*(X10 isolate) with *Acinetobacter baumannii* strain VB473 chromosome, from India.

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RESEARCH / INVESTIGACIÓN

Estimation of Vascular Cell Adhesion Molecule 1 (VCAM-1) Levels In Type 1 Diabetic Mellitus Patients

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2292

Abstract: High glucose levels in patients with diabetes are associated with increased plasma levels of soluble adhesion molecules. They could explain that the patients with diabetes mellitus will require the development of premature atherosclerosis related to hyperglycemia or hyperinsulinemia and that it not only affects vascular endothelium but also contributes to the development of microvascular complications. This study aimed to evaluate the serum concentration of VCAM-1 in type 1 diabetes mellitus patients with and without cardiovascular disease. Also, investigation the association of insulin levels, duration of diabetes, and HbA1C with VCAM-1. Include in this study a total of 60 types 1 diabetic patient. According to characteristic laboratory investigations and electrocardiogram (ECG), they were subdivided into two groups (G1) 30 T1DM patients without cardiovascular disease and (G2) 30 T1DM patients with cardiovascular disease in addition to 30 healthy subjects as a control group (G3). All subjects measured the levels of fasting blood glucose FBG, glycated hemoglobin HbA1c, and insulin levels, and VCAM-1 were also determined by ELISA technique. This study shows a highly significant difference in the average diabetic profile between G1 and G2 compared to the control group and found that VCAM-1 level was significantly higher among diabetic patients than the control group. Also, there was a significant negative correlation of VCAM-1 with the levels of FBG, HbA1c in diabetic patients G1 and G2. While insulin had a positive correlation in G1 but correlated negatively in G2.

Key words: T1DM, CVD, VCAM-1, adhesion molecule, insulin.

Introduction

Diabetes mellitus type 1 is a chronic metabolic disease caused by autoimmune pancreatic beta-cell destruction that leads to insulin deficiency and is more commonly diagnosed in young adults, and they are at high risk of micro-and macrovascular complications develop¹. Type 1 diabetes Mellitus is considered a significant risk factor for cardiovascular disease (CVD) because of poor metabolic control, long duration of disease, and existing dyslipidemia or hypertension².

Vascular infiltration affected by adhesion molecules and that released by damaged endothelial cells or inflammatory conditions entering into the arterial wall. Adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1(ICAM-1) act as atherosclerosis markers isolated from the endothelium and measured in peripheral blood, and also, VCAM-1 has a role in autoimmune diseases^{3,4}. The soluble forms of adhesion molecules found in the serum of diabetic patients, that activation suggested endothelial of a role in diabetes⁵. High glucose levels in patients with diabetes participate in increased plasma levels of soluble adhesion molecules. Therefore, they could explain that the patients with diabetes mellitus will develop premature atherosclerosis related to hyperglycemia or hyperinsulinemia and that it not only affects vascular endothelium but also contributes to the development of microvascular complications⁶.

The endothelial function is considered a marker of vascular dysfunction and is a good predictor of cardiovascular disease, irregularity in the endothelial function involves the pathogenesis of cardiovascular diseases⁷. An increase in the serum levels of cellular adhesion molecules, ICAM-1 and VCAM-1, as specific markers of endothelial dysfunction indicates an impaired endothelial function⁸. Local researches in VCAM-1 to predict complicate of type 1 diabetes mellitus on the cardiovascular are limited. So that, the aim of this study was estimated

VCAM-1 levels in serum of type 1 diabetes patients with and without cardiovascular disease. Also, investigation the association of insulin levels, duration of diabetes, and HbA1C with VCAM-1.

Methods

This study includes 60 types 1 diabetic patient diagnosed by consultant physicians enrolled in "National Diabetic Center (NDC)" Baghdad – Iraq, from December to March 2018. According to characteristic laboratory investigations and electrocardiogram (ECG), they were subdivided into two groups as follows: (G1) 30 T1DM patients without cardiovascular disease the mean age (15 ± 6.67) years and (G2) include 30 T1DM patients with cardiovascular disease their mean age (10 ± 5.81) years in addition to 30 healthy subjects as a control group with mean age (17 ± 3.12) years. This study excluded patients with autoimmune disease, any stage of heart failure, thyroid disease, and uncontrolled hypertension.

A blood sample from all subjects was drawn to measure the levels of fasting blood glucose FBG⁹, glycated hemoglobin HbA1c¹⁰. Levels of insulin and VCAM-1 were also determined by ELISA technique¹¹. And body mass index BMI was calculated for each subject in patients and control groups.

Statistical analysis

All data of this study submitted as (mean \pm SD). The student's T-test was applied to compare patient groups with the healthy group; also, Pearson's coefficient was used for correlation analysis.

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Results

This study involved sixty T1DM patients and thirty healthy controls. Table (1) listed demographic data of patient groups G1, G2, and healthy control G3. The mean age of the patient groups was (15 ± 6.67 and 10 ± 5.81) years in G1 and G2, respectively, with significant differences and distribution of sex was non-significant between studied groups. Mean BMI was normal in the patient's group and control group with significant difference between patients group when compared to control group.

Data in table 2 shown as expected a highly significant difference ($p < 0.001$) in an average of diabetic profile (fasting blood glucose (FBG), glycated hemoglobin (HbA1c) and insulin) between G1 and G2 when compared to the control group were also found, VCAM-1 level was significantly higher among diabetic patients than the control group.

In addition, there was a significant negative correlation of VCAM-1 with the levels of FBG, HbA1c in diabetic patients G1 and G2. At the same time, insulin had a positive correlation in G1 but correlated negatively in G2, as in table 3.

Discussion

This study compared mean FBG, HbA1c, and insulin with VCAM-1 between sixty T1DM patients with and without CVD and 30 healthy adolescents. As expected, levels of FBG, HbA1c, and insulin were significantly higher in patient groups. T1DM is caused by insulin deficiency; hyperglycemia, and that could damage the vascular¹². The levels of VCAM-1 was highly significantly increased in G1 and G2 compared to control similar group result in the study by other researcher^{13,14} who found

the levels of endothelial markers including VCAM-1 were higher in adults with T1DM than control, while there is no data available about levels of VCAM-1 in T1DM with CVD. Increased VCAM-1 as an adhesion molecule leads to endothelial dysfunction as a part of the inflammatory process¹⁵. T1DM may lead to increased VCAM-1, damage endothelial vascular as a result of that adhesion molecule will expression¹⁶. Therefore the risk of atherosclerosis will increase and the mortality due to cardiovascular complications in T1DM patients¹⁷.

According to the current study, VCAM-1 was significantly associated with FBG, HbA1c, and insulin in both patients and control groups. These findings disagree with the results of another researcher; they reported no correlation between serum level of VCAM-1 with FBG, HbA1c, and duration of disease¹⁸. Insulin resistance will increase the expression of adhesion molecules like VCAM-1 molecule¹⁹. So, in diabetic patients, elevated HbA1c, FBG, and increased insulin level due to insulin resistance²⁰.

Conclusions

As VCAM-1 level was significantly higher in both patient groups with and without cardiovascular disease so that, maybe we assume that it used to predictive to cardiovascular disease in T1DM patients, also; we suggested that if the patient with T1DM uses pro-inflammatory medical agents in their protocol, treatment can cause prevention complication of diabetic microvascular in future. The limitation of this study was the small sample size, and patients should be selected from a different area of Iraq. Also, HbA1c represents the average blood glucose during three months, and if the mean level of HbA1c may be more helpful.

	G1 Without CVD N=30	G2 With CVD N=30	G3 Control group N=30	P-value
Age (Yrs)	15 ± 6.67	10 ± 5.81	17 ± 3.12	NS
Sex (male/female)	15/15	14/16	16/14	S
Duration of DM (Yrs)	2 ± 0.57	1 ± 0.29	-----	NS
BMI	22.31 ± 8.26	19.90 ± 4.68	23.94 ± 4.77	HS

Statistically significant (S) considered when P-values was <0.05 and (HS) highly significant difference considered when P-values was <0.001 .

Table 1. Clinical characteristics of the patients and control groups.

	G1 Without CVD	G2 With CVD	G3 Control group	P-value
FBG (mg/dL)	274.4 ± 111.5	264.4 ± 96.07	85.90 ± 7.24	<0.001
HbA1c %	10.49 ± 2.30	10.98 ± 2.39	5.05 ± 0.46	<0.001
Insulin (MU/mL)	17.49 ± 3.74	20.88 ± 3.12	12.15 ± 2.31	<0.001
VCAM-1 (ng/mL)	26.40 ± 4.43	41.35 ± 4.47	20.17 ± 3.49	<0.001

Statistically significant considered when P-values was <0.05 and highly significant difference considered when P-values was <0.001 .

Table 2. Clinical characteristics of the patients and control groups.

		G1	G2
VCAM-1 & FBG	r	- 0.304	- 0.219
	p	0.102	0.214
VCAM-1 & HbA1c	r	- 0.224	- 0.059
	p	0.234	0.738
VCAM-1&duration of disease	r	- 0.420	0.003
	p	0.021	0.986
VCAM-1 & Insulin	r	0.088	- 0.172
	p	0.643	0.329

Statistically significant considered when P-values was <0.05 and highly significant difference considered when P-values was <0.001.

2294

Table 3. Correlate of vitamin D with some variables.

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Conflict of interest

The authors declare that they have no conflict of interest.

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RESEARCH / INVESTIGACIÓN

Evaluación temporal de sistemas agroforestales de cacao en el trópico húmedo ecuatoriano

Temporal assay of cocoa agroforestry systems in the Ecuadorian humid tropic

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Resumen: El cacao puede ser cultivado en asociación con árboles frutales, maderables y no maderables. Así, se evaluó el crecimiento de cuatro especies maderables y su comportamiento en la producción del cacao "CCN-51", obtenida a partir de plantas originadas de semillas. Se plantaron en campo las especies maderables Caoba de Montaña (*Colubrina arborescens* (Mill.) Sarg), Fernán Sánchez (*Triplaris guayaquilensis* Weed), Guayacán Blanco (*Cybistax donnell-smithii* Rose) y Laurel Prieto (*Cordia macrantha* Chodat), a una distancia de 9x9 m (123 árboles ha⁻¹), y plántulas de cacao a una distancia de 3x3 (988 plantas ha⁻¹). Cada unidad experimental tuvo nueve árboles maderables y 40 plantas de cacao, empleándose un diseño de bloques completamente al azar con cuatro repeticiones. Durante 12 años (entre 1995 y 2007) se registró el crecimiento de las especies maderables (altura de planta, DAP, tasa de crecimiento relativo y el volumen de madera). Los componentes del rendimiento de cacao (índice de semilla, índice de mazorca, número de mazorcas sanas y rendimiento de almendras de cacao por parcela) fueron registrados durante los años 2007 y 2008. El volumen acumulado de madera fue modelado mediante un análisis de regresión sigmoidal. El volumen acumulado de madera en época seca en el año 2006 y 2007 fue significativo para el Fernán Sánchez (1,992 y 1,489 m³ árbol⁻¹). Igualmente, está asociación incrementó el número de frutos sanos (228 y 133 mazorcas), rendimiento por parcela año (39,70 y 17,60 kg) y relativo de cacao (0,76 y 0,82). La asociación Fernán Sánchez + cacao es una excelente alternativa para sistemas agroforestales con cacao en la costa ecuatoriana.

Palabras clave: *Theobroma cacao*, especies maderables, rendimiento de cacao, SAFs.

Abstract: Cocoa can be cultivated in association with fruit, timber, and non-timber trees. Thus, the growth of four timber species and their behavior in producing "CCN-51" cocoa per seedling were evaluated. The timber species Caoba de Montaña (*Colubrina arborescens* (Mill.) Sarg), Fernán Sánchez (*Triplaris guayaquilensis* Weed), Guayacán Blanco (*Cybistax donnell-smithii* Rose) and Laurel Prieto (*Cordia macrantha* Chodat) were planted in the field, with a distance of 9x9 m (123 trees ha⁻¹) and were planted cocoa with a distance of 3x3 (988 trees ha⁻¹). Each experimental unit had nine timber trees and 40 cacao plants, using a Design Randomized Complete with four blocks. During 12 years (between 1995 and 2007), timber species were recorded (plant height, DAP, relative growth rate, and volume of wood). Cocoa yield components (seed index, pod index, number of healthy pods, and yield of cocoa beans per plot) were recorded between 2007 and 2008. Wood accumulated volume was modeled using sigmoidal regression analysis. The accumulated volume of wood in the dry season in 2006 and 2007 was significant for Fernán Sánchez (1.992 y 1.489 m³ tree⁻¹). Similarly, the number of healthy fruits increased (228 and 133 pods), yield per plot per year (39.70 and 17.60 kg), and relative cocoa (0.76 and 0.82). The association Fernán Sánchez + cacao is an excellent alternative for agroforestry systems with cacao in the Ecuadorian coast.

Key words: *Theobroma cacao*, timber species, cocoa yield, SAFs.

Introducción

El cacao (*Theobroma cacao* L.) en el Ecuador es un cultivo de interés económico, principalmente para los agricultores del litoral, donde se cultivan preferencialmente los tipos Nacional y CCN-51 (Trinitario)¹. Por un lado, las almendras del primero son requeridas en mercados internacionales por ser un producto de calidad denominado "fino de aroma", mientras que, el clon comercial CCN-51 desarrollado en el país, presenta tolerancia a las enfermedades, así como mayor producción de mazorcas sanas y rendimiento de almendras^{2,3}. Ambos tipos pueden ser encontrados en monocultivo, o en asociación con otros cultivos agrícolas o forestales⁴. De hecho, plantas umbrófilas como el cacao, presentan respuesta diferenciada a la asociación con especies vegetales *i.e.*, especies útiles para humanos y la fauna silvestre, capaces de proporcionar un dosel de sombra diverso y estructuralmente complejo^{4,5}.

Los sistemas agroforestales dinámicos son un método de producción alternativo practicado durante mucho tiempo en países latinoamericanos, tratando de imitar a los bosques naturales, ofreciendo múltiples beneficios como el mejoramiento de la fertilidad del suelo, la reducción de la presión de enfermedades y plagas, disminución de la erosión, y la diversificación de ingresos financieros⁶. Desde esta perspectiva, la asociación del cultivo de cacao con especies maderables de rápido crecimiento y otros cultivos como musáceas (*Musa spp.*), contribuyen a los requerimientos de sombra del cultivo de cacao, permitiendo básicamente ingresos con la venta de productos a corto (banano, plátano, y cacao) y largo plazo (producción de madera)^{4,5,7}.

Los productores cacaoteros incorporan una gran variedad de especies maderables y frutales en sus huertas. Estas

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asociaciones pueden servir para la construcción de casas y sus techos, postes para cercos, leña y otras necesidades en las fincas⁸. Especies maderables como Palo Prieto (*Erythrina poeppigiana* (Walp.) o.f. Cook), Yuca de Ratón (*Gliricidia sepium* (Jacq.) Walp.), Guabo (*Inga* spp.), Laurel (*Cordia alliodora* (Ruiz & Pav.) Oken), y frutales como Cocoteros (*Coco nucifera* L.), Naranjo Dulce (*Citrus aurantium* L., sub *sinensis*), Naranjo Agrio (*Citrus x aurantium* L., sub *amara*) y Mandarina (*Citrus nobilis* Loureiro), han sido recomendadas para la asociación con el cultivo de cacao en el Ecuador, por ser plantas que se adaptan a amplias zonas ecológicas y que proporcionan un ingreso financiero adicional al productor⁹. Sin embargo, en el país existen también otras especies maderables endémicas e introducidas que se adaptan a las zonas productoras de cacao, pudiendo ser de gran importancia económica. Por ejemplo, en la provincia de Los Ríos, se pueden observar importantes asociaciones de cacao tipo nacional con Laurel (*C. alliodora*), Fernán Sánchez (*Triplaris guayaquilensis* Weed), Pachaco (*Schizolobium parahyba* (Vell.) S.F. Blake), Guachapelí (*Albizia guachapele* (Kunth) Dugand), Moral Fino (*Maclura tinctoria* (L.) D. Don ex Steud), entre otros¹⁰.

De esta manera, el estudio de especies maderables en combinación con el cultivo de cacao es muy importante para los agricultores y para la región. Especies nativas ecuatorianas como Fernán Sánchez, están distribuidas tanto en la costa como en la sierra (estribaciones de los Andes), formando parte de la composición florística y estructural de los bosques secos, con alta demanda para la construcción, agroforestería e industrias de muebles^{11,12}. Otra especie, el Laurel Prieto (*Cordia macrantha* Chodat), una especie endémica emblemática, es encontrada en bosques secos deciduos del Pacífico Ecuatorial (provincias de Guayas y Loja) y de Perú^{11,13}, ha sido usada por mucho tiempo en sistemas agroforestales¹⁴, así como en la construcción de viviendas por la población local¹⁵. Por otro lado, tanto el Guayacán Blanco (*Cybistax donnell-smithii* Rose) como la Caoba de Montaña (*Colubrina arborescens* (Mill.) Sarg), ambas endémicas de Centroamérica, se han adaptado a la zona central del trópico húmedo ecuatoriano, encontrándolas en asociación a cacao y a café^{10,16}.

A pesar de los posibles beneficios encontrados entre la asociación de especies maderables y cultivos de importancia agronómica como el cacao, poco se conoce al respecto, más aún en Ecuador. Sobre esta base, la presente investigación se realizó a fin de evaluar el crecimiento de cuatro especies maderables y su influencia en los rendimientos de cacao CCN-51 de origen sexual.

Métodos

La investigación se realizó en la Finca Experimental "La Represa" de la Universidad Técnica Estatal de Quevedo (UTEQ), ubicada en la parte Alta de la Cuenca del Río Guayas, cuyas coordenadas geográficas son: 79° 25' 24" longitud oeste y 1° 03' 18" de latitud sur, a una altura de 73 msnm.

Fueron estudiadas cuatro asociaciones agroforestales con cacao. Primeramente, se trasplantaron las especies forestales Caoba de Montaña (*C. arborescens*), Fernán Sánchez (*T. guayaquilensis*), Guayacán Blanco (*C. donnell-smithii*) y Laurel Prieto (*C. macrantha*), a una distancia de 9 x 9 m (123 árboles ha⁻¹). Despues de seis meses del establecimiento de las especies forestales, se trasplantaron plántulas de cacao CCN-51 obtenidas por semillas, a una distancia de 3 x 3 (988 plantas ha⁻¹). Cada unidad experimental estuvo compuesta por nueve árboles maderables y 40 plantas de cacao. Se empleó un dise-

ño de bloques completamente al azar con cuatro tratamientos y cuatro repeticiones.

Para mantener las condiciones ideales de crecimiento tanto de las especies maderables como las del cacao, se realizaron podas fitosanitarias y de mantenimiento, fertilización química al inicio y al final de la época lluviosa (enero y mayo), con N, P, K, Mg, S, y B, en dosis de 18, 6, 22, 3, 4 y 0.53 kg ha⁻¹, respectivamente, en dosis de 400 g por planta, control manual de arves previo a la fertilización.

En las especies forestales se evaluaron la altura de planta (cm) y diámetro a la altura del pecho (DAP - cm), la tasa de crecimiento relativo y el volumen de madera. El crecimiento de los árboles maderables fue evaluado durante 12 años (entre 1995 y 2007). Durante los años 2007 y 2008, cuando las plantas de cacao alcanzaron la estabilidad de producción (aproximadamente a los cinco años), se registraron las variables: índice de semilla (peso de 100 semillas fermentadas y secas / 100), índice de mazorca (20 mazorcas / peso de las almendras secas de las 20 mazorcas), número de frutos sanos, rendimiento de almendras de cacao por parcela (kg) y rendimiento en escala relativa. El rendimiento de almendras de cacao se registró en escala relativa (1), al observarse que el rendimiento de almendras de cacao es variable entre un año y otro. En la relativización por año (2007 y 2008) y por época (lluviosa y seca) del rendimiento de almendras de cacao, se designó la unidad (1) al mayor rendimiento registrado, mientras que las demás producciones fueron divididas para el mayor rendimiento.

El volumen acumulado de madera por cada una de las cuatro especies forestales fue modelado a través de una ecuación de regresión sigmoidal (Ecuación 1), donde: a, volumen de madera inicial puede ser asumido como cero; b, volumen máximo de madera; c, edad en la cual la madera alcanza la mitad del volumen, delimitado entre los parámetros a y b; d, pendiente de la curva de crecimiento; x, años de edad.

$$y = a + \frac{b-a}{1 + \exp\left(\frac{c-x}{d}\right)} \quad (\text{Ecuación 1})$$

La tasa de crecimiento relativo de las cuatro especies forestales fue modelado a través de un análisis de regresión sigmoidal (Figura 1). El ajuste del modelo fue valorado a través del coeficiente de determinación (R^2), y su valor de probabilidad ($P \leq 0,05$).

Las variables: índice de semilla, índice de mazorca, número de frutos sanos, rendimiento por parcela y rendimiento relativo, se sometieron a un ANOVA. Dependiendo del resultado, las medias se compararon mediante la prueba de Tukey ($P \leq 0,05$). Además, los parámetros de la ecuación 1 entre especies forestales fueron comparados a través de la prueba de Tukey ($P \leq 0,05$).

Resultados y discusión

El volumen acumulado de madera fue diferente entre las cuatro especies maderables (Tabla 1). Se observaron diferencias significativas entre las especies maderables, donde el Guayacán Blanco (1,992 m³) y el Fernán Sánchez (1,489 m³) acumularon un mayor volumen de madera durante todos los años evaluados, en comparación a las dos especies restantes. Información similar fue reportada por Suatunce *et al.*¹⁷, quienes evaluando cuatro especies forestales tropicales de 10

años encontraron que el Laurel registró un menor volumen promedio ($0,316 \text{ m}^3$), en comparación al encontrado en la teca ($1,076 \text{ m}^3$). Estas diferencias pueden también ser observadas en otras especies forestales, lo que indicaría que es una característica genética de cada una de ellas^{18,19}.

Expresando el volumen acumulado de madera a través del tiempo (Tabla 1 y Figura 1), se observó que el Guayacán Blanco tuvo un comportamiento superior en relación con las otras especies maderables durante los primeros años de evaluación. Así también, es posible observar que esta especie y el Fernán Sánchez poseen una mayor tasa de crecimiento a lo largo del tiempo, en comparación al Laurel Prieto y la Caoba de Montaña (Figura 1, Tabla 2). De hecho, el Laurel Prieto puede presentar un menor incremento en diámetro, altura y volumen, tanto en asociación con café arábica y su monocultivo, en comparación con la asociación entre café y teca que sí registró el mayor incremento de volumen de madera a los cinco años¹⁶. A pesar de que el volumen haya sido menor en Laurel Prieto y en Caoba de Montaña, tal vez valdría la pena el establecimiento estas especies, especialmente de la última, pues su asociación con cacao es una alternativa viable para reemplazar la extracción ilegal de madera de caoba, y también puede servir para repoblar las áreas forestales que han sido degradadas por la deforestación²⁰.

El crecimiento de las cuatro especies forestales aumentó aproximadamente desde su establecimiento (trasplante) hasta el cuarto año (1999), siendo estabilizado de ahí en adelante (Figura 1). Una de las hipótesis, es que durante el tiempo inicial las plantas tendieron a crecer de forma rápida en su fase juvenil. Generalmente, especies de bosques tropicales presentan un explosivo crecimiento en edades tempranas, pero acompañada de una drástica reducción a partir de los años posteriores^{18,19}. Sin embargo, esta tendencia puede variar entre especies, indicando que el crecimiento inicial puede no caracterizar el potencial de plantación de la especie, pudiendo requerir ensayos a largo plazo¹⁸.

Con respecto a los componentes del rendimiento del cacao no se observó diferencia significativa en las variables in-

dices de semilla y de mazorca. Un elevado índice de semilla $>1,4$ y un bajo índice de mazorca <14 indican que el genotipo posee buenos atributos productivos, ya que con pocas mazorcas se obtendrá un kilo de cacao seco. Los índices de semilla durante la cosecha variaron en media de 1,27 a 1,42, mientras que el índice de mazorca fluctuó de 17,5 a 19,2 (Tabla 3). Estos resultados difieren con el de otros autores. De hecho, Pérez y Freile²², que reportaron índices de semilla y de mazorca, de 1,7 y 14, respectivamente, en clon CCN-51 pero establecidos en un sistema integrado con Plátano, Guaba (*Inga edulis* Mart), Chuncho (*Cedrelinga cateniformis* Ducke) y Laurel (*Laurus nobilis* L.). De forma semejante, Vera et al.²¹ encontró que el CCN-51 en monocultivo registró un índice de semilla y de mazorca, de 1,62 y 13,88, respectivamente. Posiblemente, los menores índices encontrados en nuestro estudio se puedan deber a la segregación del material del CCN-51 obtenido de forma sexuada.

El número de frutos sanos (133 frutos), rendimiento por parcela (17,6 kg) y relativo de cacao (0,82) fue diferente ($P \leq 0,05$) únicamente en la época seca 2007, mostrando que la asociación con Fernán Sánchez presenta un comportamiento superior en comparación a los otros sistemas agroforestales (Tabla 3). Las asociaciones entre fernán Sánchez y Caoba de Montaña con cacao durante la época seca, por lo general pueden registrar alto contenido de humedad del suelo, y producción de hojas caídas (Ramírez et al.²³). Lo que podría indicar que el Fernán Sánchez tuvo mayor disponibilidad de humedad en el suelo durante el cuajado de frutos en la época que se evidenció en una mayor producción del cacao. Se conoce incluso, que la asociación de especies maderables como Chuncho (*C. cateniformis*) y Laurel (*Laurus nobilis* L.) produce en el clon CCN-51 un mayor rendimiento de almendras, en comparación a clones comerciales como EET-95, EET-96 y EET-103 (Pérez y Freile²²). La superioridad potencial de varios componentes productivos en el clon CCN-51, comparado a clones experimentales y comerciales establecidos en monocultivo también ha sido reportado por Sánchez-Mora et al.³.

Especies forestales	Volumen acumulado de madera							
	$\text{m}^3 \text{ árbol}^{-1}$							
	1998 [†] (3) ^{††}		2001 (6)		2004 (9)		2007 (12)	
Caoba de Montaña	0,070	b*	0,248	c	0,377	b	0,528	b
Fernán Sánchez	0,123	a	0,637	a	1,043	a	1,489	a
Guayacán Blanco	0,094	ab	0,573	ab	1,054	a	1,992	a
Laurel Prieto	0,055	b	0,379	bc	0,546	b	0,691	b
EEM	0,012		0,058		0,082		0,178	
\bar{X}	0,085		0,459		0,755		1,175	

* promedios con letras iguales no difieren estadísticamente de acuerdo con la prueba de Tukey ($P \leq 0,05$). † volumen acumulado de madera desde el año 1995 (año de trasplante) hasta el año indicado en la respectiva columna (12 años en total). †† valores en paréntesis indica la edad en años de las especies forestales desde el transplante. EEM: error estándar de la media. \bar{X} : promedio aritmético ($n = 16$).

Tabla 1. Volumen acumulado de madera de cuatro especies forestales de rápido crecimiento en condiciones de bosque húmedo tropical de la latitud ecuatorial.

Especies forestales	Parámetros								R^2	
	a		b		c		d			
	$m^3 \text{ árbol}^{-1}$									
Caoba de Montaña	-0,85	ns	1,15	c	4,24	b	9,84	ns	0,875	
Fernán Sánchez	-0,50		2,16	b	7,44	b	4,13		0,956	
Guayacán Blanco	-0,24		3,75	a	13,95	a	4,58		0,995	
Laurel Prieto	-0,16		0,72	c	5,10	b	2,37		0,870	
Promedios con letras iguales no difieren estadísticamente de acuerdo con la prueba de Tukey ($P \leq 0,05$).										

Tabla 2. Parámetros (a-d) de la ecuación sigmoidal utilizada para modelar el volumen acumulado de madera de cuatro especies forestales de rápido crecimiento en condiciones de bosque húmedo tropical de la latitud ecuatorial.

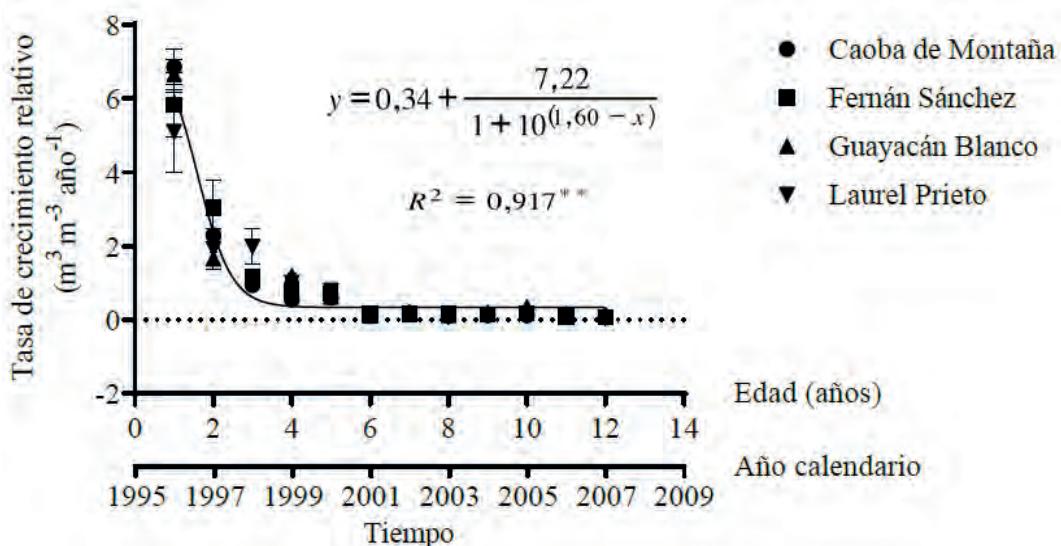


Figura 1. Tasa de crecimiento relativo de cuatro especies forestales de rápido crecimiento en condiciones de bosque húmedo tropical de la latitud ecuatorial. Cada figura geométrica representa el promedio ($n = 4$) y la barra la desviación estándar. Los asteriscos (**) representan una probabilidad significativa ($P \leq 0,05$).

Las especies maderables, tienen como función principal brindar sombra al cacao al igual que los frutales, y los productores mantienen a estas especies como un recurso a utilizar en casos de emergencia, o a su vez, para construcción en la finca (Torres *et al.*⁷). De esta manera, esta investigación arroja información relevante que podría servir tanto a productores cacaoteros, como forestales para asociar sus cultivos.

Conclusiones

Las especies maderables Fernán Sánchez y Guayacán Blanco obtuvieron el mayor volumen acumulado de madera.

El cacao en asociación con Fernán Sánchez presentó el mayor rendimiento relativo de cacao seco durante la época seca, mostrándose como una excelente alternativa para sistemas agroforestales.

Conflictos de interés

Los autores declaran que no tienen conflictos de intereses.

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Especies forestales	Componentes del rendimiento de cacao							
	Época		Época		Época		Época	
	Seca 2006		lluviosa 2007		Seca 2007		lluviosa 2008	
	----- Índice de Semilla -----							
Caoba de Montaña	1,44	ns	1,24	ns	1,35	ns	1,40	ns
Fernán Sánchez	1,45		1,13		1,33		1,40	
Guayacán Blanco	1,46		1,50		1,45		1,43	
Laurel Prieto	1,31		1,20		1,40		1,35	
EEM	0,05		0,05		0,04		0,03	
X̄	1,42		1,27		1,38		1,39	
	----- Índice de Mazorca -----							
Caoba de Montaña	-		-		20,30	ns	17,60	ns
Fernán Sánchez	-		-		19,70		16,90	
Guayacán Blanco	-		-		20,00		17,40	
Laurel Prieto	-		-		20,90		18,20	
EEM	-		-		0,47		0,22	
X̄	-		-		19,20		17,53	
	----- Número de frutos sanos -----							
Caoba de Montaña	159,00	ns	55,00	ns	84,00	b	29,00	ns
Fernán Sánchez	228,00		61,00		133,0	a	30,00	
Guayacán Blanco	138,00		56,00		68,00	b	46,00	
Laurel Prieto	151,00		54,00		100,0	ab	57,00	
EEM	13,28		4,56		8,09		4,93	
X̄	169,00		56,00		96,00		41,00	
	----- Rendimiento por parcela (kg) -----							
Caoba de Montaña	29,10	ns	9,60	ns	10,80	b	4,03	ns
Fernán Sánchez	39,70		12,10		17,60	a	4,45	
Guayacán Blanco	26,40		13,80		8,70	b	6,65	
Laurel Prieto	25,60		9,60		12,70	b	7,78	
EEM	2,26		1,05		1,07		0,68	
X̄	30,18		11,83		12,50		5,70	

Tabla 3. Componentes del rendimiento de cacao bajo sombra de cuatro especies forestales de rápido crecimiento en condiciones de bosque húmedo tropical de la latitud ecuatorial.

	Rendimiento relativo							
	0,56	ns	0,56	ns	0,50	b	0,34	ns
Caoba de Montaña	0,56	ns	0,56	ns	0,50	b	0,34	ns
Fernán Sánchez	0,76		0,57		0,82	a	0,38	
Guayacán Blanco	0,51		0,65		0,41	b	0,56	
Laurel Prieto	0,49		0,46		0,59	b	0,66	
EEM	0,04		0,05		0,05		0,06	
X̄	0,58		0,56		0,58		0,48	

EEM, error estándar de la media; X̄, promedio aritmético (n = 16); *, promedios con letras iguales no difieren estadísticamente de acuerdo con la prueba de Tukey (P ≤ 0,05).

Tabla 3. Componentes del rendimiento de cacao bajo sombra de cuatro especies forestales de rápido crecimiento en condiciones de bosque húmedo tropical de la latitud ecuatorial.

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CASE REPORTS / REPORTE DE CASO

Schizophrenia and refractory status epilepticus in a male patient with anti-NMDA auto-immune encephalitis: A case report

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Abstract: Encephalitis is the inflammation of the central nervous system cells as a result of activation of immune cells, antibodies, or proteins by a reaction with pathogens or self-body components; when this happens, a malfunction of the immune system is known as this an auto-immune disease. Auto-immune encephalitis characterizes 21% of all encephalitis and manifests with loss of memory and cognition, personality deviations, neurologic deficit, aphasia, seizures, and epilepsy. Treatment is guaranteed using steroids, immunoglobulins, and plasmapheresis, and as a second-line therapy, cyclophosphamide or rituximab is used. In cases related to tumors, surgery is part of the treatment. An unusual case of auto-immune encephalitis is reported.

Key words: Encephalitis, Auto-immune diseases, Epilepsy, Mental disorders.

Introduction

Encephalitis is the inflammation of the central nervous system cells as a result of activation of immune cells, antibodies, or proteins by a reaction with pathogens or self-body component; when this happens, a malfunction of the immune system is known as this an auto-immune disease¹.

Encephalitis's annual incidence is about 5-8 cases per 100000 inhabitants, and 21% of these cases are related to auto-immune encephalitis². Epidemiologic studies report that young patients of age 21 are most frequently affected since auto-immune encephalitis can occur between 2 months and 85 years of age³. In Ecuador, statistical data about this disease are not available.

Early diagnosis and treatment prevent the diminished quality of life with memory loss, cognition deficits, personality disorders, paresis and paresthesia, aphasia, seizures, and coma⁴.

The following case report presents a male patient with a schizophrenia-like disease followed by a refractory status epilepticus (RSE) diagnosed with anti-NMDA auto-immune encephalitis. It is considered an unusual presentation due to male gender and RSE, so it is essential to recognize presentation variants of this disease to accomplish prompt diagnosis and treatment.

Clinical case

Twenty-three-year-old male patient with a familiar history of schizophrenia in maternal aunt presented behavioral symptoms, aggressiveness, agitation, visual hallucinations, and generalized seizures without abnormalities in a head computed tomographic (CT) scan, treated with carbamazepine and phenytoin, and no improvement. After a psychiatric assessment, it was resolved to be hospitalized under a diagnosis of schizophrenia.

The patient became febrile, somnolent, agitated, and his Glasgow coma scale (GCS) changed to 12 (O4V3M5). Haloperidol and diazepam were administered to control symptoms. Cerebrospinal fluid (CSF) and blood test turned out inconclusive (Table 1), so bacterial versus viral meningitis was considered, and the patient was started on acyclovir ceftriaxone and azithromycin. Phenytoin and valproic acid were used as anti-convulsive treatments.

After eight days of hospitalization, GCS worsened to fluctuate between 11 (O3V2M6) and 7 (O2V2M3), so it was decided to protect the airway and start on mechanical ventilation. Fentanyl and midazolam infusions were indicated. He was transferred to the intensive care unit (ICU) with suspicion of auto-immune encephalitis, and methylprednisolone was initiated (1 gr IV each day for 5 days).

Three days after, the patient presented refractory status epilepticus (RSE), and propofol bolus and infusion were needed to stop it. Levetiracetam was started as the third anticonvulsive drug. A brain magnetic resonance imaging study was performed without abnormalities reported (Figure 1). Blood and CSF tests were carried to rule out herpes simplex brain infection and thyroid and testicular cancers. Nevertheless, the CSF molecular diagnosis lab reported anti-NMDA antibodies, and diagnosis of auto-immune encephalitis was achieved.

The patient showed involuntary movements despite sedative infusions, and electroencephalographic (EEG) monitoring was necessary to follow patient evolution. It was negative for persisting status epilepticus.

Plasmapheresis was indicated as the initial treatment, and 5 sessions were carried out according to institutional availability. Sedation was withdrawn while he was on EEG monitoring and GCS improved from 3T (O1V1TM1) to 10T (O4V1TM6) without new seizures. Nevertheless, superior extremities dyskinetic movements continued.

Mechanical ventilation was kept for 15 days due to infectious multi-drug resistant complications (ventilator-associated pneumonia), and tracheostomy and gastrostomy were needed to perform weaning.

ICU length of stay was 23 days, and the patient was discharged to the Neurology department. Hospitalization continued for 37 days more because of healthcare-associated pneumonia. New episodes of agitation and somnolence developed, so a relapse was identified. Second-line drug rituximab was indicated, and a good response was achieved. Agitation control and connection with the environment were accomplished. However, right arm dyskinetic movements persisted.

In the end, discharge and ambulatory follow-up were planned, but the patient didn't return.

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TEST	1	2	3
Appearance	Clear	Clear	Clear
pH	7.0	8.0	8.0
Cells	Absent	Absent	Absent
Gram stein	Normal	Normal	Normal
Red blood cells	800	Absent	Absent
Plasmocytes	0-1	Absent	Absent
White blood cells	600	Absent	6
Glucose	65	77.4	60.5
Proteins	0.09	15	14
Albumin	0.01	---	---
LDH	---	18	14
Culture	Negative	Negative	Negative
KOH	---	Negative	Negative

Table 1. Cerebrospinal fluid test characteristics in the patient.

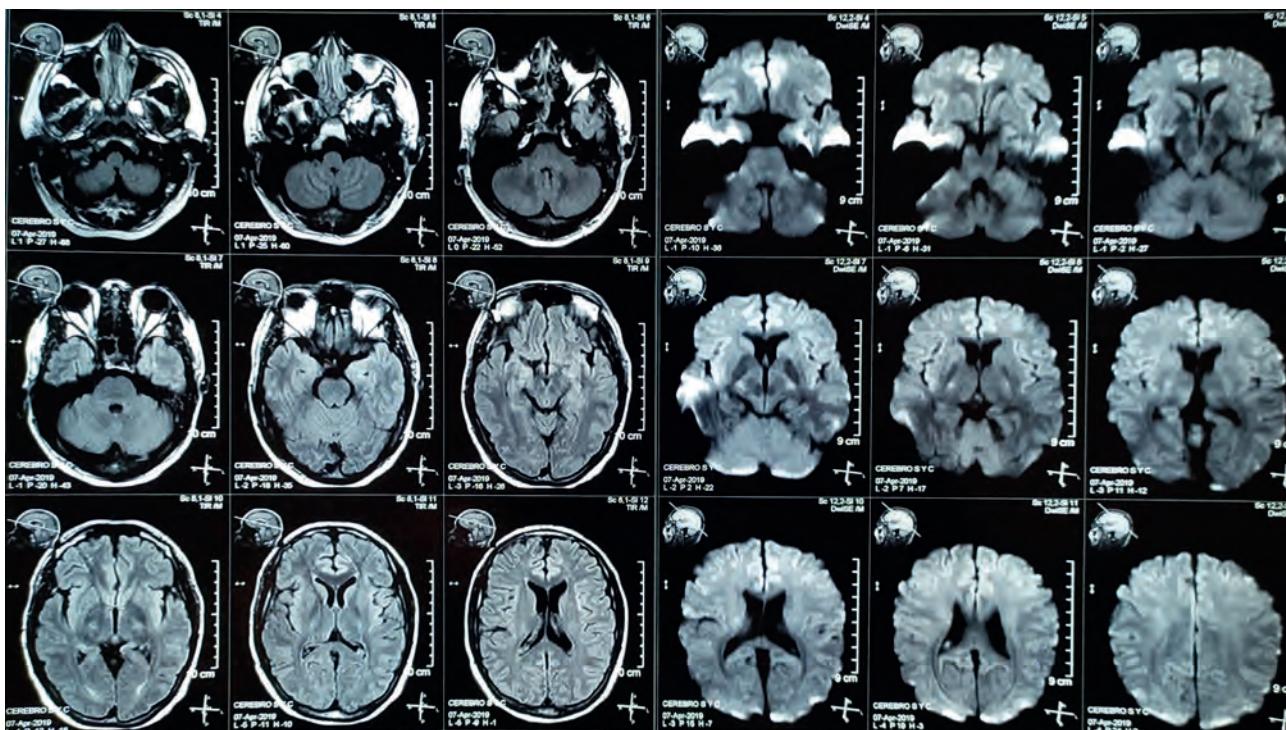


Figure 1. Simple brain MRI with FLAIR (left) and Diffusion (right) sequences without central nervous systems visible alterations.

Discussion

The case report refers to a young male patient with an initial diagnosis of schizophrenia, who developed a new onset refractory status epilepticus (NORSE), followed by suspicion of auto-immune encephalitis and confirmed by isolation anti-NMDA antibodies in cerebrospinal fluid. He is treated with plasmapheresis as a first approach, showing a slow response and a relapse. Rituximab is chosen as a second-line treatment with complete control of symptoms. Hospitalization of the patient is complicated with healthcare-associated pneumonia for two occasions.

Ecuador doesn't have a database for registry and reports of patients with a diagnosis of auto-immune encephalitis. A UK study reports that auto-immune encephalitis represents 21% of all diagnosed encephalitis; nevertheless, 37% of the encephalitis doesn't achieve an etiology⁵. In a Norwegian study, the diagnosis of encephalitis has a known cause in 43% of cases; this is because of the infectious disease focus⁶. Numbers point out a difficulty in diagnosing auto-immune encephalitis.

The first description of auto-immune encephalitis was

made in 2005 when four young women diagnosed with ovarian teratoma showed psychiatric, neurologic, and respiratory compromise⁷. In this regard, our patient was ruled out of possible testicular, thyroid, brain, and lung cancer.

In this case, the diagnosis of auto-immune encephalitis was made by isolation of anti-NMDA antibodies in cerebrospinal fluid. Antibodies against central nervous system cells are classified into 2 groups according to their mechanism of action. Antibodies like Hu-type, Ri-type, anti-fine, Ma2-type, CRMP 5-8-9-10, work against intracellular antigens and are related to paraneoplastic syndromes in lung cancer cases seminomas, and breast cancer. Immune therapy and steroids perform poorly against them, with a bad prognosis. Antibodies against the surface and synaptic proteins like anti-AMPA-GluR3, anti-NMDA-NR1, and anti-NMDA-NR2 can cause encephalic, cerebellar, and epileptic syndromes with a benign evolution and a better response to immune therapy and steroids⁸.

Young male patients with auto-immune encephalitis are unusual because anti-NMDA encephalitis is presented with a greater frequency by females in a ratio of 3:1 according to different studies^{8,9}.

The main symptoms showed by our patient were personality disorders, agitation, and seizures. According to epidemiologic studies, the most frequent sign and symptoms are seizures (88%), personality disorders (69%), fever (56%), headache (50%), and neurologic deficit (50%) (10). The most common neurologic deficits are aphasia (72%), movement disorders (63%), and autonomic dysfunction (47%). Irritability (75%), hallucinations (66%), and psychosis (59%) are the most prevalent psychiatric traces^{4,10}.

NORSE is not a common feature in auto-immune encephalitis. It is reported that 52% of NORSE cases are cryptogenic, and just 5% are related to anti-NMDA encephalitis^{11,12}.

Epidemiologic studies have reported cerebrospinal fluid characteristics in auto-immune encephalitis. They have found pleocytosis (>4/uL) in 69% of cases, proteins >0.5 g/L in 44% of cases, and a CSF/serum glucose ratio <0.5 in 27% of cases¹⁰. It was different in our patients.

Viral etiology is the most common cause of encephalitis, so tests for infectious diseases must be performed to rule out them (herpes simplex, varicella-zoster, enterovirus)^{10,13}. Our patient was negative for herpes simplex. No other test was available to be performed.

Image studies in encephalitis report: head computed tomography (CT), alterations in 17% of cases; brain magnetic resonance imaging (MRI), abnormalities in 25%-46% of cases; and electroencephalographic seizure changes in 81%-88% of cases^{10,14}. No abnormalities were displayed by head CT scan or brain MRI in the patient in the present report. EEG was not helpful because of NORSE presentation and drugs used for its treatment.

The treatment used in this case were plasmapheresis and rituximab. Case series and expert recommendations state that the first-line treatment for NMDA auto-immune encephalitis is steroids (methylprednisolone 1 g IV per day for 3 to 5 days), immunoglobulin (0.4 g/kg per day for 5 days). As second-line treatments, cyclophosphamide and rituximab are used. In cases related to cancer, surgery is part of the treatment^{3,10,12,14,15}.

It has been reported that approximately 53-75% of auto-immune encephalitis patients require ICU admission, and 41% precise mechanical ventilation. The hospital length of stay is 89 days as an average³. In this case report, ICU hospitalization was for 23 days, and the total hospital stay was 60 days.

Our patient follow-up was not possible, so neurologic and functional recovery assessment was not registered. A US multicenter observational study in 577 patients found that early treatment and no-UCI admission was related to good prognosis (OR 0.62, CI 0.50-0.76; p<0.0001; OR 0.12, CI 0.06-0.22; p<0.0001). After the first month of discharge, patients showed, according to the modified Rankin scale (Table 2), a score of 5 in

86% of cases, 4 in 12%, and 3 in 1%. After the second year, 78% of patients achieved a 0-2, and 6% died^{3,16}.

Limitations found in our case report were that it contains just one case attended in different health institutions, so difficulties were obtaining detailed psychiatric notes. NORSE as part of the patient's symptoms, didn't allow neurologic exploration and EEG record to characterize auto-immune encephalitis because of the need for sedation. And the patient was lost for follow-up.

Strengths, in this case, were early suspicion of auto-immune encephalitis after lack of response in treatment with antibiotics. The possibility of complete plasmapheresis therapy and the availability of rituximab for treatment is not standard therapy used in Ecuador. And the opportunity of confirming the diagnosis with isolation of anti-NMDA antibody because this test is not routinely accessible in the country.

Conclusions

Anti-NMDA auto-immune encephalitis is a low prevalence disease worldwide, so national reports and follow-up strategies have been created, so etiology, clinical picture, and early diagnosis and treatment are possible. This is not available in low and middle-income countries like Ecuador, so it is necessary to be familiar with the presentation and evolution of these cases to offer an opportunity for recovery. Diagnosis starts in a patient with psychiatric and neurologic symptoms, followed by isolation of antibodies in CSF. First-line treatments are IV immunoglobulins and plasmapheresis. Cyclophosphamide and rituximab are second-line drugs. Functionality recovery is slow and achieved by the second year of discharge. Good prognosis factors are early treatment and no-UCI admission.

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Grade	Description
0	No symptoms at all.
1	No significant disability despite symptoms: able to carry out all usual duties and activities.
2	Slight disability: unable to carry out all previous activities, but able to look after own affairs without assistance.
3	Moderate disability: requiring some help but able to walk with assistance.
4	Moderately severe disability: unable to walk without assistance and attend to own bodily needs without assistance.
5	Severe disability: bedridden, incontinent, and requiring constant nursing care and attention.

Table 2. Modified Rankin Scale¹⁷.

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REVIEW / ARTÍCULO DE REVISIÓN

A review on emerging micropollutants: sources, environmental concentration and toxicity

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Abstract: Every minute, the environment is filled with pollutants of various types, including physical, chemical, and biological. A new threat has emerged in recent years due to human activity, which is of significant concern. These pollutants are not like conventional pollutants but can alter the physiology of living things, and hence these are named emerging pollutants. The pollutant sources include crop protection chemicals, personal care products, antimicrobial mixtures, active pharmaceutical ingredients (API). These compounds are biologically crucial because their minute quantity can also disrupt an individual's endocrine system, and hence they are also called endocrine disruptors. This current work reviews many aspects, including source, problems, and legislative solutions that have been farmed to cope with the current situation of emerging micropollutants.

Key words: Emerging pollutants; chemicals, Active pharmaceutical ingredients (API), endocrine disruptors, microplastics.

Introduction

Micropollutants are chemical compounds usually found in lower concentrations in the aquatic environment^{1,2}. Their amount in water may be significantly from undetectable to a few nanograms per liter³. The term micropollutants (M.P.s) are those, which were previously not considered nor had no significance in the quality of both ground and surface water by distribution and concentration⁴. These compounds were previously not available but are found widely in the recent past and can cause known or suspected adverse effects on human health and ecology. These compounds include natural and synthetic, such as pharmaceuticals and personal care products (PCPs), detergent, steroids, hormones, industrial compounds, pesticides, nanoparticles, and many other contaminants, increasing the environmental concern⁵⁻¹⁶. Bacteria with Antibiotic-resistant genes are also considered as emerging pollutants¹⁷.

Micro pollutants are called 'emerging' because they were not considered and were not included in monitoring programs as their fate, eco-toxicological behaviors are unknown¹⁸. However, it is a challenge to understand EMPs as less information is available. These EMPs started to be detected after the development of sophisticated chromatographic and mass spectrometric techniques¹⁹ at the traces levels, which led to large numbers of research to identify, quantify and catalog the EMPs by publishing them²⁰⁻²⁴.

EMP regulation in various countries

There is a large gap of scientific data relevant to the impacts and fates of EMPs with different concentrations. This has become a hurdle to various Governments around the world to control their usage and handling. It is a matter of more significant concern to handle the EMPs which have already entered the aquatic ecosystem. Specific laws concerning EMPs have not been framed globally, which would mandate concentration limits in any water source treatment plants or nature. The USA prepared an archive of observation techniques to handle enzyme-disrupting compounds and reduce their contact with human or animal life. This document did not have any permissible limits of these compounds in the water but reduced the concentration in consumer products²⁵. The European commis-

sion in 2000 framed a water framework Directive to achieve the good qualitative and quantitative status of all water bodies in the European Union (E.U.)²⁶. In 2013, it released a directive to list 45 priority compounds for their quality in the aquatic environment. In 2015, it added 10 more compounds to the watch list to assess the water quality²⁶.

The E.U. set the permissible limit from 10 ppm and 10mg/kg in surface water and soil to pharmaceutical drugs in the drinking water. In 1998 USFDA gave detailed protocols to assess human drugs, which led to the preparation of an environmental report on each compound and restricted their expected introduction to aquatic environments to 1 ppb²⁷. Switzerland proposed a similar mechanism to European Union's Environmental Quality standards (EQS) for water quality criteria for pharmaceutical compounds and pesticides²⁸. These regulatory bodies of E.U. & USA have tabulated the disinfection byproducts transformed from EMPs and published for general awareness²⁹. But India has no such mechanisms to identify or regulate EMPs³⁰.

In the case of pesticides, every country has its pesticide regulations regarding production, distribution, and utilization. In most countries, their occurrence in drinking water is not regulated through laws even though there are risks to human life and ecology^{31,32}. In the case of India, these are considered as pollutants under the Water (Prevention & Control of Pollution) Act 1974, but their transformed byproducts are not included as pollutants. European Union in 1998 specified the maximum permissible limit of pesticide & their byproduct in water is 1ppb and 0.5 ppb, respectively³³. No specified pesticide was mentioned in this directive but created a monitoring authority to determine the pesticides present in drinking water. This led to identifying several pesticide risk indicators and developing various methodologies with different parameters, including the toxicity of aquatic organisms, assessment of health risks to humans on contact both by occupational exposure and by contaminated water or food³⁴⁻³⁸.

The Environmental Protection Agency (EPA) is an independent body in the USA that takes care of air, water, and soil problems. This agency has evolved over a century along with

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regulations related to the environment. EPA started to look into the adversity of M.P.s in 1985 by preparing detailed guidelines for national water and protecting aquatic life^{38,39}.

Various researches showed the endocrine-disrupting activities by EMPs; EPA published a White paper in 2008. This includes the effect of chemicals on aquatic life during various stages of growth, toxicity, and specific mode of action. This white paper on aquatic life criteria for emerging contaminants was developed to modify the 1985 guidelines^{40,41}.

Japan is a developed country with many water pollution episodes, such as mercury poisoning, cadmium poisoning, organic pollutants in ports, and nuclear pollution. But it has managed to overcome these problems by well-planned regulations and stringent action⁴². The umbrella act, Basic law of environmental pollution control, was enacted in 1967, which concerned human health, control of pollution, and conservation of natural resources. In 1970, a water pollution control law was enacted to protect water resources and was amended in 1995. By mode of follow approach and tightened regulations have given fruitful results^{42,43}.

In this work, we tried to identify some significant chemicals used in various fields, EMPs in the Indian case scenario. Our focus was on chemicals used for crop protection, pharmaceutical drugs and personal care products. Here we tried to quantify them based on their availability to the public and identified their derivatives. In most cases, chemical derivatives were not identified not because of their ability to degrade but because of their insignificant amount in the water sources.

Sources and Movement of EMPs in Environment

When Non-Pathogenic, hazardous material is exposed to humans can cause a significant health risk. This effect increases if these compounds enter into the aquatic source from which drinking water is obtained. In many cases, these EMPs have been seen in drinking water; hence, an attempt is made to identify the pathways. These EMPs all transported through the general model as

1. Source: Agricultural land or Industrial outlet, effluent or Sewage sludge in open land.

2. Flow of EMPs: Through fractures of aquifers, Run-off from Rain Water.

3. The receiver: Crops or tap water for drinking.

Sources of EMPs include wastewater derived from domestic, industrial, hospital premises and waste disposal sites widespread with various openings to the aqueous sources like surface or ground water sediments including pesticides from the agricultural farm, horticultural gardens parks (Figure 1), golf course, urban infrastructure and transport systems, discharge from hospital and industrial waste water which contains various pharmaceutical and PCPs (Figure 2 and 3)⁴⁴⁻⁴⁶. Other sources include sewage and industrial sludge application on land, treatment of animals and pets with pesticides and pharmaceuticals⁴⁷.

Potential sources of E.P.s are Agricultural farms includes crop protection compounds like pesticides, pheromones, hormones sprayed to the crops to protect the harvest. These compounds are deposited on either leaves or soil, leached into groundwater during raining or watering the crops⁴⁸. Thus, a large number of pesticides and their derivatives enter into groundwater or run-off.

Hospital and industrial waste disposal sites in urban areas

are also considered as the sources of EMPs. Polychlorinated biphenyls (PCB), polyaromatic hydrocarbons, alkyl phenols, dioxins furans have been identified in waste water for a long time. Many studies are conducted on waste water treatment effluents, sewage septic tanks, and artificial recharge treated effluents. These waters possess EMPs, majorly pharmaceutical from human excreta and unused pharmaceutical products, rare earth minerals, heavy metals, and contrast Medias⁴⁹⁻⁵⁴.

In many parts of Asia, Europe, and the USA, animals are fed with a concentrated antibiotic diet, an essential source of environmental contamination with EMPs. These antibiotics are seen in waste lagoons, groundwater below lagoons, surface water such as ponds and rivers where this livestock is pastured, and the areas where animal waste is applied to fields as manure⁵⁵⁻⁶¹.

In urban landfills have been a source of polluting ground water. The leachate from the landfill reaches ground water, including caffeine, nicotine, phenols, sterols, and phthalates⁶¹⁻⁶³. Industrial pollutants are also found, such as detergents, antioxidants, plasticizers, fire retardants, and personal care products like antibiotics, anti-inflammatory, and barbiturates⁶⁴.

The path of EMPs in the transmission stage from source to receptors is always unclear. Various factors are involved during the transmission, such as aquatic environment, pH, the solubility of compounds in water, biotic components in an aqueous ecosystem^{65,66}. Considering these factors, the injection of EMPs to groundwater and surface water is studied. EMPs may enter through sewage leakages from septic tanks, wastewater application to agricultural fields, and leachate from landfills. These enter into ground water bypassing the soil zone⁶⁷.

In many cases, treated effluents from sewage and industries are discharged to surface water. The groundwater and surface water interaction is another method of transmission where the difference in density of waters leads to the exchange of compounds. Atmospheric transmission is also a possible case of nonvolatile dust compounds, especially in veterinary and agriculture⁶⁸.

The end-user of water is humans for drinking and farming purposes. In the world, 11% of the population does not have a water supply, and 25% do not have proper sanitation facilities⁶⁹. Hence every government agency utilizes the water available in the area without looking at the quality. Household connectivity of water and public standpipe bore well facilities are provided to improve the drinking water problem and sanitation. Hence the risk of exposure to pollutants is very high. So to reduce the risk, the drinking water treatment is carried out, but the effectiveness of the treatment is significantly less since the concentration is very less can escape from the detectors or indicators⁷⁰.

Wastewaters from animal shelters and agriculture are directly utilized for agriculture, where chemical compounds such as solvents, pesticides, and hormones are taken up by crop plants and lead to poisoning. This issue has become a global concern as most food items are grown or processed in this water source and transmit the EMPs to far ends of the world^{71,72}.

Not just water and soil, even emerging pollutants in the air have gained attention recently. It is very well-acknowledged in recent reviews that study on micropollutants in the air is marginally studied.

It is known that the primary source of air pollution is the activities happening on soil and water through anthropogenic activities. Having said that, there exist natural sources of pollution such as dust and emissions from wildfires and volcanoes. In totality, particulate matter accompanied by volatile organic

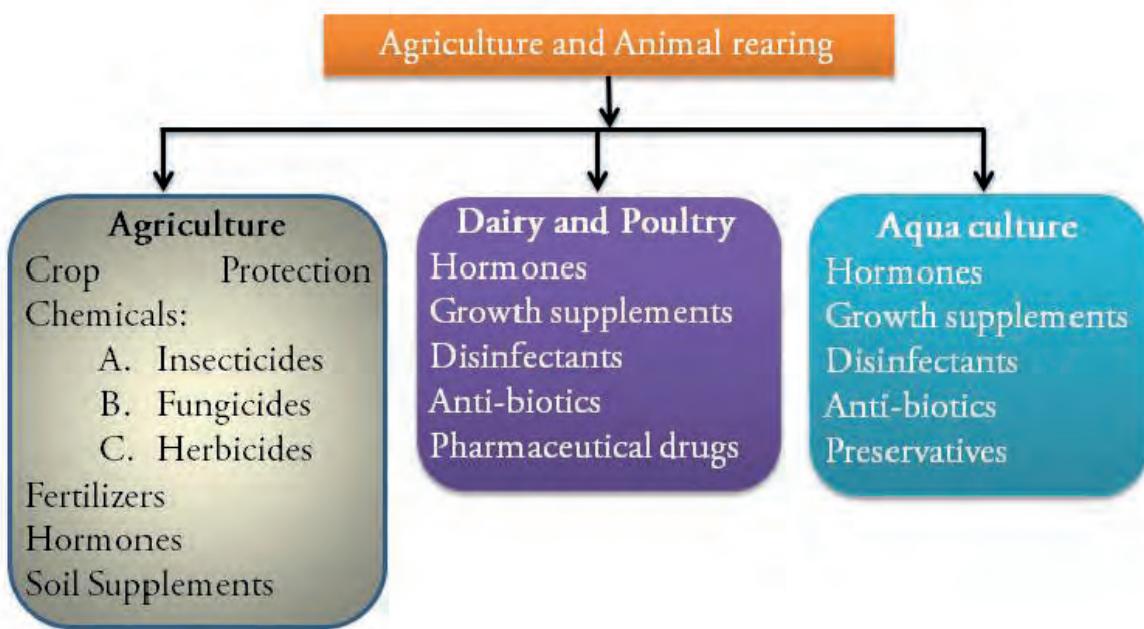


Figure 1. EMPs from Agricultural and allied Source.

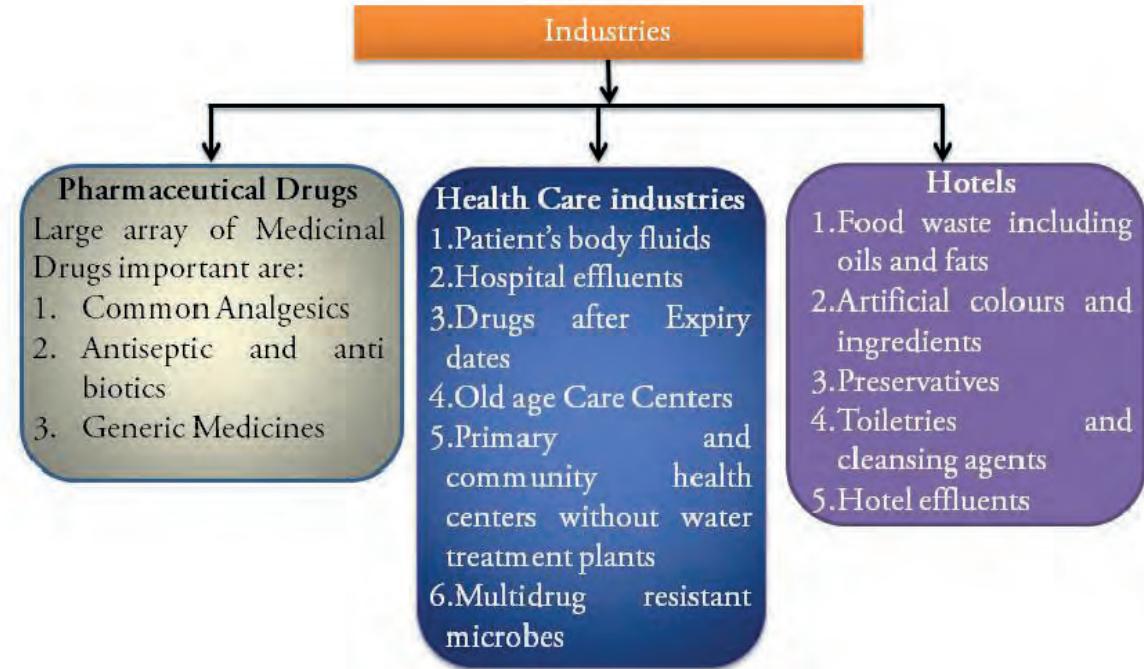


Figure 2. EMPs from various industrial sources.

compounds (VOCs), and other gases form the emerging pollutants in the air⁷³.

Effects of EMPs on different organisms

Hazardous materials are more toxic in lower concentrations and can cause acute and chronic disorders depending on the exposure. EMPs have nanoparticles, antibiotics, endocrine disrupting substances, pesticide derivatives, and hormones that can impact the organisms' daily activities and internal biology. The effects may be invisible in the initial stages, but continuous exposure can cause severe cellular imbalance. The list of EMPS and their adverse effects are tabulated in Table 1.

Crop Protection chemicals

Indian sub-continent has extended from equatorial to sub-

tropical regions 8° N to 38° N and spread through 70° E to 98° E. It has compressed every ecosystem. Tropical Region is the primary structure of India, so the condition is favorable to grow the majority of crops and fruits. Hence, agriculture is the principal occupation that contributes 18% to the GDP of the Indian economy. Approximately 58% of the Indian population depends on agriculture for their daily livelihood. Export of cotton and production of sugar is expected to be increased by 9% and 23% by the end of 2018. India is the second-largest producer of fruits and the largest producer of spices globally⁸⁵.

Crops are exposed to various pests, and to protect them from loss, hundreds of pesticides are produced and imported. Pesticides are of various types in the market depending upon the mode target-based and molecule-based. These are broadly classified into insecticides (Action on insects, mainly

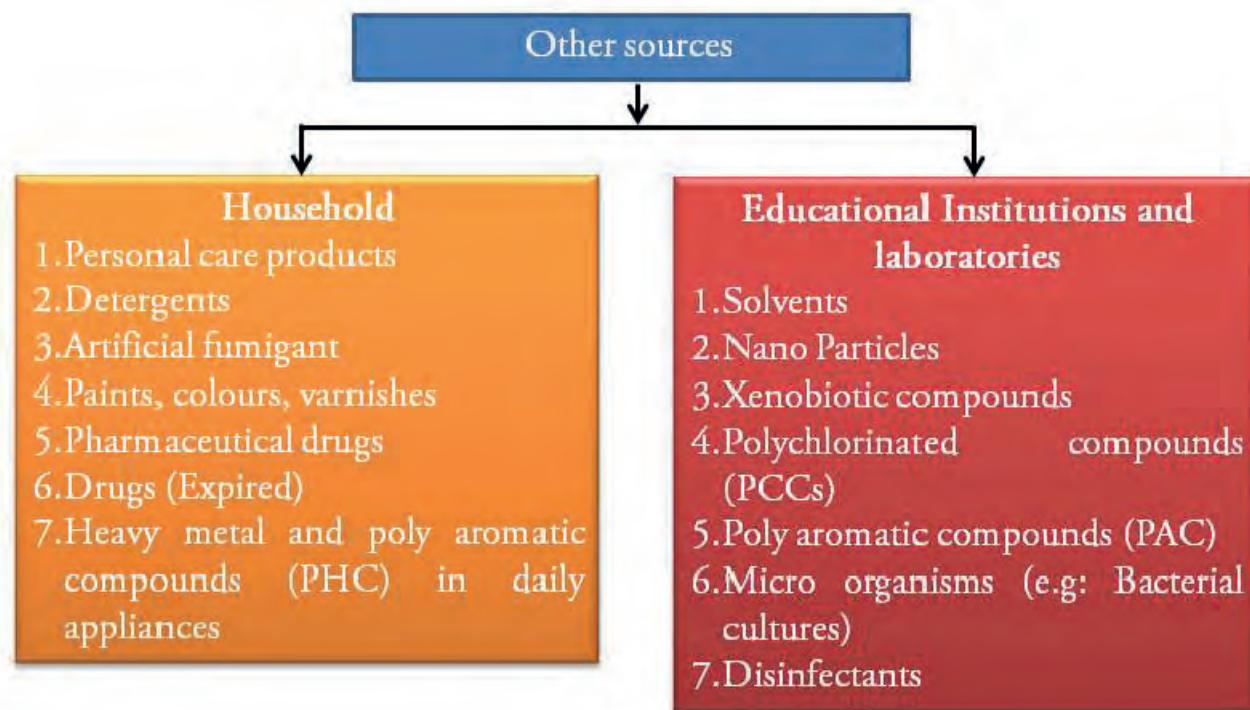


Figure 3. EMPs present in sewage and other wastewaters

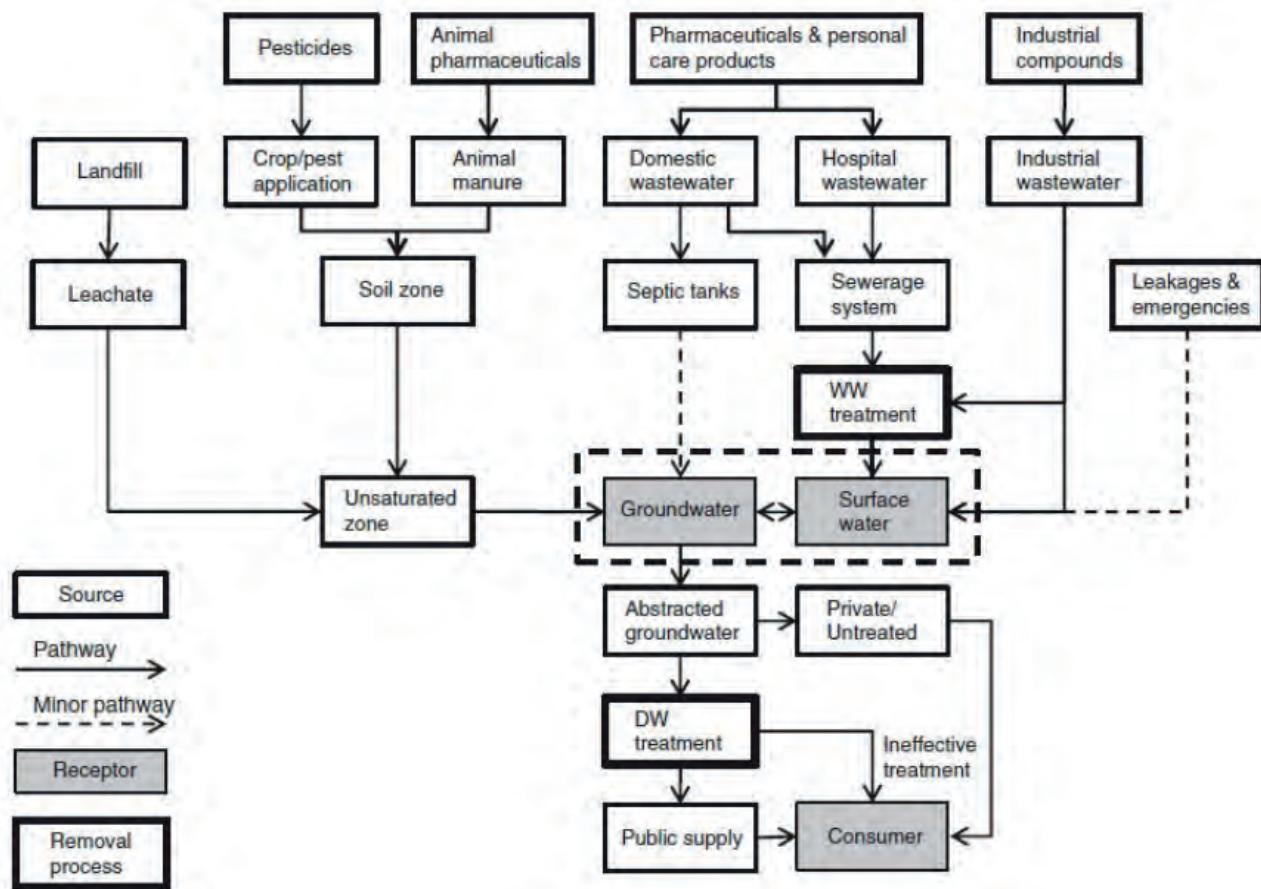


Figure 4. Expected pathway of EMPs from source to receiver.

Compounds	Adverse effects	References
Penicillin, sulfonamides, tetracyclines (Antibiotics)	Resistance among bacterial pathogens that leading to antibiotic-resistant strains, reduction of efficiency of treatment plants by reducing the microbial number	^{74,75}
Caffeine (Stimulant)	Endocrine disruption of goldfish (<i>Carassius auratus</i>)	⁷⁶
Diclofenac (Nonsteroidal anti-inflammatory drug)	Renal lesions and gill alterations of rainbow trout (<i>Oncorhynchus mykiss</i>)	⁷⁷
Triclocarban (Antimicrobial agents)	Growth inhibition of algae (<i>Pseudokirchneriella subcapitata</i>)	⁷⁸
Bisphenol A (BPA) (Endocrine-disrupting compound)	Show estrogenic effect and hormonal disruption leads to increase in risk of breast cancer in humans	^{79,80}
Estrone and 17-β estradiol (steroidal estrogens) and 17-α ethynodiol (synthetic contraceptive) – contained in contraceptive pills	Causes change of sex in fishes (feminization), mimics estrogen hormone	^{69,77}
Preservatives, i.e., parabens (alkyl-p hydroxybenzoate) – used for anti-microbiological preservatives in cosmetics, toiletries, and even foods	Mimic Estrogenic Activity	^{77,81}
Disinfectants/antiseptics, i.e., (triclosan – used in toothpaste, hand soaps, acne cream)	Reduction of the number of microbial in treatment plants by acting as biocides agent.	^{82,83}
Benzalkonium Chloride (Disinfectant)	The observed increase in antibiotic resistance	⁸⁴

Table 1. List of EMPs and Adverse effects.

arthropods), fungicides (activity on the unwanted and harmful fungi species), and herbicides (action of unwanted herbs and bushes). Other categories include rodenticides (mammals like rats, mice, and bats) and bactericides. The pesticides used in India over the year 2012 have been shown in figure 5⁸⁶.

Pesticide usage pattern in India

Currently, India supports 18% of the world population within 2.4% of the land and 4% of the water resources. India planned a green revolution to feed the enormous population and had successes. Indian Agriculture depends on Monsoon rains. Hence, the requirement to procure and maintain stock happens during pre-monsoon and post-monsoon seasons⁸⁸. The requirements of pesticides are regularly checked during a zonal conference on the information of Karif and rabi by individual states. The Government of India will store these pesticide data to plan its agriculture policies for the next financial years. In the figure, 6 data of overall pesticides consumption and consumption per hectare are given from 1992-1993 to 2012-2013. The figure shows that the consumption of pesticides has declined from 72,130 tonnes to 56,090 tonnes. In 2015-2016 and 2016-2017, the consumption of pesticides was 53,719

and 56,215 tonnes, respectively. The consumption of pesticides has been fluctuating because of rainfall, pest infestation, and market availability. The main reason for the reduced use of pesticides is the awareness given by the states for their hazardous nature, and the importance of organic farming has yielded favorable results. And the development of new generation products that have shown to be highly effective at lower quantities is also a reason for the lower use of pesticides⁸⁷⁻⁸⁹.

Though the loss of crops due to pest attacks is high in India that is 15-25% of the produced is lost, the intensity of pesticides consumption is significantly less than 600 g/ha. This number has varied through the years. It has not crossed 1kg/ha. This fluctuation from the year 1992-1993 to 2012-2013 has been shown in the figure. The other countries with high intensity of pesticides consumption are China (14 kg/ha), Japan (11kg/ha), and the USA (4.5 kg/ha), and the world average is 3kg/ha. Indian share of pesticides is about 3% of overall production, used for 16.4 million hectares under cultivation (9% of the total land)⁸⁶.

State-wise use of Pesticides

The variations of pesticides consumption in different sta-

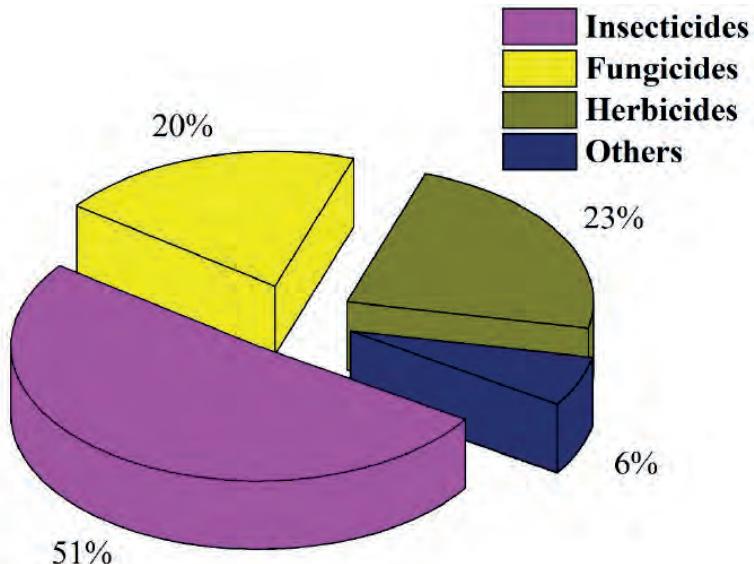


Figure 5. Overall Share of Pesticides Consumed in 2012⁸⁷.

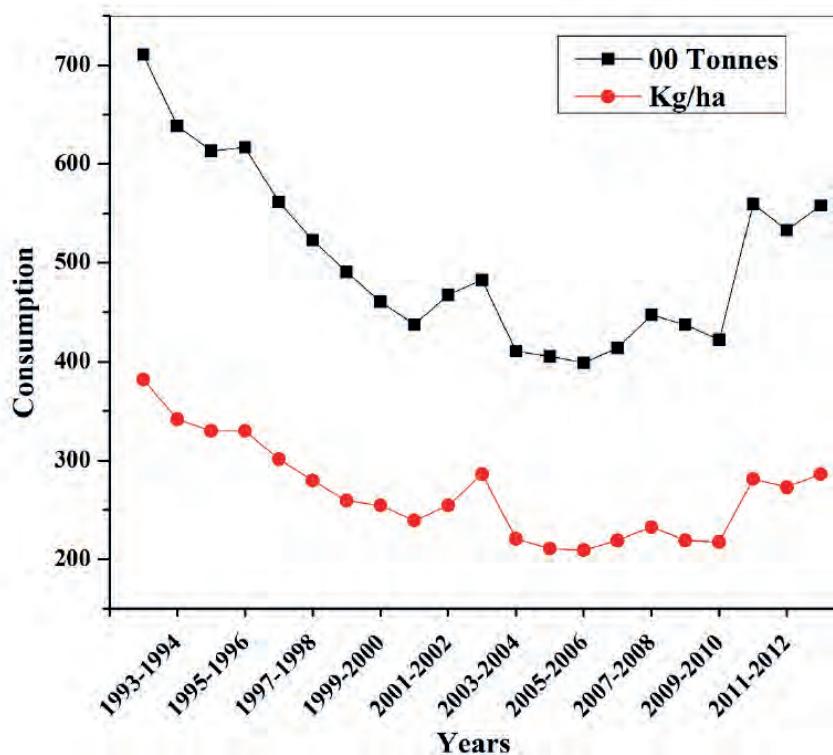


Figure 6. Consumption of pesticides in India (Black overall consumption and red the intensity of consumption)^{87,90}.

tes are shown in figure 7. Maharashtra, Uttar Pradesh, and Punjab are consuming higher pesticides with 13,500 tonnes, 10,200 tonnes, and 5,842 tonnes, respectively. These areas of Uttar Pradesh, Punjab, Haryana are in the plain of perennial rivers; hence annual crops are grown in turn requiring pesticides year long. Maharashtra is a state of cash crops and horticultural crops which require pesticides regularly. The states of South India utilize fewer pesticides due to organic farming that have been popularized in these states. In Kerala and a few parts of Karnataka, the victims of endosulfan pesticides are residing. Assam is the only state using many pesticides where Paddy is grown⁹⁰. Both acute and chronic poisoning of endosulfans in humans is reported extensively. The reports majorly

suggest the toxicity and harmful effects of endosulfans in Kalsangod.

Crops and pesticides

The agricultural field has many single species of crops (Monoculture), increasing the chances of damage. Cash Crops are fruits, vegetables, cotton, plantation crops like coffee, tea, ginger etc., where a significant amount of crop protection chemicals are used. India is a tropical country and is exposed to a vivid type of climate which facilitates the parasites to easily infect and transmit to the whole crop field in no time. Thus, large numbers of these chemicals are put to the field where a part controls the pest, but the remaining part is leached out

to soil or water. In figure 8, the number of pesticides used in various crops is mentioned. About 50 % of the pesticides are used on cotton because on every 15-day cycle; the pesticides are applied to kill and control large numbers of pests such as American bollworm, tobacco caterpillar, jassids etc. About 18% of crop protection chemicals are used on paddy fields. Paddy fields are exposed to insects such as plant hopper, rice bug, stem borer, fungal infections such as root and stem rots etc., and other pests include snails and birds mice, which are potent in reducing the production of crops.

Following paddy, crops of fruit and vegetables are primarily exposed to pests. In the preliminary stages, they include bacterial and fungal infections. During the fruiting season, insects, rodents, and mammals such as squirrels and bats attack the plantation. These are also exposed to a variety of weeds since these plantations are sparsely cultivated. Thus large quantities of chemical compounds are sprayed to the plantations to overcome this menace.

Following them, plantation crops (8%) and cereals and millets (7%) stand in the line. These plantation crops like coffee and tea are cultivated in elevated areas where rainfall is high, and the cereals and millets are cultivated in a dry land with less water availability where both the conditions will increase the risk of pest infestation; chemical compounds are used⁹⁰.

Top pesticides produced in India

In the table above, 3, Insecticides, fungicides, and herbici-

des produced are listed. Since India is a tropical country, most of the crops are infested with arthropods. Therefore, insecticides are majorly produced in the country compared to herbicides and fungicides. Organo-Phosphate pesticides are increasing in number because they are effective. Their residence time is 6-8 months. This gives the farmer an interval between two sprays.

Top pesticides used in India

Since India is a tropical country, most of the crops are infested with arthropods. Therefore, insecticides are majorly produced in the country compared to herbicides and fungicides. Previously Organo-chlorides dominated the pesticide field due to their effectiveness and persistence for longer durations. These were banned after various researches showed the cause of cancer and other endocrine disruption activity. Recently organo-Phosphate pesticides are being replaced, and residence time is hardly 6 months. This gives the farmer an interval between two sprays. In figure 9, major insecticides, fungicides, and herbicides produced in India in 2016 are given.

Comparison of Pesticide Regulation in India with the developed country

According to the Insecticide act 1968, more Chemical formulations and 110 combinations of crop protection chemicals have been registered. This act looked into the registration, manufacture, and distribution of pesticides⁹². Under this act, Central Insecticide Board and Registration Authority (CIBRA)

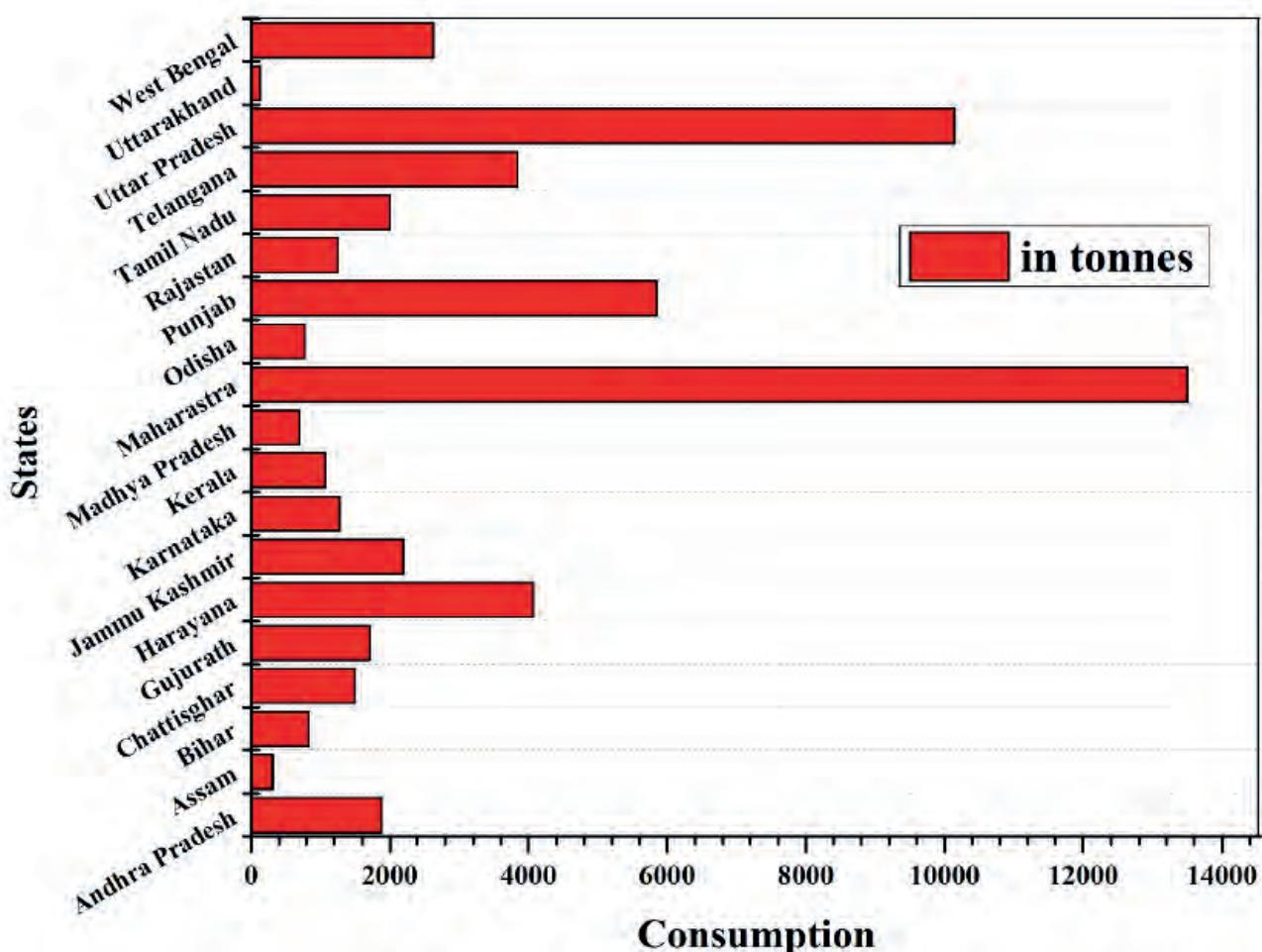
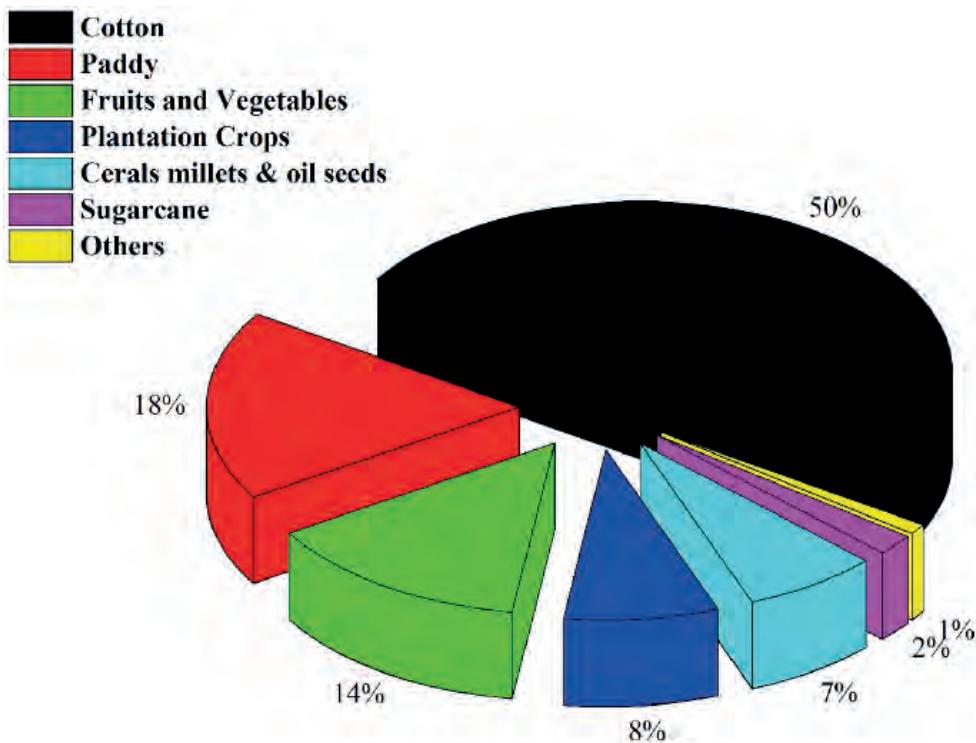


Figure 7. State-wise consumption of pesticides in the year 2016-17^{88,91}



2312

Figure 8. Percentage of Pesticides used on different crops⁸⁶.

Crop Protection Chemicals	Type	Mode of Action	Volume of Production (in Metric tons)	Residues Expected
Cartap	Carbamate	Insecticide	50000	4-(N,N-dimethylamino)-1,2-dithiolane
Acephate	Organophosphate		20000	Methamidophos
Chlorpyriphos	Organophosphate		10000	3,5,6-Trichloro-2-Pyridinol
Clodinofop	pyridyl phenyl ethers	Herbicide	10000	Persistent
Glyphosate	Organophosphate		8000	Aminomethylphosphonic acid
Pretilachlor	Chloroactamide		5000	2-chloro-N-(2,6-dimethylphenyl)-acetamide
Mancozeb	Dithiocarbamate	Fungicide	6000	Ethylenethiourea (ETU)
Copper Oxychloride	Metallic		4000	copper oxalate
Hexaconazole	-		4000	1,2,4-Triazole, 1H-1,2,4-Triazol-1-yl acetic acid

Table 2. Top 3 insecticides, herbicides, and fungicides produced in India along with their residual derivatives in water^{86,90,91}.

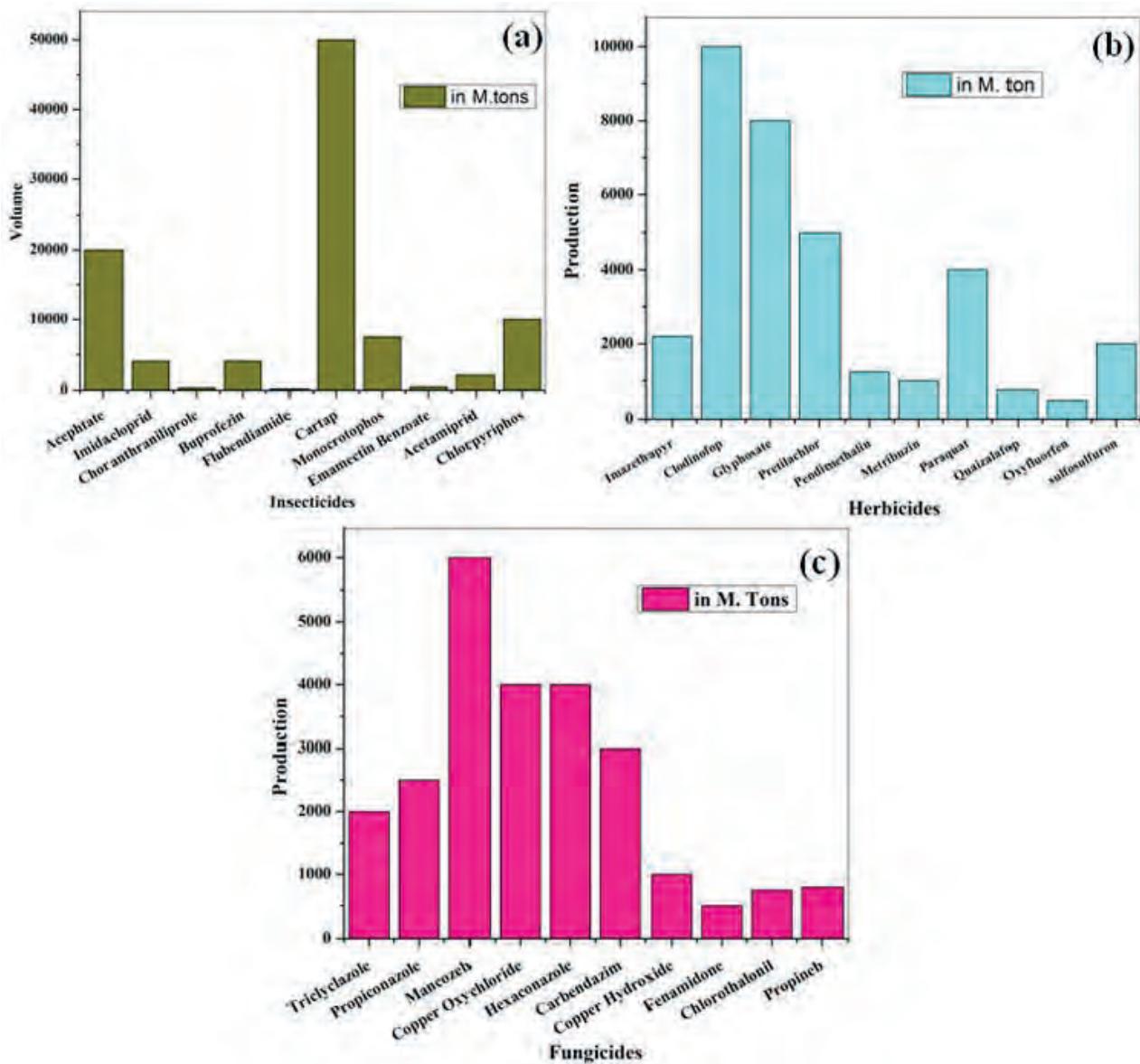


Figure 9. Different pesticides produced in India in 2016 (a. Insecticide, b. Herbicide, c. fungicide).

was set up, which carried out pesticide registration, evaluation related to the product chemistry, bio-efficacy, and toxicity. This regulatory body has banned 70 pesticides from importing and exporting or manufacturing in India^{93,94}.

In 2006, the Food Safety and Security Authority of India (FSSAI) was formed under Food Safety and Security Act to look into the quality of food material. Under this act maximum, residual limits of pesticides were set. To strengthen the law on insecticide, in 2008, a new regulation pesticide management bill was introduced to limit tolerance limits to pesticides according to the Food Safety and Security Act^{89,95,96}.

European Union

European Union has been straightening regulations regarding pesticides and plant protection chemicals. They have been evolving over the years with the current thematic strategy for pesticides. The European Union's health and Consumer Protection Directorate-General (under European Commission) is the regulatory body responsible for looking after the matter related to food health, pesticides and the member states authorize the use or ban of the products in their territories.

The lowest analytical permissible value of pesticides is set to 0.01mg/kg. The E.U. rules do not specify the pesticide or the crop in which they are used. A detailed protocol for sampling and determination of pesticide residue concerning maximum residual level is Gina in SANCO/12571/2013^{89,97,98}.

USA

In the USA, the United States Environmental Protection Agency is in-charge of pesticide registration and regulation. USEPA establishes the permissible risk limits of pesticide exposure, both occasional and occupation and child health on exposure. In most cases, tolerance exemptions are not allowed and are maintained consistently. Other Agency U.S. Department of Agriculture handles the pesticide detection in Agricultural products, meat, and poultry eggs. USFDA takes care of food material produced and imported into the country where it checks for the detection of pesticide and restricts the product to enter^{97,99-103}.

Japan

Japan is the largest consumer of pesticides in the world.

The regulatory body is the Ministry of Health, Labors, and Welfare. Japanese laws were general on food safety regarding poisonous and determined substances and did not specify any pesticides. In 2003, the pesticide limit was approved with a positive list. Under food sanitation law in 2006, maximum residue limits of pesticides were set under a positive list system. After the implementation, all agricultural products and imported products had to follow the procedures as per the positive list system. The highest tolerance limit was set to 0.01 ppm^{42,43,104,105}.

Pesticides found in Indian Waters

At the source, near the agricultural field, their concentration is very high. When the water enters the stream, these compounds dilute, thus reducing the chance of detection, which cannot be treatable. These untreatable, untraceable species cause emerging pollution in drinking water. In Tables 3 and 4, a list of pesticides found in groundwater and surface water in India is given.

Pharmaceutical drugs

Pharmaceutical drugs are the compounds used as medicines in the cure of illness. These come in various types and forms, such as tablets, syrups, injection solutions, powder, inhalers. Illness may be to the man or animal; these are considered as lifesavers. These pharmaceuticals are used broadly and progressively are leading to ecological contamination. Broad-spectrum Antibiotics, legal and illicit drugs, analgesics, steroids, beta-blockers, and others are causing environmental issues¹⁰⁷⁻¹¹⁰. Pharmaceutical pollutants are creating secondary pollutants called antibiotic-resistant microorganisms, which are also the reason for concern. The Active pharmaceutical ingredient (API) and their biotransformation are least studied, are the reason which nobody can anticipate their consequences to the ecosystem¹¹¹⁻¹¹³. These compounds have entered the ecosystem in the past 100 years, but the study on their adverse effects started recently¹¹⁴.

Pharmaceutical drugs were not previously considered pollutants as they were entering the environment in lower concentrations; hence, they were considered pseudo pollutants. Over 160 pharmaceutical drugs are being detected in the water at lower concentrations of ng/L to ppm. Hence their eco-toxicological analysis and impact studies are inadequate to handle them^{112,115}.

In table 5 list of top 20 drugs prescribed in India which includes both generic and bulk drugs. In India, the highest prescribed drugs for lifestyle disease, specifically diabetes, then paracetamol, antibiotics, analgesics, and antiallergic drugs are followed by them¹¹⁶.

In the case of the USA, the condition is different. Paracetamol is a highly prescribed drug followed by Anti hypertension, antacids, antibiotics, and antidiabetics¹¹⁷. We could not find the number of drugs prescribed, but their revenue generated was identified.

India and Pharmaceutical Industry

Indian has a well-established pharmaceutical and healthcare industry. India is the third-largest pharmaceutical supplier, and about 40% of these drugs are generic. Pharmaceutical industries have been developed such that India supplies about 50% of global vaccines. India supplies 80% of the anti-retroviral drugs used for combat acquired immune deficiency syndrome (AIDS).

The Healthcare industry is well spread in India with hospitals, telemedicine, medical tourism, health-related finance

solutions. Both the Government and private health care delivery systems are operational. As of 2015, more than 1.8 lakhs hospitals are operational in India, including government hospitals, private nursing homes, primary and community health care centers (PHCs and CHCs)^{8,17,118,119}.

Along with these credits, India is also the world's most significant consumer of Antibiotics. Carbapenem, a lifesaving antibiotic drug available, is sold on a large scale without prescription. Reports related to illicit prescription drugs are used for narcotics drugs leads to illegal production houses and dumping.

EMPs generated by pharmaceutical industries and hospitals are detected in the Indian environmental samples at higher concentrations. Waters and sediments of Hyderabad have turned into poisons because of pharmaceutical wastes dumped into nearby water bodies and landfills¹²⁰⁻¹²⁴.

Detection of Pharmaceutical drugs in various regions

Northern states

Pharmaceutical drugs have been recorded in various water sources. The river Ganga (Haridwar, Kanpur, Allahabad), Yamuna, and at specific points of groundwater (Uttarakashi, Varanasi, Bhagamarg), wastewater (Kanpur, Ghaziabad, Delhi) STPs and secondary sludge (Delhi, Haridwar), sediment and drinking water (Gomathi river and Delhi), these EMPs were detected. Reports show that traces of drugs like caffeine, ibuprofen, paracetamol are found in River Ganga and Yamuna, along with higher concentrations of amoxicillin (Nondetectable levels to 172.6 ng/L).

Antibiotics such as amoxicillin, Ciprofloxacin, gatifloxacin are detected in STP effluents in Delhi. Anti-inflammatory drugs like ibuprofen, diclofenac, antiepileptic drugs (carbamazepine), and other drugs were found in various samples at Ghaziabad and Lucknow in Uttar Pradesh. At SAS Nagar in Punjab, groundwater is contaminated with diclofenac¹²⁵⁻¹³¹.

Southern states in India

These states are highly polluted with pharmaceutical wastes. The study starts at Patancheru near Hyderabad, turns out to be a point source of APIs. This area has a vast number of pharmaceutical manufacturing situated here made an important center for bulk drug production. High concentrations of APIs were found around the effluent treatment plant, of which 21 APIs are above 1ppm Ciprofloxacin, an antibiotic, was detected with the highest concentrations (31ppm), which is higher than the maximum therapeutic human plasma level. It was found even in sediment, surface, ground, and drinking water around 3 km. The effluent is released into Musi River, and the concentration of Ciprofloxacin is 35.4 - 6058 µg/L in water and 11.4 - 4763.3 ng/l in sediments. Other than antibiotics, antifungal agents, fluconazole was found in the sewage of nearby areas. The famous Hussain Sagar Lake of Hyderabad has norfloxacin, and sulfamethoxazole was found^{53,124,125,128,132-136}.

Other categories of pharmaceutical drugs are detected in South India, such as Udupi, Bangalore, Coimbatore, Chennai landfills. It is reported that 5.1kg of caffeine and 2.1kg ibuprofen are discharged annually to SIP in Udupi¹³⁷⁻¹³⁹.

Eastern and western zones

Few reports found in these regions, indicating gaps in pharmaceutical pollution research in these areas. Most illicit drugs and pharmaceuticals are found in the surface waters of Saidpur and Bener in Bihar. Ibuprofen and amphetamine were

Place	Pesticide	Pesticide detected	Quantification technique
Jaipur, Rajasthan	Heptachlor epoxide,	0.943 mg/kg	GC-ECD
	Heptachlor,	0.736 mg/kg	
	Aldrin	0.336 mg/kg	
Dug wells, Unnao, UP	Σ Aldrin	BDL-1355.2 ng/l	GC-ECD and GC-MS
	Σ Chlordane	BDL-7.2 ng/l	
	Σ Endosulfan	BDL- 54.4 ng/l	
	Σ DDT	BDL-266 ng/l	
	Σ HCH	0.56-2920.9 ng/l	
	Σ Heptachlor	BDL-303.6 ng/l	
Hyderabad, A.P.	Lindane	0.78-1.39 ppb	GC-ECD
	DDT	0.15-0.19 ppb	
	α Endosulfan	1.98-2.86 ppb	
	β Endosulfan	0.30-0.32 ppb	
Hisur, Harayana	Σ HCH	0.41-2.31 ppb	GC ECD and NPD
	Σ DDT	0.05-0.86 ppb	
	Σ Endosulfan	0.02-0.37 ppb	
	Cypermethrin	0.02-0.09 ppb	
	Deltamethrin	0.17-0.06 ppb	
Sonapur Greater Kolkata WB	Aldrin/dieldrin	0.01-0.03 ppb	GLC
	Dicifol	0.02-0.03 ppb	
Nanded, Maharashtra	Endosulfan	1.50-4.00 ppb	GC-ECD
	Heptachlor	0.00-0.03 ppb	
	Aldrin/dieldrin	0.01-0.04 ppb	
	HCB	1.00-2.00 ppb	
	DDT	2.00-4.00 ppb	
Bore wells of Delhi region, Delhi	α -HCH	0.003-0.005 ppb	GC ECD and GC MS
	β -HCH	0.008-0.037 ppb	
	γ -HCH	0.007-0.014 ppb	
	δ -HCH	0.013-0.039 ppb	
	Heptachlor	0.028-0.032 ppb	
	Aldrin	0.011-0.034 ppb	
	α Endosulfan	0.034 ppb	
	Heptachlor epoxide,	0.016-0.026 ppb	
	dieldrin	0.009-0.058 ppb	
	DDE	0.018-0.046 ppb	
	Endrin	0.029-0.054 ppb	
	β Endosulfan	0.031-0.060 ppb	
	DDD	0.024-0.055 ppb	
	Endosulfan Sulfate	0.036-0.057 ppb	
	DDT	0.068-0.093 ppb	
	Methoxychlor	0.066-0.094 ppb	

Table 3. Pesticide found in Ground water and their analytical techniques¹⁰⁶.

Lake/River/ Place	Pesticides Detected	Detected concentration	Detection method
Unnao, UP	Aldrin	BDL-1.88 ppb	GC ECD and GC MS
	Σ Chlordane	BDL-0.04 ppb	
	Σ DDT	BDL-0.23 ppb	
	Σ HCH	1.88-1.95 ppb	
	Σ Heptachlor	BDL-0.11 ppb	
Greater Kolkata, W.B.	Methoxychlor	2.63-3.72 ppb	GLC
	Lindane	0.01-0.05 ppb	
	HCH	0.01-9.90 ppb	
	Aldrin/dieldrin	0.01-0.90 ppb	
	Dicofol	0.01-14.03 ppb	
Baratagi lake, Bijapur, Karnataka	DDT	0.01-1.40 ppb	GC ECD and GC MS
	Endosulfan	0.00025 mg/kg	
	Chlorpyrifos ethyl	0.0002 mg/kg	
Hisar, Haryana	4-bromo-2-chlorophenol	0.0003 mg/kg	GC ECD
	Σ DDT	50.1-332.2 ng/l	
	Σ HCH	2.3-560.6 ng/l	
Khindsi lake, Bhandara, Maharashtra	Σ Endosulfan	BDL-206.3 ng/l	GC ECD and GC MS
	α -HCH	0.019-0.120 ppb	
Tamiraparani river basin (TN)	β -HCH	0.031-0.061 ppb	GC MS
	Σ HCH	0.01 ng/l	
	Σ DDT	0.01-0.72 ng/l	
	Heptachlor	0.06-2.1 ng/l	
	Aldrin	0.02-1.5 ng/l	
	Trans-chlordanne	0.01 ng/l	
	Cis-chlordanne	0.01 ng/l	
	Dieldrin	0.03-7.5 ng/l	
Rishikesh, Ganga river, Uttarakhand	Endrin	0.02-58.0 ng/l	GC ECD and GC MS
	Mirex	0.01-0.47 ng/l	
	Σ HCH	5.54 ng/l	
	Σ DDT	1.01 ng/l	
	Σ Endosulfan	0.92 ng/l	
Cauvery river, Karnataka	Σ Aldrin	1.89 ng/l	GC ECD
	Σ Heptachlor	0.32 ng/l	
Other rivers	α -HCH	0.87 ppb	
	β -HCH	0.56 ppb	
	γ -HCH	0.93 ppb	
	δ -HCH	0.78 ppb	
	p, p'-DDT	0.64 ppb	
	p, p'-DDE	0.96 ppb	
Other rivers	p, p'-DDD	0.53 ppb	
	Endosulfan	0.84 ppb	

Table 4. Pesticides residues in Surface water¹⁰⁶.

Sl. No.	Brand Name	Active Pharmaceutical Ingredient (API)	Class of Pharmaceutical	Company	Moving Annual Turnover (in Rs. Cr)
1.	Mixtard	Soluble Insulin	Anti-Diabetic	Novo Nordisk, India	512
2.	Glycomet	Metformin	Anti-Diabetic	USV	415
3.	Spasmo Proxyvon Plus	Dicyclomine, Paracetamol, Tramadol	Anti-Cholinergic and Analgesic	Wockhardt	377
4.	Lantus	Insulin glargine	Anti-Diabetic	Sanofi India	341
5.	Galvus	Metformin-Vildagliptin	Anti-Diabetic	Novartis India	328
6.	Liv52	Natural Ingredients	Hepato Protective	Himalayan Grugs	296
7.	Janumet	Sitagliptin, Metformin	Anti-Diabetic	MSD Pharma	285
8.	Augmentin	Amoxicillin, Clavulanate potassium	Anti-Biotics	GSK	283
9.	Clavam	Amoxicillin, Clavulanic Acid	Anti-Biotics	Alkem Labs	281
10.	Monocef	Ceftriaxone	Anti-Biotics	Aristo Pharma	277
11.	Voveran	Diclofenac	Analgesic and Anti-inflammatory	Novartis India	266
12.	Novomix	Protamine-crystallised Insulin	Anti-Diabetic	Novo Nordisk India	263
13.	Betadine	Povidone-iodine	Antiseptic	Win Medicare	253
14.	PAN	Domperidone, Pantoprazole Sodium Sesquihydrate	Anti-ulcer	Alkem Lab	250
15.	Volini	Diclofenac Diethylammonium Topical, Menthol Topical, Methyl Salicylate Topical, Oleum Lini Topical	Analgesic and Anti-inflammatory	Ranbaxy	248
16.	Synflorix	Pneumococcal Polysaccharide Conjugate	Vaccines	GSK	237

Table 5. Top pharmaceutical drugs produced in India with APIs and category¹¹⁷.

17.	Phensedyl Cough Linctus	Promethazine, Codeine, Ephedrine	-	Abbott	228
18.	Aciloc	Ranitidine	Anti-ulcer	Cadila Pharma	225
19.	Foracort	Formoterol Fumarate, Budesonide	Anti-Allergic	Cipla	224
20.	Becosules	Multi Vitamin	-	Pfizer India	223

Table 5. Top pharmaceutical drugs produced in India with APIs and category 118

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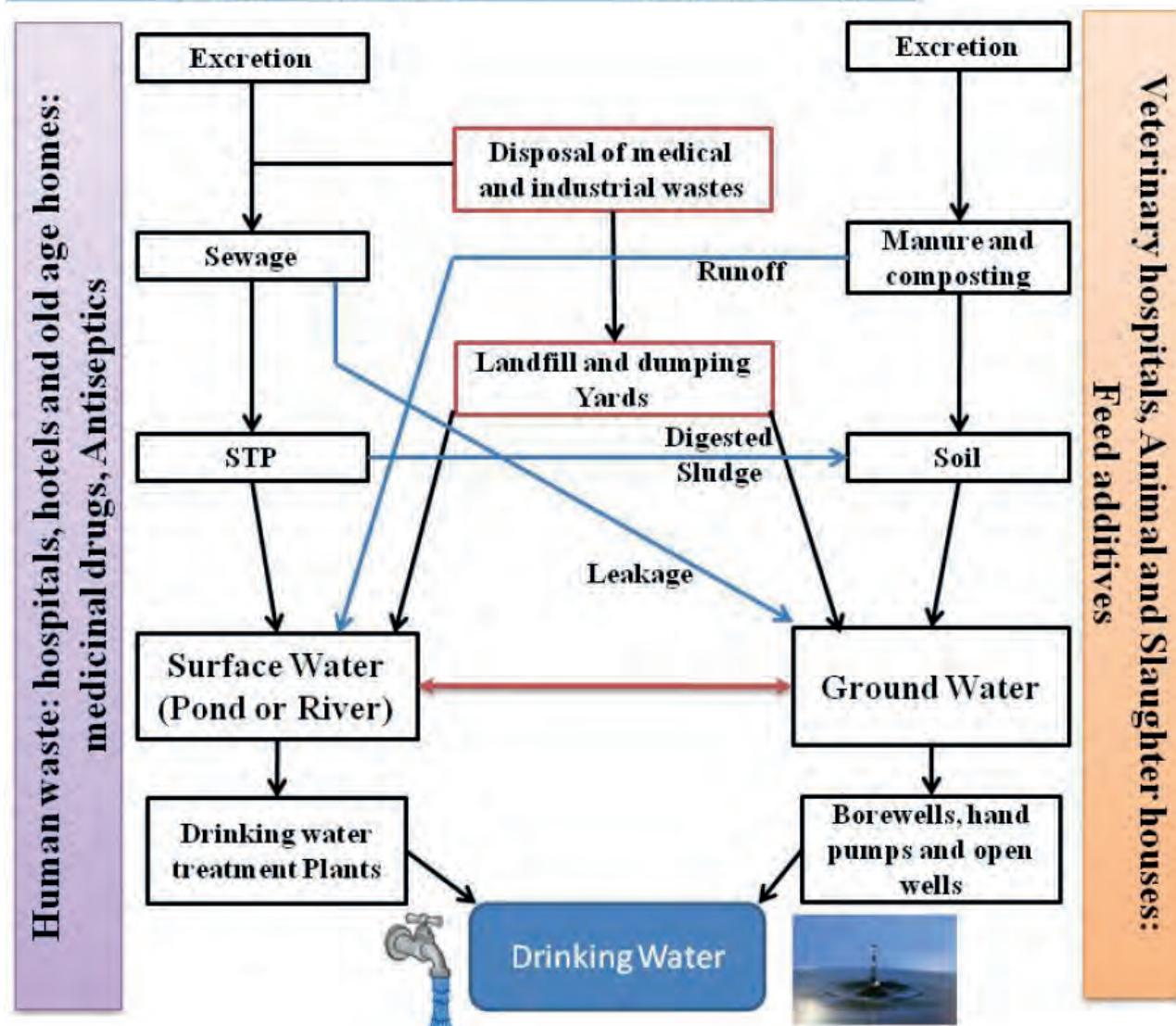


Figure 10. Pathway of transmission of pharmaceutical drugs..

found in the effluent (1130 ng/L) and sludge samples (1230ng/L)¹⁴⁷⁻¹⁴⁹. In western parts of Maharashtra's Nagpur, antibiotics and antidepressants were found in Nag and Pili River. More than 12 APIs are detected in 2 wastewater treatment plants of one of the largest metropolitan areas of western India. The concentration of pharmaceutical drugs varies in treatment plants because of zonal planning, seasonal diseases, medicinal patterns, lifestyle-related disorders, and rainfall^{140,141}.

Antibiotic use in India

India is the largest consumer of antibiotics. From the past decade, 62% of usage has been an increase of antibiotics. In-

dia has consumed 12.6×10^9 units of antibiotics during the past decade, and Per capita antibiotics consumption was 10.7 units¹⁴². The reason is poor public health systems, the high rate of tropical infectious diseases and the availability of antibiotics at cheaper rates. Indian health care system depends on doctors who routinely receive remuneration on prescribing antibiotics, and another reason is that patients are not willing to give time for the disease to cure by the body's innate immunity¹⁴³⁻¹⁴⁶.

Antibiotics in animal husbandry have also resulted in more excellent pathogens because of increased demand for poultry, meat, and related products. According to a report, the

demand for poultry will be raised by 312% by 2030. To cater to that demand, this sector is expected to be investing in Antimicrobial chemical drugs to nearly \$ 1.2billion.

Dairy Industry has developed two-fold in India over the past 2 decades because of the increase in demand for milk and dairy-related products. Milk is considered a complete food because of its protein and fat contents. Nowadays, antibiotics are being used frequently in lactating animals to promote animal health, treatment and control of the infectious disease. Mastitis is a significant disease in dairy animals where the treatment involves antibiotics to mammary glands. Other reasons include using dosage deviations from recommended prescriptions, using antibiotics as preservatives, maintaining incorrect cleaning orders, and veterinary errors¹⁴⁷⁻¹⁴⁹.

Consequences of Excessive use of Antibiotics

Antibiotics are used to cure diseases that are caused by microorganisms, especially bacteria. Bacteria undergo fast mutation to resist the activity of antibiotic medication is called Antibiotic resistance (A.R.) organisms. In some instances, bacteria that resist multiple antibiotics are called multi-drug-resistant (MDR). If bacteria survive in an extensive antimicrobial environment, they are called superbugs or extensive drug-resistant organisms (XDRs). For bacteria with Antibiotic resistance activity, *Staphylococcus aureus*, *Enterococcus* sp., and Multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) are a few examples¹⁴⁹.

The evolution of antibiotic resistance (A.R.) and expression of AR Gene in nature has led to significant public health problems around the Global¹⁵⁰⁻¹⁵². If the A.R. gene is integrated into a gene transmission element and can spread to non-resistant species, turn them into Antibiotic-resistant organisms (ARO); for this character, these organisms are making their names in emerging pollutants (EMPs) list¹⁵³⁻¹⁵⁵.

This crisis has led to the differential use of anti-microbial drugs in therapeutic and non-therapeutic purposes at clinical and non-clinical settings. Previously researches were focused on the emergence of these bacteria in hospitals and dumping sites of pharmaceutical wastes. From the last 5 years focus of research shifted to the overall environment, which acts as a site for antimicrobial resistance evolutions. Researchers have found the various bacteria with A.R. genes in environmental samples in natural ecosystems such as sediments, soils, ground, surface, marine and drinking water¹⁵⁶⁻¹⁶⁵.

Personal care products (PCPs)

According to E.U. Regulation 1223/2009 (article 2), "cosmetic product means any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odors"¹⁶⁶.

Substances such as pharmaceuticals, personal care and other endocrine-disrupting compounds products enter the environment from different sources such as:

- (i) Effluents from wastewater treatment plants
- (ii) Leakage from septic tanks or landfill sites
- (iii) Surface water run-off

- (iv) Direct discharge into waters.

These species can also be chemically degraded by microorganisms or by U.V. light action¹⁶⁷.

Personal care products can reach the wastewater system through multiple routes. Products such as shampoos, body washes, and toothpaste are directly washed down the drain during and after use. Products like cosmetics and hand lotions can be washed down the drain as well during a routine. The chemicals that can penetrate the body and are then excreted can also enter the wastewater system through toilet¹⁶⁸.

The environmental safety of household products is assessed based on the ecological properties of their many components. Two fundamental issues determine their environmental safety: the environmental fate and potential effects on the environment. The environmental fate of chemical substances depends mainly on the physicochemical properties, such as water solubility, adsorption behavior, and volatility, and on their degradability, which is overwhelmingly affected by microorganisms (biological degradation) present in sewage treatment plants, surface waters, and soils. These fate-relevant properties control the distribution of a chemical in the environmental compartments (water, soil, air) and its final removal by degradation processes.

The second assessment aspect, the potential impact of a chemical on the organisms living in the environmental compartments, also depends on substance-specific properties, i.e., ecotoxicity. Data from standardized tests on representative organisms are required by European chemical legislation¹⁶⁹.

Disinfectants

Triclosan (TCS) and triclocarban (TCC) are diphenyl ethers used as anti-microbials in soaps, deodorants, skin creams, toothpaste and plastics. TCS and TCC are among the top 10 most commonly detected organic wastewater compounds for frequency and concentration. A study monitoring 95 compounds in surface water throughout the United States found TCS to be one of the most frequently detected compounds with surface water concentrations as high as 2.3 ppb TCC has been used in PCPs since 1957 and has been observed in surface water at concentrations up to 6.75 ppb. It is believed that TCC occurs as frequently in WWTP effluent and surface water as TCS; though, until 2004, TCC could not be detected at low levels (ppb). However, TCC has been detected at higher concentrations and more frequently in WWTP effluent and surface water than TCS or M-TCS over the last 10 years. Acute toxicity of TCS and biphenylol has been examined in invertebrates, fish, amphibians, algae, and plants^{170,171}.

Fragrances

The most used fragrances are synthetic musks. Synthetic musks are used in a wide range of products, including deodorants, soaps, and detergents. The most commonly used nitro musks are musk xylene (MX) and musk ketone (M.K.), whereas musk ambrette (M.A.), musk moskene (MM), and musk tibetene (M.T.) are used less frequently. Nitro musks however are slowly being phased out due to their environmental persistence and potential toxicity to aquatic species. Polycyclic musks are currently used in higher quantities than nitro musks with celestolide (ABDI), galaxolide (HHCB) and toxalide (AHTN) used most commonly and traseloid (ATII), phantolide (AHMI), and cashmeran (DPMI) used less often. HHCB and AHTN production alone has been estimated at 1 million pounds per year and has thus been placed on the High Production Volume List

by the USEPA (the United States Environmental Protection Agency). Nitro and polycyclic musks are water-soluble, but high octanol-water coefficients ($\log K_{ow} = 3.8$ for M.K. and 5.4–5.9 for polycyclic musks) indicate high potential for bioaccumulation in aquatic species^{75,170–172}.

Insect repellents

N, N-diethyl-m-toluamide (DEET) is the most common active ingredient in insect repellents and is routinely detected in surface waters. DEET is relatively persistent in the aquatic environment, but unlike many other PCPs (i.e., fragrances and U.V. filters) DEET has a low BCF and is likely not accumulated into aquatic organisms. DEET has been regularly detected in the effluent (95% of analyzed samples) and surface water (65% of all analyzed samples) with median concentrations of approximately 0.2 ppb and 55 ppb, respectively. The only other insect repellent detected in WWTP effluent or surface water is 1,4-dichlorobenzene. 1,4-dichlorobenzene has been detected in surface water (40% of surface water screened), receiving significant inputs of WWTP effluent at concentrations up to 0.28 ppb^{170,173}.

Preservatives

Parabens (alkyl-p-hydroxybenzoates) are antimicrobial preservatives used in cosmetics, toiletries, pharmaceuticals, and food. There are currently seven different types of parabens in use (benzyl, butyl, ethyl, isobutyl, isopropyl, methyl, and propyl). Only a handful of studies have examined paraben concentrations in WWTP and surface water. The most significant concentrations of parabens have been identified in surface water with concentrations ranging from 15 to 400 ppb depending on paraben, whereas effluent had lower concentrations ranging from 50 to 85 ppb¹⁷².

U.V. filters

U.V. filters are used in sunscreen products and cosmetics to protect from U.V. radiation and can be either organic (absorb U.V. radiation, e.g. methyl benzylidene camphor) or inorganic micropigments (reflect U.V. radiation, e.g. ZnO, TiO2). U.V. filters are well known to bioaccumulate, and recent studies have also indicated the potential for estrogenic activity. In vitro assays using fish, MCF-7 cell lines indicate five UV-A and UV-B sunscreens (BP3, homosalate (HMS), 4MBC, octyl-methoxy-cinnamate and octyl-dimethyl-PABA) have the potential to cause estrogenic effects¹⁷³.

Additional compounds

Three additional PCPs have been identified in surface water in the U.S. by United States Geologic Survey researchers. The fixative benzophenone was detected most frequently (67.5% of samples in one study), whereas the flavorant menthol was detected at the highest concentrations (1.3 ppb). The other compound detected in the surface water is methyl salicylate (wintergreen flavoring and liniment), although it has only been detected at low concentrations and in a few environmental samples⁷⁵.

Conclusions

Emerging micropollutants are the problems of present living conditions and lifestyle changes of human beings. The EMPs generated from every human activity like agriculture, healthcare, and sanitation which most of them ends them in water through the sewer lines or at water bodies. Fortunately,

the distribution of EMPs through the air is negligible except during the aerial dispersions. The detections of these compounds are significantly less or available in smaller quantity makes them harder to regulate.

Pesticides and other crop protection chemicals are used extensively in agricultural practices and gardening, entering the soil and reaching water through run-off. Air dispersion of pesticides are not much into concern as most of them disintegrate due to sunlight and eventually settle on to the soil.

Health care and hygienic products from hospitals or residential areas are directly entering into sewers, like APIs antibiotics or endocrine disrupting substances that show amplified effects even at lower concentrations. These compounds are usually used in an unregulated manner and disposed of without treatment increases the risk of exposure.

Over the few years, nations have been working on identifying and regulating these EMPs. Countries in Europe and the USA are training their human resources and capacities for identifying and handling these pollutants. Developing countries like India are facing the problem of EMPs in various parts and are coming forward to create legislation to reduce the generation of EMPs.

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REVIEW / ARTÍCULO DE REVISIÓN

Composition, hydrology, and health benefits of Zamzam water

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2326

Abstract: Since before, many religions have used Zamzam water because they used it for treatment and other spiritual activities. This study reviews the composition, hydrology, and the effect of Zamzam water on human health. Zamzam water differs from regular water. Mineral deposits show it is alkaline and biologically has no biotic growth, and does not show toxicity signs. Zamzam is well-focused based on the expenditure of Makah hill. Zamzam well has been used for about 4000 years. Zamzam water is used for the treatment of many diseases. The data on Zamzam water composition, hydrology, and health benefits have been narratively reviewed. Future research is needed to investigate the other benefits and uses of Zamzam water on human health as antioxidants, antimicrobial, and its effects on cancer patients.

Key words: Zamzam water, antioxidants, antimicrobial, spiritual activities.

Introduction

Water is secured for human society from either surface water or groundwater surface water includes all forms of water bodies above the earth's surface; such as streams, rivers, lakes, wetlands, and oceans, while groundwater refers to water that seeps through the layers of the earth consisting of gravel, sand, or crushed rocks such as limestone, which contain materials with pores and spaces between their grains that make them permeable, and the speed at which groundwater flows depends on the size of the voids and pores in the earth layers¹. All living organisms need water to survive, starting from simple organisms to more complex organisms such as humans, as water is used as a carrier and solvent, as it works to dissolve all the vitamins and essential nutrients from food and transfer them to cells. These organisms vary in how much they need water to survive, as bacteria, for example, need smaller amounts of water to survive compared to other organisms, and no organism can survive without getting water. Humans can live with no food for 30 days, but they can live with no water for only seven days. Water is a suitable medium for mixing organic compounds, and water is one of the main factors that helped form the first life forms on earth; in addition, water is also necessary for protecting the earth from sunlight. Water is used to keep plants alive, such as those found in gardens and parks. Water is necessary for the production of various crops, and it is also necessary for the manufacture of many products. Zamzam water is natural alkaline water that contains many essential mineral salts for the body, supporting the body with energy; it also helps to neutralize its pH, which protects it from disease and makes it a potent antioxidant agent². Zamzam well is located in the Kingdom of Saudi Arabia, in the holy city of Makkah. It is filtered by micro-filters and sterilized by ultraviolet light before it is disseminated to the

consumers. Microbiological investigations set that there were no signs of microbial progress in the water gained from the Zamzam well³.

Background

Many religions have used Zamzam water since before that belief was used for treatment and other spiritual activities. Many Muslims considered that Zamzam water is delightfully deified and can fulfill together starvation and thirstiness, at the same time to treat illness. Pilgrims sort a durable activity to take this water throughout their hajj, besides individuals alive neighboring, might take the water regularly². Zamzam water has special optical measures that differ from bottled drinking and distilled water, and it differs from standard water in minerals qualities⁴. Many or even millions of pilgrims drink it annually during the Hajj season and take it to their countries. Many Muslims and pilgrims drink Zamzam water for medicinal or religious use².

Source of Zamzam water

Zamzam water was found in Makkah AlMukarramah, kingdom of Saudi Arabia (KSA). The well of Zamzam (Figure 1) was hand excavated, and it has a depth of about 30.5 meters and a diameter from 1.08 to 2.66 meters⁵. The Arab historians said Zamzam was well used for about 4000 years. Allah's mercy sent Gabriel to rub off the earth to make the spring when Hajar

Prophet Abraham's wife and her son Ismail went after a water search with dying thirst. When he found the spring, Hajar surrounded it with stones and sand as it ran out⁶. Zamzam water would have been a stream flowing on the surface of the earth (Sahih Bukhari). Muslims contemplate that the Zamzam water well seemed to Hagar, mother of Ismail Son of Abraham.

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Figure 1. Historic Zamzam well mouthpiece.

As she was pointed with gloom for water for her infant and other Muslim historians say Ismail rub the earth with his heel, and after that, Zamzam water sprang up².

Location and hydrogeology of the Zamzam well

In hydrogeological expression, the well located in Ibrahim hill, which surrounds Makkah and pipes groundwater of the hill silt to a much lower range and from the undeveloped fresh bedrock 13.5 m of the upper well dig in the sandy build-up of the Ibrahim valley and the lower 17 m in the underlying diorite bedrock. There is a thinner layer (0.5 m) of porous weathered rock between sandy alluvium and diorite bedrock. The silt part of the wall stuff with rock structure, except for the topmost 1.0 m, has a fortified solid hoop. The weathered stone part is filled with stone and supply the main water entree into the well⁷.

The Zamzam well was drilled manually long ago, to a depth of 30.5 meters and a diameter ranging from 1.08 to 2.66 meters. Hydrogeological, the well is located inside Wadi Ibrahim, which passes through the holy city of Makkah and extracts water from it. The well of Zamzam is located in a room in the basement, and the well is surrounded by glass panels that allow it to be seen.

They used ropes and buckets to draw water from inside the well in the past, but now electric pumps are used. Visitors are not allowed to pass into the well room. Cold Zamzam water fountains and drinking bottles are provided in the service area outside the room. Upon the recent expansion of the Haram, this area was covered and is no longer available to pilgrims. Instead, cold Zamzam fountains and distribution vessels are now located in the vicinity of the Tawaf area⁸.

Quality parameters of water compared to Zamzam water

Physical parameters

Zamzam water has many characteristics that make it unique and different from other wells, as the Zamzam well has not been contaminated throughout history. However, it still supplies everyone who visits the Holy Mosque in Mecca with water. It is abundant enough for all visitors; the follow-ups have shown that on the seventh of Dhu al-Hijjah for several consecutive years, approximately ten thousand cubic meters per hour are withdrawn from it. One of its characteristics is the ability to drink it over time and with different visitors' domiciles. There was no complaint from any visitor that his health was affected after drinking it, and no biological growth was proven in the well, such as algae, compared with other wells². Comparing Zamzam water with other water, numerous scientific studies have proven that, Zamzam water has chemical properties that distinguish it from others, where the percentage of mineral salts in it reaches 2000 mg/liter, while the treated mineral water ranges from 150 to 350 mg/liter. Its percentage is 200 mg/liter. It is also considered digestive soft water as it contains 366 mg of bicarbonate per liter, and the normal water is carbonated if it includes more than 357 mg, and it should be noted that mineral water helps treat many diseases⁹.

The properties of magnetized water are close to the properties of Zamzam water, and this concludes that Zamzam water may have been magnetized in origin, which acquired these unique properties, and this may be due to the nature of the strong rocky stones surrounding the well of Zamzam, on the other side, as is common the majority of the physical and chemical

properties of the two water molecules are responsible for the covalent bonds between the two atoms. Therefore, this arrangement of hydrogen and oxygen atoms, where they form angle magnitudes 104° angle in the two molecules of Zamzam water in a particular form that differs entirely from what is blind in normal water, which makes Zamzam water consistently superior in physical properties to the rest of the magnetized models of water. The most important conclusion is that Zamzam water has important optical properties that it cannot characterize, even if it is magnetized. This means that Zamzam water can be used as an optical fluid and has optical properties⁹.

The scientist Emoto, M.¹⁰ showed that Zamzam water has unique scientific properties not found in normal water through many studies and analyses conducted on the water. He used his nanotechnology research; he found that it could not change Zamzam's water properties. The world was also surprised at adding one drop of Zamzam water to a thousand drops of regular water makes the latter acquire the blessed Zamzam water characteristics Emoto¹⁰ explained that Zamzam water is unique. Its crystals do not resemble any water in the world, regardless of its source, the resulting crystals after refining give beautiful shapes, and when water crystals were exposed to Basmala (In the name of God, the Most Gracious, the Most Merciful) through reading, it made a tremendous effect on it and formed stunning crystals in the formation of water. When the Holy Qur'an was recited on the water, it is crystals formed with a symbolic design, in serenity and purity. That might be due to the different geometric shapes in which the water crystals are formed on which the Qur'an or supplication was recited form vibrations resulting from reading in the form of a picture of energy, indicating that the memory of water is a form of the potential energy that enables it to hear, see and feel and emotion and storing information, transmitting it and being affected by it. In addition to its effect on strengthening human immunity and possibly treating organic and psychological diseases as well. Emoto¹⁰ also reported that every word uttered at any point of Zamzam water makes it take a particular shape when it is frozen at high speed when it turns into frozen water crystals under magnification.

Emoto¹⁰ captured the expression of water and developed a technique for photographing the newly formed crystals from frozen water samples using a mighty microscope in a cold room. He indicated that controlling the interaction of the molecules involved in the reaction and directing these molecules through the production of a specific substance, this type of response is known as molecular fabrication and placing the atoms during the reaction in their right or appropriate place¹¹.

Zamzam water bears the name water, while it differs radically from the water compounds, as all waters are acidic while Zamzam water is alkaline. Drinking it frequently gives the human body a strong immunity against viruses because it does not live in an alkaline environment. That is why pilgrims used to drink Zamzam water as much as they could to keep not being infected with the pilgrims' transmitted diseases. Many chemical analyses were performed to find out that Zamzam water contains high amounts of Ca, Mg, and other minerals. Also, other waters contain between 130-160 mg/liter of mineral salts; in contrast, the total mineral salts in Zamzam water are 2000 mg/liters, and for this reason, Zamzam water refreshes exhausted pilgrims².

Chemical parameters

Shomar² examined thirty Zamzam water samples using four tools IC, ICP-OES, ICPMS, and the HGAAS. The results in-

dicated that the water quality did not change for 24 months; the results showed that Zamzam water was alkaline (average pH was 8), the average NO₃ concentrations showed values three times higher than the standards of the World Health Organization.

While the mean calcium and potassium were 95 and 50 mg L⁻¹, respectively. A progressive study of multi-elemental and hydrochemical study of Zamzam water was carried out by (5), who used inductive couple plasma and other available traditional methods. The concentration of thirty-four elements reported, including Ca, Mg, Na, and Cl, was higher in Zamzam water than in natural water. The concentrations of Sb, Be, Bi, Br, Co, I, and Mo in Zamzam water were more minor than 0.01 ppm. There were a bit of Cr, Mn, and Ti noticed in Zamzam water.

Arsenic, cadmium, lead, and selenium, as harmful elements, were found below the risk level for human drinking water⁵.

Biological parameters

Zamzam water demand has always been universal, as it does not change the color, taste, smell, as usually occurs in other waters. That makes water stodgy due to the expansion of moss for most alterations in taste and odor. However, many studies reported no mark of microbial growth in Zamzam water and never been chemically conserved or chlorinated, as in normal water⁵. Average growth and flora are usually present in most water resources. Nanotechnology was used to investigate Zamzam water; the study revealed that when mixing one drop of Zamzam water with 1000 drops of normal water, the average water will have the quality of Zamzam water^{5,12}. Mashaat stated that Zamzam water has no signs of biotic growth. Results showed that *E. coli* had not polluted Zamzam water, and the entire settlement sums fall within the accepted parameters for all samples¹³. Muslims attend the Zamzam well, from all their ethnic backgrounds, drink, search for restoration, do their ablution, and clean. This means that a lot of this water goes to the sewage system. As a result, this enormous amount of water needs to be invested in farming, as some plants can be planted either as nutrient crops or as valuable street trees, especially in holy places such as Arafat and Mina place¹⁴. A study done by Alsokari¹⁵ investigated the impact of Zamzam water on growth and biochemical parameters in Lentils compared to tap water (as control). The study revealed that the irrigation of lentil seedlings with various quantities of Zamzam water significantly increased the germination level and augmented the content of protein and RNA. In general, Zamzam water has beneficial impacts on plant irrigation near Makkah lands due to the form of nutrient in the water and the proportion of this nutrient that has served as synergistic agents for useful nutrients or as antagonistic agents for harmful elements to postpone their harmful effects.

Antioxidants activity

Many diseases in animal and human bodies are related to oxidative stress, so antioxidants are essential for animals and human health¹⁶. The antioxidant capacity of animal bodies was reinforced by water¹⁷. Alkaline water is considered to be with high antioxidant activity, which is due to it is alkalinity¹⁸, the anti-radiation effect of Zamzam water on mice bone marrow after being subjected to gamma radiation was investigated by (18) and resulted that, Zamzam as alkaline water reduced the clastogenic and cytotoxic effects, this attributable to the previous believes that the natural antioxidant activity of the natural radioprotectors has less toxicity and no side effects than the chemical.

(19) conducted his study on the effect of Zamzam water on diabetic type 2 patients, according to their oxidant-antioxidant status, glycaemic control, and lipid profile; his results revealed that drinking Zamzam water effectively increased antioxidant efficiency and improved wellness. Zamzam as alkaline water was investigated by (20) in terms of antioxidant mechanisms; the experiment was conducted on two groups the first contains normal rats as control and the second group was subjected to a high dose of gentamicin then Zamzam water was given to both for 21 days and demonstrated that Zamzam water does not show any sign of toxicity in normal rats and total antioxidant in rats stressed with gentamicin overdose. Investigation of Zamzam water as a potential antioxidant agent was conducted by (21) in terms of decreasing rats' liver toxicity caused by carbon tetrachloride and the results indicated that Zamzam water has a potential protective effect against carbon tetrachloride liver toxicity in rats.

Antimicrobial activity

Microorganisms are the most well-known reason for irresistible infections in humans. Diversity of bacterial pathogens, such as *Escherichia coli* and *Salmonella*, leads to soft-viscer mortality in many humans, which is a problem in such diseases²². Also, fundamental fungal diseases caused by *Candida albicans* have risen as significant reasons for bleakness and mortality. Zamzam water is water consumed by a lot of Muslims worldwide. It contains neither microscopic organisms nor molds that cause changes in smell and taste. Many studies reported it as free from any microorganism growth and confirmed it is suitable for drinking purposes. The Prophet Mohamed of Islam (peace and blessings of Allah be upon him) said: "The best water in the world is the water of Zamzam; it is a piece of food and source of healing from disease"²².

Zamzam water has a superior composition that makes it beneficial and contains a large amount of calcium and magnesium elements compared to the other types of water. It also contains fluorides that have germicidal effects, which makes the growth of algae so challenging to produce changes in taste and odor. All these criteria give Zamzam water characteristics of no growth of bacteria and make it an antioxidant to fight against so many diseases and their complications^{5,7}.

Anticancer effect of Zamzam water

As Zamzam water is alkaline and contains many minerals, it is considered an effective anticancer agent. (23) investigated the cytotoxic effects of Zamzam water on human lung cancer (A549) cell lines, and they compared it with human skin fibroblasts (HSF). The study showed that Zamzam water treatments reported a reduction in the cell viability of A549 cells, and they assured the effectiveness of using Zamzam water in treating lung tumors. (7) investigated an experimental mouse fed with 500 ml Zamzam water once a day for 30 days for treating colon cancer. Their results revealed that a decrease in the tumor mass was detected significantly. The probable cause for the loss of cancer is the natural composition of Zamzam water. Zamzam water contains 34 different components. The concentrations of sodium (Na), calcium (Ca), chloride (Cl), and magnesium (Mg) are higher than normal water. Rare traces of chromium (Cr), manganese (Mn), and titanium (Ti) exist in Zamzam water. Likewise, the quantity of toxic 34 estimates of the probable anticancer activity of Zamzam Water in human colon cancer cell Line components including, cadmium, lead, arsenic and selenium, is below the level of danger of human consumption.

Additionally, Zamzam water has a higher pH value (7.9–8.0) than the pH value of normal water (pH 6.5–7.0)⁸. Earlier studies confirmed that specific doses of some toxic minerals such as selenium, arsenic, and lithium inhibited the proliferation of cancer cells lymphocytes in the colon and were directed by Zamzam water. Cancer origin is affected by a definite immune mechanism, causing a reduction of cancer mass. In the same manner, (24) studied the effect of Zamzam water as colon anticancer; their results showed that Zamzam water treatments reduced the cell viability of cancer cells, they concluded that the death of cells happened through the cell death pathway in the two treatment situations. The early cell death was (3.0%, 3.5%), and 2.8% in control, respectively. The late phase of cell death was significant (4.2%) after one day of cure with Zamzam water. (25) studied the effect of Zamzam water on breast and ovarian tumor cell lines; their results revealed that using Zamzam water as a treatment raises cell production compared to drinking water. The chemotherapeutic agent diminishes and drops the sustainability of the cell by Zamzam water. Treatment with Zamzam water inhibited the influence of chemotherapy-induced reduction of CRAF, MEK1/2, ERK1/2, and P38 phosphorylation in breast and ovarian tumor cell lines. Equally, their results revealed that the silencing of ERK1/2 raises the chemotherapy-influence cell death in breast and bowel tumors. These data recommend that MAPK proteins primarily activated ERK1/2 showed a role in Zamzam water refereed defeat of chemotherapy- influence cell death.

Zamzam water, diabetes, and hypertension

Using Zamzam water as a treatment for hypertensive patients exaggerated excellent and bad cholesterol levels as verified significantly between intervention groups ($p < 0.05$)²⁶. These authors showed that the hypothesis testing using paired T-test revealed significant prior and afterward cured with Zamzam water ($p < 0.05$). While the study that was conducted by (27) showed that drinking Zamzam water leads to a significant rise in systolic pressure, diastolic pressure, heart rates, and the average pressure in patient arteries during one cardiac cycle within a few times after consumption. Also, they reported that drinking Zamzam water leads to a substantial rise in parasympathetic activity but does not affect cardiac sympathetic activity. Compared to carbonated water, Zamzam water has no significant influence on parasympathetic and sympathetic activity. In their distinguished study on the effect of drinking Zamzam water on the level of insulin and cholesterol in experimental rats, (28) fed them a diet with a tap or Zamzam water as the only source of fluids. Ten weeks later, these researchers measured fasting blood glucose, serum insulin, insulin resistance, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, super-dismutase, and lipid oxidation. Their study concluded that consuming Zamzam water for ten weeks reduces fasting blood sugar, blood insulin, and insulin resistance. However, Zamzam water does not affect lipids, redox homeostasis, and body composition. A study was conducted by (19) who studied the impact of drinking Zamzam water for non-insulin-dependent diabetes mellitus patients. His result showed that the group of patients who drink Zamzam water has great significance in the antioxidant capacity, superoxide dismutase, and glutathione concentration. This author emphasized that a diabetic patient who drinks Zamzam water has a significantly a lower glycated haemoglobin but not in fasting plasma glucose. Both glycated haemoglobin and fasting plasma glucose were not change significantly in the control group.

Other health benefits of Zamzam water

A study was conducted by (29) on patients with inflammatory bowel disease (IBD), they found that there was extreme simple guidance regarding the usage of complementary and alternative medicine (CAM) by their families (66%) and (62%) used honey, (54%) Zamzam water and physical exercise (32%). The most common of patients prefer the use of complementary and alternative medicine when they have a severe complaint.

In a comparative study between Zamzam water and other water sources and their effect on the prevalence and severity of tooth decay among school girls, by dividing the children into two groups, the first group of mixed dentition and the second group of permanent dentition, the results of the clinical examination indicated that there were no statistically significant differences in the first group, while the mean decayed missing filled teeth (DMFT) score is the lowest among all children in all the children used Zamzam water. A possible justification for that is the high level of fluorides in Zamzam water. Many studies have referred to the role of fluoride in preventing tooth decay, as they mentioned that, there is an inverse relationship between the spread of tooth decay and drinking water according to the percentage of fluoride in it, as it was found that 1.0 part per million of fluoride in drinking water reduces the experience of caries by 50%, this confirms the importance of adding a certain percentage of fluoride to drinking water and that it has beneficial effects⁶.

Conclusions

This review clarified much important information about the chemical composition of Zamzam water, and it became clear that there are many benefits of drinking Zamzam water compared to regular tap water. Perhaps this is because Zamzam has many benefits water is alkaline. This study summarized the results of many studies about the great benefits of this water on humans and animals. But also, on plants, as studies conducted on many experimental rats have shown, the resistance or reduction of various human diseases and reduce cancerous growth by Zamzam water. This mini-review study confirmed the mineral and nutritional balance in the composition of Zamzam water.

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REVIEW / ARTÍCULO DE REVISIÓN

Study review of camelid and shark antibodies for biomedical and biotechnological applications

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Abstract: The antibodies of camelids and sharks are about one-half of the conventional ones while regular antibodies have four protein chains: two light and two heavy, these small antibodies studied have just two heavy chains; they lack a light chain. In recent years, nanobodies have been the focus of attention because they can recognize epitopes that are usually not antigenic (hidden) for conventional antibodies. On the clinical side, researchers are testing nanobodies (Nbs) in the fight against diseases and disease diagnosis. Nanobodies also are attractive because they can prevent protein aggregation and clear the already existing aggregates. Furthermore, new treatments using these Nbs can neutralize the severe acute respiratory syndrome coronavirus (SARS-CoV-2) for preventing COVID-19. In this review, we sum up recent findings of the proposed nanobodies for their potential application.

Key words: Nanobodies, Camelids, Sharks, Therapeutics, Diseases.

Introduction

Immunoglobulins play a central role in the adaptive immune system by recognizing the specific molecular patterns of external antigens, and through these interactions, they can neutralize and eliminate pathogens from the host.

The three main types of humoral immunoglobulins (Igs) are IgG, IgM, and IgA in the milk of mammals. The concentration of each type of Igs depends on several factors. These factors include the lactation stage and the type of neonatal transfer of antibodies from the mother to the offspring (placental, colostrum or mixed)¹.

While regular antibodies are made of four protein chains (two light and two heavy), the Camelidae family and cartilaginous fishes produce functional antibodies which naturally lack light chains². These antibodies (Abs) were named single domain antibodies or heavy chain antibodies. Using affinity chromatography techniques performed by Hamers-Casterman³ in 1993, they realized that these Abs have about one-half of the size of the conventional ones.

The heavy chain (VHH) variable region has excellent solubility, stability, tiny size, and a great capacity to recognize unique epitopes, thus having a high specificity and antigen affinity⁴. It is thought that this super-lightened version of antibodies has evolved independently several times, suggesting that they are highly effective in this animals⁵.

Immunizing a camelid (camels and llamas) with soluble, adequately folded proteins raises an affinity-matured immune response in the unique camelid heavy-chain only antibodies (HCabs). The peripheral blood lymphocytes of the immunized animal are used to clone the antigen-binding antibody fragment from the HCabs in a phage display vector⁶.

To get these antibodies tailored to a specific molecule, the molecule must first be injected into the animals. Animals then make small antibodies over weeks or months. Researchers use their blood cells for the small antibodies to obtain genes, then use bacteria to produce the antibodies in the laboratory⁷.

Unlike conventional antibodies, VHH can be cloned, expressed and purified in a prokaryotic system such as *E. coli*⁸. The most popular approach to select recombinant antigen-recognition antibody fragments is the modified panning phage display libraries. This method is based on the selection of DNA sequen-

ces that are cloned in a phagemid vector (located within phage particles) and is coding for recombinant proteins, recombinant antibodies, which are able (being expressed as a part of a filamentous phage surface protein on the surface of phage particles) to bind the defined ligand (antigen)⁷ specifically.

It might soon be easier to obtain nanobodies since a team has made a nanobody library in yeast cells, allowing researchers to skip the animal-inoculation step. iCAN is built as the first comprehensive nanobody database to provide an integrative analysis tool for academic and industrial researchers to expand and accelerate nanobody research².

Nanobodies and their derivative forms can serve as nano tracers in intracellular bioimaging¹ and help visualize the structure of complex proteins due to their nanoscopic size and high affinity against intracellular signaling molecules. They can bind tightly and deeply; this can help stabilize ornate molecules whose flexibility thwarts the imaging process.

On the clinical side, the application of nanobodies in targeting therapeutics shows good prospects in the therapy of acute thrombotic thrombocytopenic purpura⁹, infectious disease¹⁰, rheumatoid arthritis¹¹, central nervous system disease¹², breast¹³ and ovarian cancers¹⁴, and so on². Next on the horizon for the nanobodies is getting them to stick to tumors for imaging purposes¹⁵ or using them to allow drugs to cross the blood-brain barrier to tackle diseases of the brain¹⁶.

It is essential to know the characteristics of each nanobody to find its proper biomedical or biotechnological applications. This paper review shows the specific characteristics of nanobodies that makes them more potent than the conventional antibodies and will also collect all the advances made in the field of the different species of sharks and camelids' antibodies, such as the new information collected from ongoing studies of these animals' immunoglobulins and their potential applications in immunotherapies and fighting diseases.

Methodology

In this study, we critically examined 83 papers published in various journals. The paper selection strategy and evaluation criteria considered in this paper are in Figure 1.

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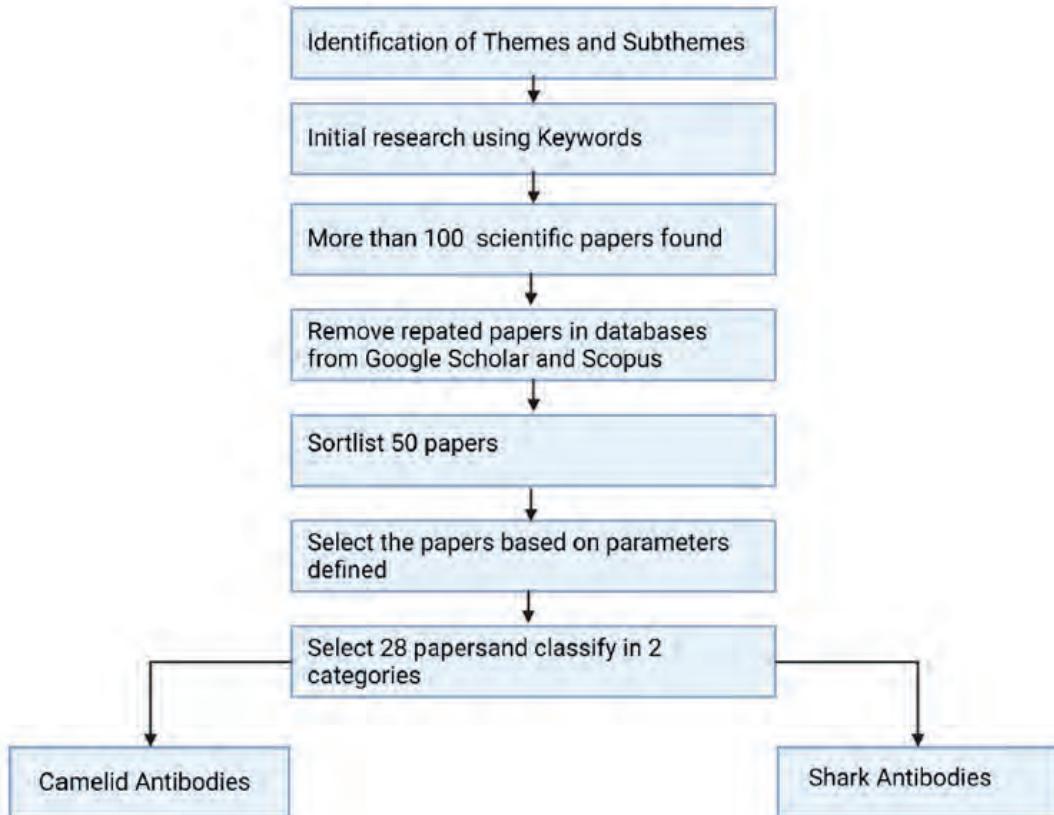


Figure 1. Review methodology for paper selection.

The papers were obtained from Scopus and Google Scholar using keywords' camelids', 'sharks', 'nanobodies', 'biomedical application', 'therapeutic application', 'antibodies', etc.

In this study, we downloaded papers published available online. We found 123 papers and selected 83 papers for review, and filtered out 40 papers that were not useful or not relevant.

In this literature review, 83 papers and 5 books published were critically examined. The information was published in different journals, and some were entrepreneurial web pages (3) since some nanobodies are already in the market.

The parameters used for the paper selection were if the study topics such as the nanobody's therapeutic use, its functional characteristics, biomedical application, biotechnological methods.

The papers selected were separated into two categories: camelid antibodies and shark antibodies.

Results

Camelids antibodies

The camelids of South America are a family of artiodactyl mammals belonging to the order Artiodactyla, suborder Ruminantia, family Camelidae¹⁷. The single-domain antibodies are antibodies of the IgG type present in the serum of species of the Camelidae family that lack both the light chain and the CH1 region of the heavy chain^{18,19}.

During pregnancy, some camelids, such as alpacas, cannot transport immunoglobulins from the mother to the fetus, so the offspring are born agammaglobulinemia and require maternal antibodies that will be transferred thanks to colostrum²⁰⁻²². After the ingestion of colostrum, there is an increase

in the IgG concentration in the blood serum of the offspring, which generates resistance to infectious agents^{23,24}.

These antibodies were named single-domain antibodies or heavy chain antibodies because they did not have a light chain, using affinity chromatography techniques performed by Hamers-Casterman³, where the affinity of the antibodies present in the serum of camels for protein A was studied^{25,26}.

Camelids have a single antigen recognition domain so that their unique domain contains a complete antigen-binding site and is the smallest functional antigen-binding fragment (around 15 kDa)²⁷. As is known, antibodies are generally tetramers, composed of 2 dimers called heavy chain (H) of 55 kDa each, and two dimers called light chain (L) of 25 kDa, generally linked by disulfide bridges, each forming a protein in the shape of Y^{28,29}, shown in figure 2.

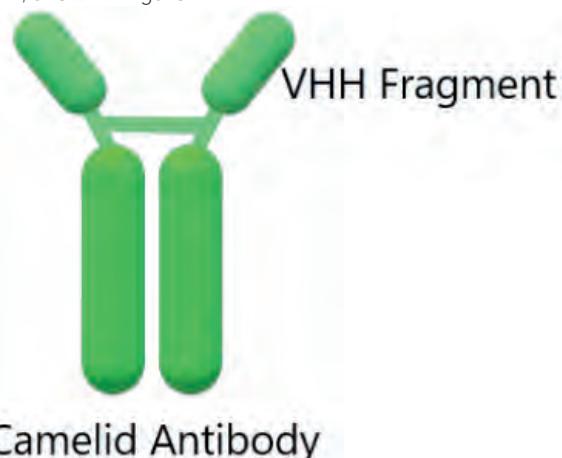


Figure 2. Structure of an IgG camelid immunoglobulin. At the top of this structure is the single-domain antibodies (VHH).

Immunoglobulins of camelids

Currently, these immunoglobulins and especially their simple variable domains (VHHs for short) have various applications in biotechnology²⁹. It has been observed that antigenic sites tend to be more hydrophilic³, flexible, and accessible than the rest of the protein, often protruding from the antigen surface. An early study suggested that conventional Abs have lower surface shape complementarity than other protein-protein complexes³⁰.

Single-domain antibodies or heavy chain antibodies (HCabs) are G-type immunoglobulins that lack a light chain. In camelids, there are 5 isotypes of antibodies: IgG, IgM, IgA, IgE, and IgD, which differ as much in sequence as in the function of the heavy chain³¹.

Faced with the invasion of an infectious agent, camelids and other animals activate a humoral response through immunoglobulin G (IgG), preventing the invasion of this infectious agents³². The immunoglobulins, in figure 3, has both the heavy and light chains have a constant region (CH and CL, respectively) and two variable regions (VH and VL, respectively).

Variable region (VH, VL)

The variable region is responsible for recognizing foreign agents or antigens that are molecules that activate the immune system; the amino acid sequence of this region has a significant variability to recognize different antigens^{33,34}.

The variable region of single-domain antibodies, also called VHH, is the minor antibody that can interact with the antigen. It has the advantage of behaving as a monomer independent of the rest of the HCAb; it is very stable and has a low molecular weight of approximately 12-15 kDa. The VHH domain is a fragment of only 15 to 18 KDa³⁵.

Unlike conventional antibodies, VHHs fold independently of the rest of the protein and maintain the ability to have high specificity and affinity for their antigen; they can also be cloned, expressed and purified in a prokaryotic system such as *Escherichia coli*⁸.

VHHs are known as nanobodies (Nbs); these Nbs possess properties that allow them to be an ideal tool in research and various therapeutic applications.

Due to these characteristics, VHH has been the object of several studies, mainly for biomedical purposes.

There are many direct applications of VHH in scientific research, for example, as membrane protein stabilizing proteins

to facilitate their crystallization³⁴. There is also a significant advance as a therapeutic agent, and even several VHH are in advanced clinical stages for use in humans. However, there are no studies that consider VHH in personalized medicine, such as tissue engineering³⁶.

Constant region (CL, CH)

While the constant domains of the antibodies are not involved in the recognition of antigen. Type G antibodies have three constant domains called CH1, CH2, and CH3 of the heavy chain, and one of the light chains called CL. The IgG is constituted only by heavy chains²⁹.

Potential Biomedical Application of camelids immunoglobulins

The VHH, due to its small size and high stability, has the property of being functional in the interior of living cells and even detecting conformational changes in proteins^{37,38}. In addition to allowing the identification of intracellular elements, VHH can mark proteins for their degradation through the ubiquitination pathway, as the main characteristic of VHH to bind to antigens^{39,40}. This capacity is used in crystallography, where the crystallization chaperones also participate^{41,42}.

The VHH does not require post-translational modifications, so the production of these recombinant VHH is an alternative to monoclonal antibodies for therapeutic use, and they are also very economical and of minimal size with tiny stable molecules⁴³. The nanobodies of camelids have become very popular in cancer treatment since they have a more significant advantage than conventional antibodies^{44,45}. While conventional antibodies are difficult to penetrate and expensive large-scale production, camelid antibodies are more minor, very soluble, stable, easier, and cheaper to produce⁴⁶.

Studies conducted in mice already show that nanobodies counteract specific tumors in these rodents. Also, these nanobodies have been used in other treatments against life-threatening diseases. More than 85% of human cancers are solid tumors⁴⁷. In most solid tumors, the necessary first step is to access sufficient amounts of the therapeutic antibody to various tumor regions to obtain a maximal therapeutic effect⁴⁸. The transfer of macromolecules in tumors is mainly by diffusion, and the speed of diffusion through tumors is inversely proportional to molecular size. In agreement with this, small antibody fragments penetrate the solid tumors more efficiently than the whole antibody molecule⁴⁹.

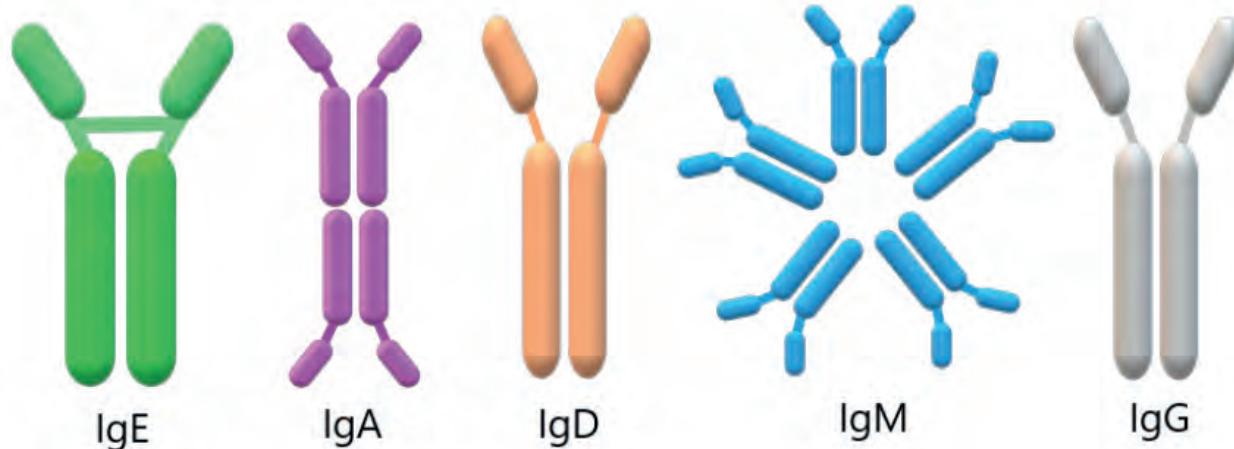


Figure 3. Structure of the types of antibodies found in camelids. According to their H chains, camelids antibodies are classified into five isotypes (IgE, IgA, IgD, IgM, and IgG from left to right), which provide each isotype with distinct characteristics and roles.

Furthermore, to be effective for cancer treatments, nanobodies are also attractive therapeutic options for disorders such as Alzheimer's and Parkinson's diseases since they can prevent protein aggregation and, even more interesting, clear the already existing aggregates⁵⁰. To increase target-to-background signal ratios for high detection sensitivities in imaging and reduce non-specific toxic effects of antibody conjugates, the unbound antibodies should be cleared rapidly from the non-target organs⁵¹. When compared to the whole antibody molecule, the small antibody fragments have a faster clearance rate⁵⁰. The main differences between conventional nanobodies and camelid nanobodies are summarized in table 1.

Furthermore, a recent study shows that single-domain antibodies (VHHs) were isolated from a llama immunized with prefusion-stabilized coronavirus spikes. These VHHs neutralize MERS-CoV and SARS-CoV-1 S pseudotyped viruses. The findings provide a molecular basis for VHHs' ability to neutralize pathogenic beta coronaviruses, implying that these molecules could be effective therapeutics during coronavirus outbreaks¹⁰. A summary of this application is in figure 4.

Another study reported by Xiang *et al.*⁵⁵ identified a vast repertoire of extremely powerful neutralizing nanobodies

(Nbs) to the SARS-CoV-2 spike (S) protein receptor-binding domain (RBD) using camelid immunization and proteomics. They found Nbs that blocked viral infection with picomolar to femtomolar affinity and determined the structure of one of the most potent complexes with RBD. They created multivalent Nb constructs with ultrahigh neutralizing potency (IC_{50} s as low as 0.058 ng/mL) and mutational escape resistance.

Furthermore, a recent study engineered anti-RBD nanobodies from llamas and "nanomice" to produce VHHs cloned from alpacas, dromedaries and camels. They identified two sets of highly neutralizing Nbs using cryo-electron microscopy. These findings show that multivalent nanobodies can overcome SARS-CoV-2 variant alterations through two mechanisms: increased avidity for the ACE2 binding domain and identification of conserved epitopes that are difficult to reach with human antibodies. While additional SARS-CoV-2 mutations will continue to arise, nanobodies are promising strategies for preventing COVID-19 mortality if vaccinations are damaged⁵⁶. This potential alternative of using camelid nanobodies promises to be a revolutionary therapy in searching for new drugs against Coronavirus disease (COVID-19)^{10,36,57,58}.

Conventional nanobodies	Camelid Nanobodies
Light chain and heavy chain	Lack light chain ⁵²
Common size	About one-half of the size ²⁹
Hydrophobic binding interactions	HCAb in camelids is hydrophilic ⁵³
Are challenging to penetrate the solid tumors in cancer	Easier to penetrate the solid tumors in cancer ⁵⁴

Table 1. Table of main differences between conventional nanobodies and camelid nanobodies.

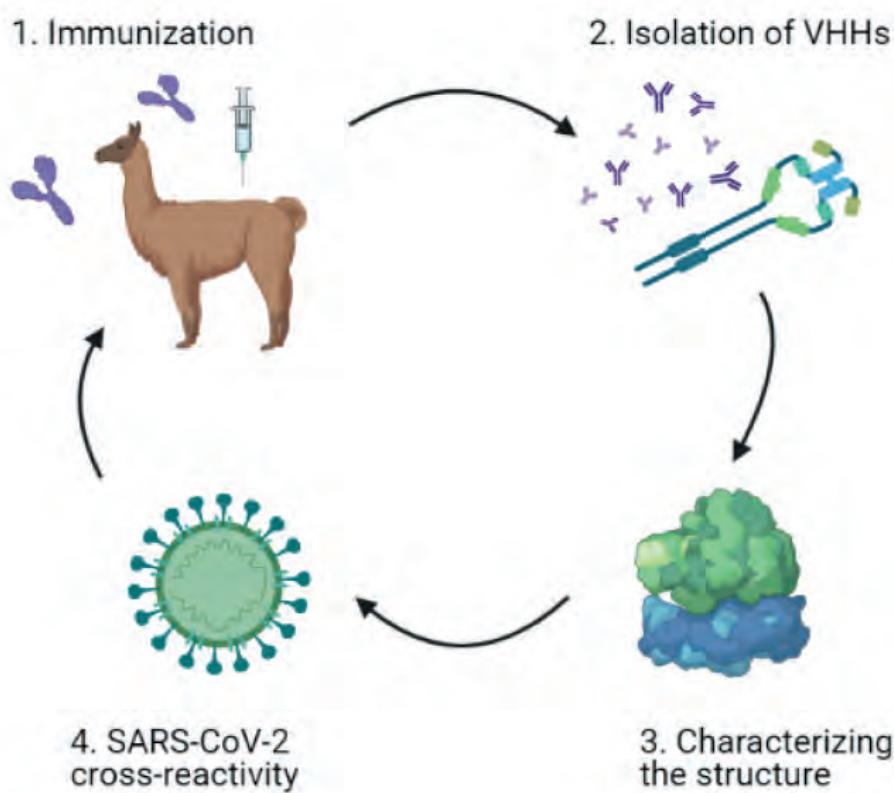


Figure 4. General graphic of single-domain antibodies (VHHs) application in SARS-CoV-2, based on Daniel Wrapp *et al.*, 2020. The schematic representation shows the identified neutralizing cross-reactive single-domain camelid antibodies in llamas immunized with prefusion-stabilized betacoronavirus spike proteins, which may serve as valuable reagents for researchers studying the viruses that cause MERS, SARS, and COVID-19¹⁰.

Cartilaginous fishes antibodies

Sharks are a group of Elasmobranch fish from the class Chondrichthyes or Cartilaginous fish. The evidence of their existence dates back to 450-420 million years ago, placing them in the Ordovician Period⁵⁹; this makes them the "oldest vertebrate taxon to possess an adaptive immune system"⁵ that includes immune factors like immunoglobulins, T cells, Major Histocompatibility Complex (MHC), sites of intense cellular activity, recombinase- activating gene (RAG) activity and also have the earliest version of Ig switching where Somatic Hypermutation (SHM) and Class Switch Recombination (CSR) are contiguous activities⁶⁰. However, since sharks are cartilaginous species, they do not have bone marrow. Instead, it has been proven that they have an epigonal organ that is the site where the lymphopoiesis occurs, and the B cells are secreted, showing the analogous function of a mammalian bone marrow⁵.

Cartilaginous fishes immunoglobulins

Sharks have an adaptive immune system based on lymphocyte antigen receptors generated by V(D)J recombinants. Shark B cells express two classical immunoglobulins (Ig), IgM and IgW, encoded by an early, alternative gene organization consisting of numerous autonomous miniloci, where the individual gene cluster carries a few rearranging genes segments and one constant region, μ or ω ⁶¹. Adult cartilaginous fish express the three immunoglobulins (Ig) isotypes, IgM, IgNAR and IgW. Newborn nurse sharks produce multimeric and monomeric IgM, a germline-joined, IgM-related VH, and meager amounts of monomeric IgM and IgNAR proteins⁶².

IgW

IgW transcripts, shown in figure 5, are found in secretory full-length, long-form; secretory truncated, short form that is probably derived by alternative splicing⁶³; and trans-membrane long and short forms⁶⁴.

Additionally, a molecular characterization of Ig Heavy chains identified both IgW forms in the African lungfish, *Protopterus aethiopicus*, a lobe-finned bony fish, which phylogenetic studies suggest are closely related to land vertebrates (tetrapod)⁶³.

The trans-membrane IgW long heavy chain alternative spliced to produce a shorter heavy chain with five domains: one variable and four constant domains, or an even shorter three domains heavy chain: one variable and two constant domains, have been found in the nurse and horn sharks⁶⁴. And the secreted IgW long heavy chain is composed of seven domains: one variable and six constant domains, and the IgW short heavy chain is composed of three domains: one variable and two constant domains. It is also called IgX or IgNARC^{64,65}. Both IgW secretory forms have also been found in nurse and horn sharks⁶⁴.

There are multiple-spliced trans-membrane cDNA forms of IgW and IgNAR, possibly selected over evolutionary time to limit proteolysis of the putative cell surface proteins⁶². There is good evidence of a class switch recombination between IgM and IgW and that IgW(ω) in sharks is orthologous to IgD of vertebrates^{61,66}. IgW short is most highly expressed in the spleen. Also, in unimmunized adults, IgW short transmembrane and secreted forms are produced in the pancreas, suggesting a role in gut mucosal immunity⁶⁷.

IgM

In B cell receptor systems, the sharks' IgM exists in both trans-membrane (for activation of lymphocytes) and secre-

tory forms (to bind antigen and induce effector functions of the humoral system)⁵. This IgM humoral system of sharks was initially thought to be very different from mammals, mainly because the kinetics of the climb in titter was slower to rise than in endotherms⁶⁷. Serum IgM of immune responsiveness is found in a monomeric 7S and a pentameric (19S) form, both shown in figure 6, present in roughly equal amounts and constituting half of the total serum protein in adult animals^{5,65,67}. A subclass of the IgM, IgM1gj, was reported in nurse sharks in high amounts as a monomer in the sera of neonates with only three C domains. IgM is expressed in the epigonal organ, but its levels decline in the spleen with age⁶⁷.

Cartilaginous fish express high monomeric and multimeric serum levels of the same Ig isotype⁶⁵ in approximately equal amounts despite independent production from the same set of IgH loci^{26,67}. Immunoglobulins of the IgM class generally have disulfide-linked pentamers of total molecular weight near 900000. Each monomer unit is similar to an IgG molecule, containing two light (L) and two heavy chains linked by disulfide bonds and noncovalent interactions⁶⁸.

IgM has been found in the eyes, gills, intestine, liver, pancreas, peripheral blood leukocytes, spleen and testis⁶⁷.

IgNAR

Surface Ig New Antigen Receptors (IgNARs) are formed by two identical heavy chains composed of five or three constant domains, shown in figure 6, a variable domain (V-NAR) and one amino-terminal V domain^{65,66}. The first and third constant domains homodimerize and lend to the antibody stability, and the resolution of the stable region structure showed stabilizing motifs^{66,69}.

The IgNAR is quite an unconventional antibody being a homodimer of H chains that do not associate with L chain⁷¹, and since it does not fit the profile of an actual antibody although it is structurally similar⁵. The variability within the CDR1 and CDR3 regions⁶⁹ certainly suggested so, as did the general organization into a variable-constant domain format and the occurrence of transmembrane and secretory forms of the protein⁷². Nevertheless, it follows an analogous target-specific kinetics of expression in response to an immunogenic challenge^{5,73}.

The IgNARV is the smallest antigen-binding domain known in the animal kingdom and is also particularly water-soluble^{66,74}. VNARs are classified into types based upon the number and location of additional noncanonical cysteine (cys) residues in their variable domain. These cys form inter-domain disulfide bonds that dictate the structure of the VNAR domain^{71,72}. Type I VNARs have germline-encoded, noncanonical cys residues in framework regions (FR)2 and FR4, that pair with D region encoded cys residues in CDR3; this folds the long CDR3 loop over the surface that would associate with VL in conventional Igs, pinning it there.

In contrast, type II VNARs either have a single germline-encoded, noncanonical cys residue in CDR1 that pairs with a lone cys present in CDR3, forming a stabilizing disulfide bond between the protruding antigen-binding loops, or no extra cys (sometimes called type IIb or type IV) allowing CDR3 to move away from CDR1. Type I CDR3's are longer on average than those of type II, undoubtedly a result of selection due to their unique disulfide bonding patterns.

A third VNAR type, type III, so far found only in nurse sharks, has a structure very similar to type II VNARS; however, CDR3 diversity in this type is drastically reduced due to germline fusion of two (D1 and D2) of the three D segments present in this cluster⁷¹. There is a limited sequence diversity of type III

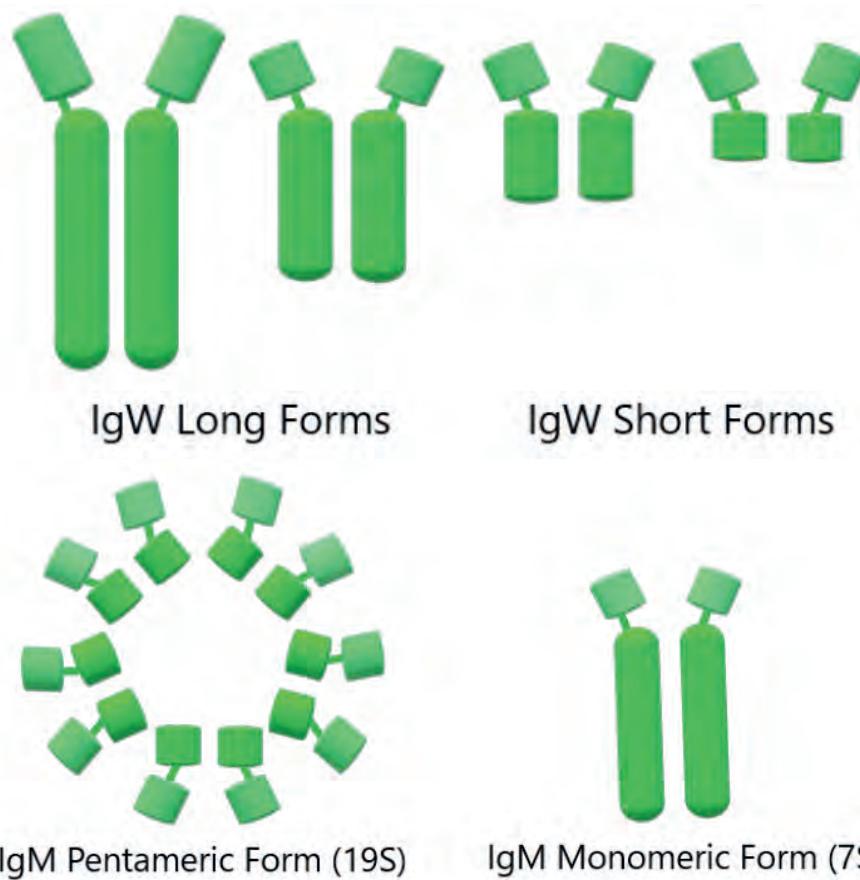


Figure 5. Schematic representation of the IgW architecture in long and short forms (from left to right)⁶².

Figure 6. Schematic representation of the overall IgM architecture. The first represents the pentameric form of IgM, and the second represents the monomeric form of IgM (from left to right)⁶⁰.

CDRs structural modeling; however, it has been revealed invariant stabilizing aromatic residues in CDR1 (tryptophan 31) and CDR3 (phenylalanine 96), which could allow a more excellent range of structural diversity than would be predicted for this VNAR type. Expressed early in development and retaining expression only in the epigonal of adult sharks, type III is hypothesized to mediate the first line of immune defense in neonates; however, the antigens it targets still require investigation⁷³.

Much remains to be understood about the role of IgNAR in the immune system of cartilaginous fishes. So far, isolation of antigen-specific VNARs from libraries has primarily been conducted via bacteriophage (phage) display²¹. In this case, the library of VNAR sequences is cloned in-frame with a phage coat protein (usually the gene III protein) encoded in a phagemid vector. However, it is abundantly clear that VNARs present a singular molecular framework that can be exploited to deve-

lop reagents for scientific research, disease diagnosis, and/or potentially therapeutic intervention^{65,72}.

Potential Biomedical Applications of Cartilaginous fishes immunoglobulins

The IgW is an Ig isotype that exists in two forms, one of full length and one lacking the Fc region⁶¹. This isotype can bind antigen without evoking Fc-mediated responses, thus limiting inflammation⁶⁵. The possibility of an anti-IgW antibody is being investigated that will provide an essential research tool to studying cartilaginous fishes' adaptive immune system⁷⁵. Sharks can produce an IgM-based response following immunization, evidence for memory and affinity maturation⁶⁵.

IgM might have a role in blood osmoregulation⁷⁶ analogous to the physiology of albumin in other vertebrates and have been shown to be active in lytic, opsonic and antibody-induced cytotoxicity-like reactions⁷⁷. This killing is observed through phagocytosis, which is mediated by both 7S and 19S IgM antibodies. Throughout evolution, IgM reacts with particulate antigen and through binding Fc- receptors on the surface of leukocytes, enhancing phagocytosis⁶⁷.

The relative stability of the IgNAR heavy chain homodimer, the extraordinary stability and resistance to irreversible denaturation of VNAR domains⁷⁴ and the somewhat simpler genetics encoding the single variable domain have fuelled applied research to adapt this molecule to biotechnology and immunotherapy⁶⁶. IgNAR has a small structure and an excellent binding ability. Its single variable domain represents an opportunity to bind different epitopes than traditional antibodies and can also work as an autonomous paratope⁷⁴.

For diseases like protein aggregation disorders such as Alzheimer⁷⁸ and Parkinson⁷⁹, nanobodies can prevent protein aggregation and, even more interestingly, clear the already existing aggregates⁵⁰. Ossianix Inc⁸⁰ has utilized transferrin

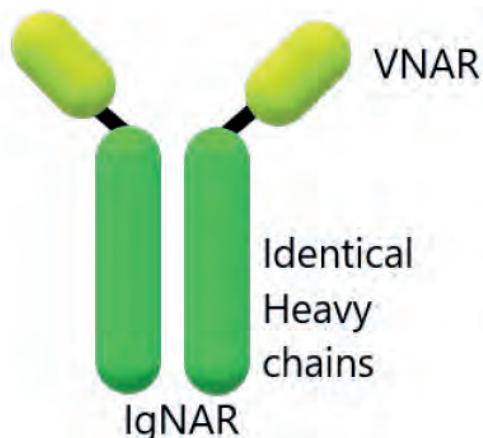


Figure 7. Schematic representation of the overall IgNAR architecture. Shark antibody-containing identical heavy chains^{69,70}.

receptors to demonstrate that they can transfer VNAR antibodies across the blood-brain barrier⁸¹ shown in figure 7.

In addition, they are also developing VNAR-therapies for neurological, neuromuscular as well as autoimmune diseases^{74,80}. Antigen-specific VNAR molecules have been isolated against a wide range of disease-related targets, including proteins involved in cancer⁸² and arthritis⁸³, cytokines⁸⁴, toxins⁸⁵, and viral targets. However, with their small size, high stability, and possible advantages in cryptic epitope recognition, VNARs are natural candidates for future niche biotechnological and diagnostic applications^{71,72}.

There has been a recent study⁷³ to evaluate the VNARs' potential for therapeutic development, where researchers chose a variety of human tumor biomarkers and virus antigen proteins as selection targets, including glycan-3 (GPC3), HER2 and PD1, the spike proteins of the MERS and SARS viruses, and *Pseudomonas* exotoxin (PE38). They developed a method to construct phage display libraries based on PCR extension assembly followed by self-ligation. The selected binders, Type I and II VNARs, were produced successfully in *E. coli* as soluble proteins for antigen-binding validation. This study validates the diversity of the nurse shark VNAR library and the utility of the shark VNAR library as a platform for therapeutic antibody discovery.

A naive wobbegong VNAR library was used to identify two natural clones of micromolar (M) affinity for the protease gingipain K (Kgp), a virulence factor of *Porphyromonas gingivalis*, which causes periodontal disease in humans⁷¹. Researchers used panning a semisynthetic wobbegong shark library to isolate a VNAR capable of targeting Hepatitis B virus (HBV) pre-core protein, which is secreted as Hepatitis B e antigen (HBeAg)⁷¹. It also may work as half-life extension tools in di-

sease treatment⁷⁴. Engineering stability into a shark VNAR combined strategies of CDR grafting and consensus sequences mutagenesis have been developed in a VNAR specific for the nucleoprotein of the Ebola virus⁸⁶.

Recently, a group of researchers tested the VNAR domains for the treatment of COVID-19. They used the neutralizing properties of the VNAR against the Spike protein from the SARS-CoV-2 Wuhan variant. The antibodies showed great affinity and high blocking of the ACE2 receptors' interaction with the virus variant. This study expands the alternative treatments against the new virus⁸⁷.

To produce VNARs for the above applications, individual clones that bind the target of interest with high specificity and good affinity must first be isolated from an extensive repertoire of diverse sequences. Two strategies have been utilized to generate natural VNAR libraries: 1) amplifying the VNAR repertoire from one or more naive animals or 2) from animals immunized with the target antigen⁷¹. The application of each immunoglobulin explained in this review is summed up in table 2.

Conclusions

As presented previously, the nanobodies of the camelids not only have characteristics that have made them very popular for cancer treatments, but they are also desirable therapeutic options for disorders such as Alzheimer's disease and Parkinson's disease. These studies in sharks and camelids can also be performed in Ecuador since these animals live within the fauna of this biodiverse country and can boost biomedical and biotechnological research to get better and effective therapies and treatments for better and effective therapies and

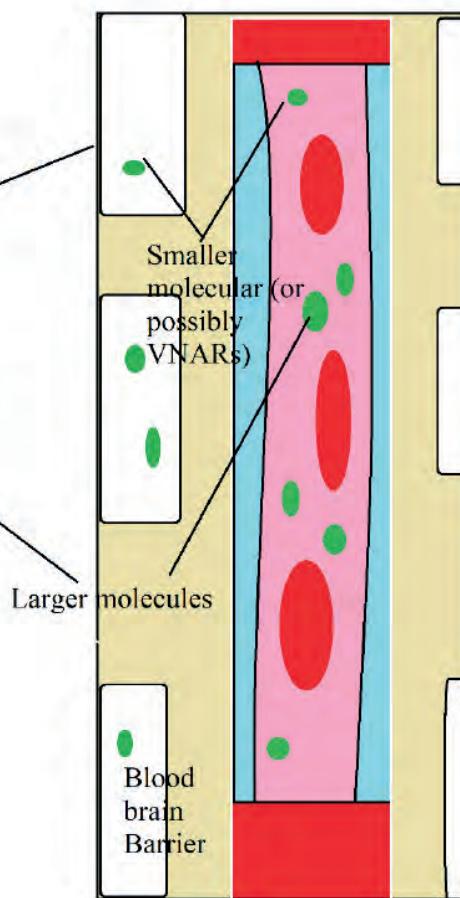
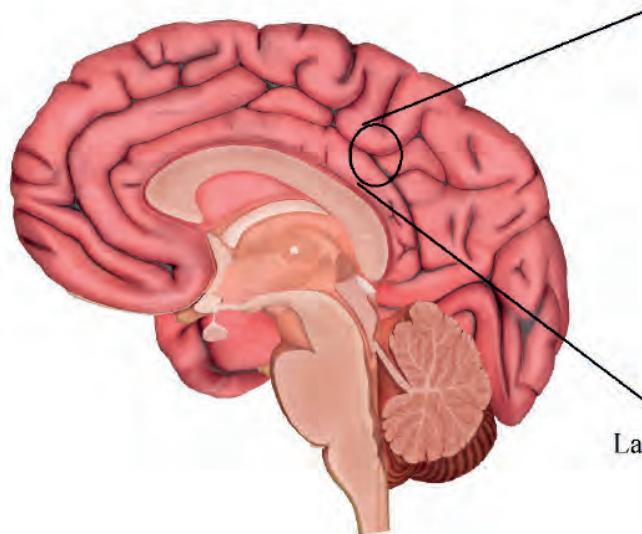


Figure 8. Mechanism of therapeutic use for nanobodies in neurological diseases. Nanobodies can pass the blood-brain barrier⁷⁴.

treatments in hospitals and clinics. Nanobodies have reached many publications that are increasing in recent years; one of the fields very interested in these investigations of nanobodies are the large pharmaceutical industries, which reflect high expectations for nanoantibody technology.

Despite the exhaustive study of the sharks' immunoglobulins, there is still a lack of information about the possible applications of the recently deeply studied immunoglobulins IgW, IgM and IgNAR. Although their characteristics seem very promising on the clinical side, especially in the therapeutic and antiviral treatments, few experiments have not gotten further than mice experimentation. For example, while VNARs have been raised against a diverse array of targets, most studies have confirmed antigen specificity, affinity, and stability; very few studies have proceeded as far as testing the VNARs in relevant disease models. Further work is needed to establish the structural and functional repertoire of VNAR domains and their potential immunogenicity.

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Igs	Potential applications
IgNAR-VNAR and IgM	Alzheimer ⁷¹ , Hepatitis B ⁷² , periodontal disease in humans ⁶⁵ , cancer ¹² and arthritis ¹¹ , cytokines ⁷⁴ .
IgW short form	Immunity gut mucosa ⁶⁷ , limiting inflammation ⁶⁵ .

Table 2. Table of Sharks immunoglobulins and their application.

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REVIEW / ARTÍCULO DE REVISIÓN

Pruebas moleculares para el diagnóstico de COVID-19: La respuesta de Sudamérica

Molecular tests for the diagnosis of COVID-19: South America's response

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Resumen: Actualmente a nivel mundial se utiliza la reacción en cadena de la polimerasa con transcriptasa inversa (RT-PCR) como una técnica de alta precisión en la detección y amplificación de secciones específicas de la estructura génica. En tal sentido es considerado como prueba diagnóstica molecular en la detección del SARS-CoV-2. Se realizó una revisión de la literatura en artículos publicados en los últimos 5 años (2015 – 2020). Se consultaron las bases de datos: PUBMED (Medline), PUBMED CENTRAL, y SCIELO. Se recuperaron artículos en español, portugués, e inglés, mediante los términos de búsqueda SARS-CoV-2, RT-PCR, y COVID-19. Las RT-PCR que utilicen especímenes de hisopado nasal y faríngeos son considerados como gold standard en casos sospechosos de COVID-19. Mediante lo revisado a nivel de Sudamérica, la RT-PCR resultó ser la prueba de elección durante el período menor a 7 días de infección, resaltando la producción *in house* en escala por Uruguay y la Prueba de amplificación isotérmica mediada por bucle (RT-LAMP) adaptado en Perú, como una alternativa diagnóstica rápida con principios similares a la RT-PCR, solo que para establecimientos de salud con menor equipamiento, e infraestructura y personal entrenado. La disponibilidad de pruebas diagnósticas moleculares también es crucial para el aislamiento de casos positivos y el seguimiento de la cadena epidemiológica de transmisión.

Palabras clave: COVID-19; Virus SARS; Diagnóstico; RT-PCR (Fuente: DeCS BIREME).

Abstract: Currently, worldwide, the reverse transcriptase-polymerase chain reaction (RT-PCR) is used as a high-precision technique in detecting and amplifying specific sections of the genetic structure. In this sense, it is considered a molecular diagnostic test in the detection of SARS-CoV-2. A review of the literature was carried out on articles published in the last 5 years (2015-2020). The following databases were consulted: PUBMED (Medline), PUBMED CENTRAL, and SCIELO. Articles in Spanish and English were retrieved, according to MESH terminology: SARS-CoV-2, RT-PCR, COVID-19. RT-PCR tests that use nasal and pharyngeal swab samples are considered the standard gold test in suspected cases of COVID-19. Through the South American review of molecular tests, RT-PCR turned out to be the test of choice in the countries of South America during the period less than 7 days of infection, highlighting the in-house production in scale by Uruguay and the Loop-mediated isothermal amplification test (RT-LAMP) adapted in Peru as a rapid diagnostic alternative with principles similar to RT-PCR, only for health facilities with less equipment, infrastructure, and trained personnel. The availability of molecular diagnostic tests is also crucial for isolating positive cases and monitoring the epidemiological chain of transmission.

Key words: COVID-19; Virus SARS; Diagnosis; RT-PCR (Source: DeCS BIREME).

Introducción

Los coronavirus son un grupo de virus ARN altamente diversos de la familia Coronaviridae que se dividen en 4 géneros: alfa, beta, gamma y delta, y que causan enfermedades de leves a graves en humanos y animales¹. Sin embargo, tres coronavirus zoonóticos que causan enfermedades graves en humanos han emergido: el coronavirus del síndrome respiratorio agudo grave (SARS-CoV) en 2002-2003, el coronavirus del síndrome respiratorio de Oriente Medio (MERS-CoV)²; y, con un impacto aún mayor, el síndrome respiratorio agudo severo tipo-2 (SARS-CoV-2)³. En enero de 2020, el agente etiológico responsable de un grupo de casos de neumonía grave en Wuhan, China, fue identificado como un nuevo betacoronavirus inicialmente llamado 2019-nCoV, actualmente SARS-CoV-2, distinto del SARS-CoV y MERS-CoV⁴. La secuencia genómica

completa de este nuevo agente está disponible y se han desarrollado diferentes protocolos y técnicas para su detección, aunque aún no se han validado por completo⁵. La introducción de un caso sospechoso relacionado con el 2019-nCoV en la Región de las Américas colocó en alerta a la Organización Panamericana de la Salud/Organización Mundial de la Salud (OPS/OMS) recomendando a los Estados Miembros garantizar su identificación oportuna, el envío de las muestras a laboratorios nacionales o de referencia y la implementación del protocolo de detección molecular para 2019-nCoV^{6,7}. La OMS puso por su parte a disposición dos protocolos de diagnóstico molecular en la página web "Pruebas de laboratorio para casos humanos sospechosos del nuevo coronavirus 2019 (2019-nCoV)"⁸. Por tanto, hay una necesidad inmediata de un procedimiento de

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prueba de detección de primera mano, que debe estar disponible para personas sintomáticas y asintomáticas que tengan contacto estrecho con un individuo infectado⁹. De esta manera se puede determinar si la persona probada es negativa o potencialmente positiva para la presencia de SARS-CoV-2. Las plataformas de detección moleculares disponibles actualmente para el SARS-CoV-2, se basan en un concepto simple de detección indirecta de ARN por una prueba de reacción de cadena de polimerasa con transcripción inversa (RT-PCR) y la posterior clonación y uso de ADN complementario (ADNc) en otras técnicas moleculares. En resumen, una persona portadora potencial es sometido a una colección de frotis faríngeo (frotis de garganta) y/o esputo, esta muestra recolectada luego se procesa a través de una serie de procedimientos para purificar ARN viral, convertir ARN en ADNc mediante una transcriptasa inversa y medición del número de copias mediante gPCR utilizando conjuntos de cebadores específicos obtenidas de la secuencia genómica de SARS-CoV-2^{10,11}. Es así que, utilizando estos recursos disponibles, es posible que se pueda probar la presencia de SARS-CoV-2 de forma segura en un laboratorio certificado para Bioseguridad de Nivel II, mínimamente equipado con una máquina gPCR¹². Con el fin de desarrollar una plataforma molecular rápida, de alta precisión, de bajo costo, lista para el laboratorio y protocolos de laboratorio seguro para evaluar sujetos asintomáticos como un procedimiento de detección de primera mano para la detección del SARS-CoV-2.

El objetivo de este estudio fue identificar todos los recursos disponibles que se han desarrollado en los países de Sudamérica para el diagnóstico molecular de SARS-CoV-2.

Virología del coronavirus

El genoma del SARS-CoV-2 contiene elementos específicos que facilitan la replicación del virus y la formación de la nucleo-

cápside y la proteína S de anclaje. El genoma está compuesto de segmentos estructurales y no estructurales que son utilizados como dianas en pruebas moleculares mostrada en la Figura 1.

Pruebas moleculares sars-cov-2 en el mundo

El examen molecular que se está empleando en todo el mundo para la detección directa de la infección con el SARS-CoV-2 es la prueba de gPCR sobre los genes expresados por este virus. Se realizó el secuenciamiento del fragmento génico ORF1ab y de un fragmento de la proteína de la nucleocápside (NP). Para un diagnóstico confirmatorio de SARS-CoV-2, la OMS indica la detección del gen de la proteína E, seguida de la expresión del gen RdRp y la expresión del gen N solo si se requiriese un ensayo confirmatorio adicional^{13,14}.

Wang, *et al.* analizaron diferentes muestras: nasales, hisopados faríngeos, sangre, esputo, heces y orina provenientes de pacientes infectados con el virus en 3 hospitales de China. En pacientes graves en ventilación mecánica se recolectaron muestras del líquido de lavado broncoalveolar y de biopsia de cepillo de fibrobroncoscopio, para ser utilizado como fuente para la posterior extracción de material genético (ARN), RT-PCR y diagnóstico. Se realizó la técnica de reacción de cadena de polimerasa con transcripción inversa (RT-PCR) en la cual se determinó dos genes dianas del fragmento génico para la detección de SARS-CoV-2. El secuenciamiento incluyó el marco de lectura abierto 1ab (ORF1ab) y fragmento de proteína de la nucleocápside (NP). Se tomaron 1070 pruebas provenientes de 205 pacientes infectados por SARS-CoV-2, en la cual el lavado broncoalveolar demostró tener un 93% de positividad para diagnóstico de SARS-CoV-2, seguido por esputo, 72%; hisopado nasal, 63%; biopsia con cepillo de fibrobroncosopia, 46%; hisopado faríngeo, 32%; heces, 29% y sangre, 1%; mientras que ninguna prueba con muestra de orina resultó positiva^{15,16}.

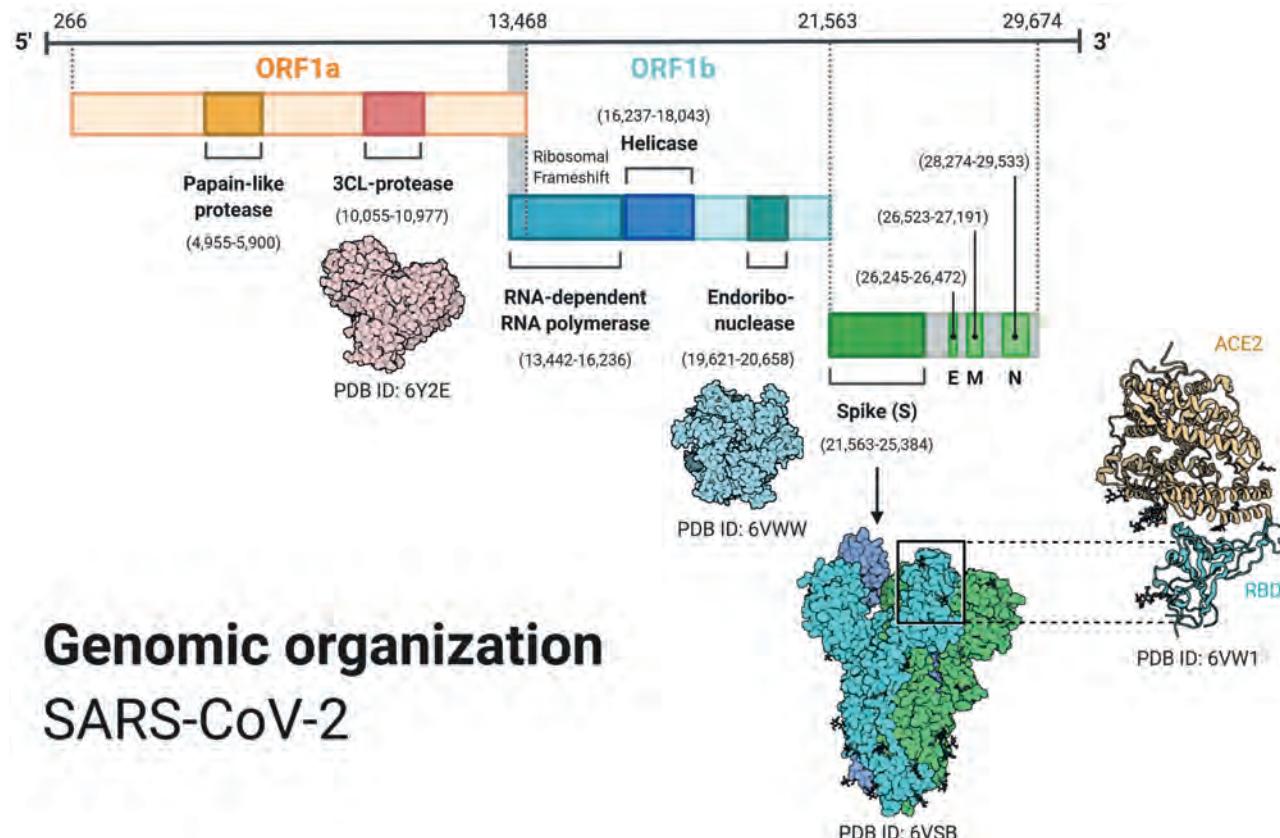


Figura 1. Estructura genómica del SARS-CoV-2

RT-PCR real time	<ul style="list-style-type: none"> ● Gen 5 ● Gen ORF1 SARS-CoV-2 ● Gen ORF1ab y N ● Gen ORF1ab, N y S ● Rp, E, P1 ● RdRp y N ● Gen N y E ● Gen E y N2
ELISA	<ul style="list-style-type: none"> ● Ig A ● Ig G
Immunocromatografía	<ul style="list-style-type: none"> ● Ig M/ Ig G
Quimioluminiscencia	<ul style="list-style-type: none"> ● Ig M ● Ig G

Tabla 1. Métodos Diagnósticos para SARS-CoV-2 en BRASIL.

Bajo la misma técnica, Liu *et al.* estudiaron una población de 4880 personas, las cuales 1875 fueron positivas para SARS-CoV-2, determinando diferentes tipos de detección dependiendo a qué gen diana iba dirigido. Para el gen ORF 1ab fueron positivos 40.98% y para el fragmento de proteína de la nucleocápside (NP) fueron positivos 39.80%. Siendo la muestra de lavado broncoalveolar de mayor positividad, 80% para NP, 100% para ORF1ab y 80% para ambos genes; seguido de esputo 49.12% para NP, 50.88% para ORF1ab y 49.12% para ambos genes; y hisopado nasal y faríngeo 39.64% para NP, 40.81% para ORF1ab y 38.25% para ambos genes¹¹.

Adicionalmente, la utilización de primers para la detección de SARS-CoV-2 para poder diferenciar de otros SARS-CoV fue propuesta por Corman *et al.* En las cuales se diseñan fragmentos de secuenciamiento genético para ORF1ab RdRp para la posición 15361-15460 nts; para el fragmento de la proteína N en la posición 28555-28682 nts; y para el gen de la proteína E en la posición 26141-26253 nts. Para la muestra obtenida proveniente de Wuhan en el 2019 (NM908947 Wuhan-Hu-1) comparadas con otras muestras virales (NC_004718 Sars-CoV) y según los primers diseñados se logró demostrar que todos eran una especie de SARS-CoV y de la subfamilia betacoronavirus. Ante ello se propuso analizar con otras muestras virales de la misma subfamilia, entre ellas, muestras que provenían de la ciudad de Wuhan como, Beta-CoV/Wuhan/PBCAMS-WH-01/2019|EPI_ISL_402123; Beta-CoV/Wuhan/IVDC-HB-01/2019|EPI_ISL_402119; BetaCoV/Wuhan/IVDC-HB-04/2020|EPI_ISL_402120; BetaCoV/Wuhan/IVDC-HB-05/2020|EPI_ISL_402121 y BetaCoV/Wuhan/WI-V04/2019|EPI_ISL_402124; y muestras virales provenientes de murciélagos, MG772933 Bat SARSrelated Cov (bat-SL-CoVZC45) y NC_014470 Bat SARS-related CoV (BM48-31/BGR/2008). Demostrando variación en la secuencia de nucleótidos para los genes RdRp y la proteína de la nucleocápside, pero sin encontrar diferencia para los genes de la proteína E, por lo cual se concluye la variabilidad con respecto a la secuencia de nucleótidos entre la misma familia de SARS y su importancia para el diseño y estandarización de primers para la detección específica de SARS-CoV-2 para su diagnóstico¹⁰.

Sin embargo, el Centro para el Control y la Prevención de Enfermedades (CDC por sus siglas en inglés) en los Estados Unidos utiliza un método de detección diferente a lo establecido por la OMS. Esta está enfocada en la detección principal del gen N1, N2, N3 y RP dando resultado positivo con valores de < 40.00 Ct¹⁷.

Métodos

El estudio presenta un registro nacional de investigación en salud con código EI00001613. Es de tipo observacional, descriptivo, transversal y retrospectivo. La revisión sistemática del presente estudio abarca criterios para la inclusión de las diferentes pruebas moleculares realizadas en Latinoamérica para la detección del SARS-CoV-2.

Criterios de elegibilidad

Se cuenta con revisión bibliográfica internacional de artículos publicados en las bases de datos en línea PUBMED (<https://pubmed.ncbi.nlm.nih.gov/>) y SCIELO (<https://scielo.org/>), seleccionando artículos de los últimos 5 años (2015-2020). Se utilizaron los siguientes términos de búsqueda según terminología DeCS BIREME: COVID-19; Virus SARS; Diagnosis; RT-PCR. Se incluyeron estudios en idiomas de inglés, portugués y español.

Estrategia de búsqueda

Se realizaron las siguientes búsquedas: (COVID-19 OR SARS-CoV-2 OR Coronavirus OR COVID-19 Testing) AND (RT-PCR OR Diagnosis OR Polymerase Chain Reaction OR COVID-19 Nucleic Acid Testing OR Real-Time Polymerase Chain Reaction OR Reverse Transcriptase Polymerase Chain Reaction OR Multiplex Polymerase Chain Reaction OR PCR). Con filtros de búsqueda para: Abstract, Free full text, Classical Article, Review, in the last 5 years, Humans, English, Spanish, Adult: 19+ years. Las búsquedas se complementaron con revisiones específicas de las siguientes páginas web: Organización Mundial de la Salud (<https://www.who.int/es>), Agencia Nacional de Vigilancia de Salud-Brasil (<https://www.gov.br/anvisa/pt-br>), Federación Médica de la Provincia de Buenos Aires-Argentina (<http://www.femeba.org.ar>) y Ministerio de Salud de Perú (<https://www.gob.pe/minsa/>). Ministerio de Salud de Bolivia (<https://www.minsalud.gob.bo/>).

Pruebas moleculares en Sudamérica

Las situaciones variadas de cada país en sudamérica los ha llevado a la utilización de pruebas diagnósticas como parte de un plan estratégico complementario para el SARS-CoV-2 en caso de que los reactivos para la prueba de RT-PCR no se puedan sustentar durante el tiempo.

Para realizar las diferentes pruebas de detección de SARS-CoV-2 deben primero tener la presencia de criterios clínicos, como la afectación de vía respiratoria asociada a fiebre, o sensación febril, tos, secreción nasal, dolor de garganta o di-

Ensayo	Gen	Alcance	Límite inferior detectable	Muestras	Condiciones de almacenamiento
OMS (20,23)	E	Primer ensayo	3,9 copias/reacción	Hisopo nasofaringeo y orofaringeo o muestras de tracto respiratorio inferior (esputo, aspirado endotraqueal y/o lavado broncoalveolar)	≤5 días (2 a 8 °C)
	RdRp	Primer ensayo confirmatorio	3,6 copias/reacción	Hisopos nasofaringeos y orofaringeos, esputo, aspirados del tracto respiratorio inferior, lavado broncoalveolar y lavado nasofaringeo o aspirado nasal	5 días (≤70 °C; hielo seco)
	N	Segundo ensayo confirmatorio	N/A		
CDC (17)	N1/N2/N3	Ensayo Combinado	1,0-3,2 copias/µL	Hisopos nasofaringeos y orofaringeos,	≤4 días (4 °C)
	RNase P	Ensayo Control	N/A	esputo, aspirados del tracto respiratorio inferior, lavado broncoalveolar y lavado nasofaringeo o aspirado nasal	4 días (≤70 °C)
Brasil (19) Argentina (20)	E	Primer ensayo	3,9 copias/reacción	Hisopo nasofaringeo y orofaringeo y muestras de tracto respiratorio inferior (esputo, aspirado endotraqueal y/o lavado broncoalveolar)	≤5 días (2 a 8 °C)
	RdRp	Primer ensayo confirmatorio	3,6 copias/reacción		5 días (≤70 °C; hielo seco)
	N	Segundo ensayo confirmatorio	N/A		
Uruguay (21)	N	FAM SARS-CoV-2	Ct<<35	Hisopado nasofaringeo y/o hisopado orofaringeo. Lavado / aspirado nasofaringeo o aspirado nasal (2ml.-3ml.)	2-8 °C por hasta 72 horas después de la recolección. Retraso en la extracción del ARN, almacene las muestras a -70 °C o menos.
	RNasa P	HEX (Control Interno)	N/A		
Perú (23,24)	RdRp GAPDH	Kit colorimétric LAMP	Positivo: Color amarillo Negativo: Color rojo	Hisopado nasofaringeo	Cadena de Frio 2 a 8 °C
Colombia (10) Chile (10)	E	Primer ensayo	5.2 copias RNA/reacción	Hisopado Nasofaringeo e Hisopado	
Ecuador (10)	RdRp	Ensayo confirmatorio	3.8 copias RNA/reacción	Orofaringeo en tubo MTU (medio de transporte universal) + Muestras de vía aérea baja (esputo, succión endotraqueal (Parte II) o lavado broncoalveolar)	

Tabla 2. Comparación de pruebas moleculares para diagnósticos para SARS-CoV-2

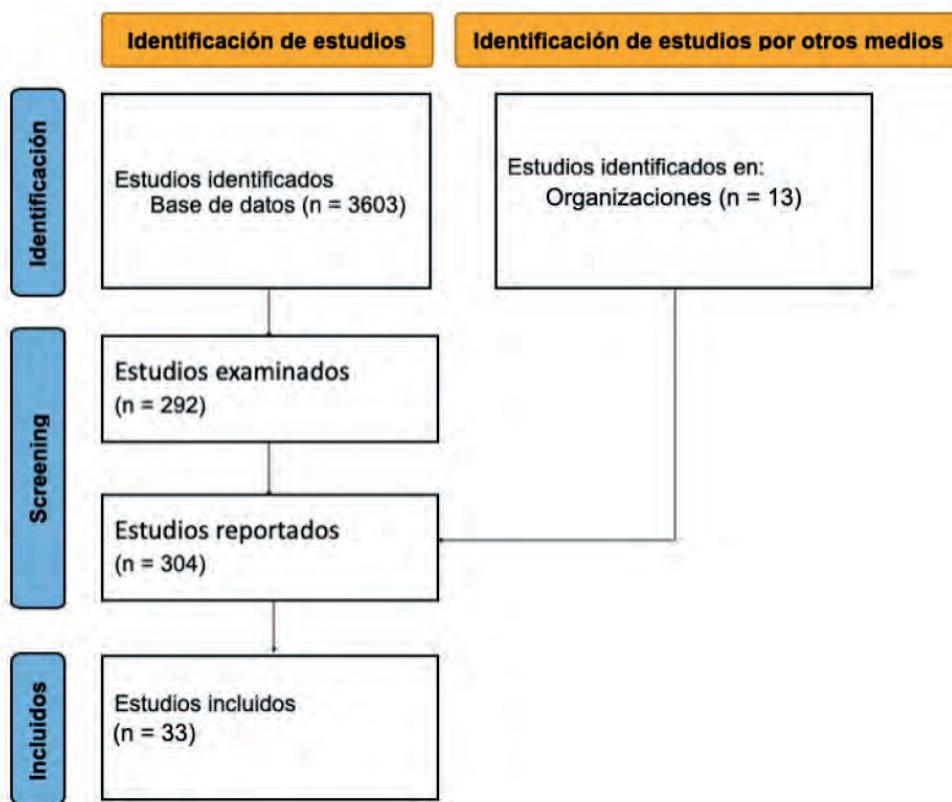


Figura 2. Bases de datos y estudios consultados para la realización de esta revisión.

ficultad para respirar. En aquellos casos se realizará la prueba de RT-PCR para detección de SARS-CoV-2, influenza y presencia de virus sincitial respiratorio¹⁸.

En Brasil se utilizan diferentes pruebas de diagnóstico aprobados en un informe de la Agencia Nacional de Vigilancia Sanitaria (ANVISA) en la fecha del 12 de abril del 2020, en la cual se detallan diferentes pruebas recolectadas en base de datos de ANVISA¹⁹ (Tabla 1).

En un informe aprobado el día 21 de abril del 2020 en Argentina por la Administración Nacional de Medicamentos, Alimentos y Tecnología Médica (ANMAT) se utiliza como método de diagnóstico la técnica de RT-PCR. Los genes a los cuales apuntan la detección son; N, E, S, y RdRp, utilizando gabinetes de bioseguridad en instalaciones BSL-2 o su equivalente²⁰.

En Uruguay se ha planteado la utilización de una prueba RT-PCR Real TM Fast (HEX) desarrollada *in house* para la detección y diagnóstico de SARS-CoV-2. El principio de la técnica se basa en la detección del gen que codifica la proteína N, gen humano de la RNasa P (control humano interno), pudiendo utilizar dos tipos de termocicladores, ROTOR GENE y ABI 7500. Dando una muestra positiva ct <35 con un control positivo de <35²¹.

En Perú a mediados de septiembre se empezó a utilizar la prueba molecular rápida del tipo LAMP (amplificación isométrica mediada en lazo), la cual disminuye el tiempo de espera para el diagnóstico de SARS-CoV-2, la cual lleva un resultado en dos horas. Esta prueba de PCR-LAMP se enfoca en la detección también del gen N, ORF 1ab y S, demostrando una sensibilidad entre 80 y 100%, y una especificidad entre 73 y 100% para el diagnóstico de SARS-CoV-2²².

Países como Chile, Ecuador y Colombia en la detección del SARS-CoV-2 emplean la técnica de RT-PCR elaborada por Cormann y colaboradores en Berlín, la cual se basa en la detección del gen de la proteína E principalmente para luego dar una confirma-

ción de un segundo ensayo para el gen RdRp (10). Los diferentes ensayos establecidos en cada país se detallan en la tabla 2.

En Bolivia las pruebas de diagnóstico han sido manejadas con métodos moleculares, entre ellos la prueba de PCR que detecta 2 marcadores del genoma del virus SARS-CoV-2 el gen E y gen RdRp.^{25,26} En pruebas rápidas moleculares el país adquirió xpert Xpress SARS-CoV-2 test (cepheid) y el abbott ID NOW COVID-19 test (Abbott)^{27,28}.

En Trinidad y Tobago las pruebas de mayor alcance y uso han sido las pruebas de antígeno para las cuales se piden muestra tipo nasal y nasofaringeo, y las pruebas de amplificación de ácido nucleico para el SARS-CoV-2 que identifican específicamente las secuencias de ARN con muestras de tipo nasal, nasofaringeo, orofaringeo, esputo y saliva^{29,30}. Sin embargo, se han implementado métodos diferentes para amplificar los ácidos nucleicos y detectar el virus que incluye reacción de amplificación de endonucleasa de mellado (NEAR), amplificación mediada por transcripción (TMA), amplificación isotérmica mediada por bucle (LAMP), amplificación dependiente de helicasa (HDA), repeticiones palindrómicas cortas agrupadas regularmente interespaciales (CRISPR) y amplificación de desplazamiento de hebra (SDA)³¹.

En Paraguay en asociación con la OPS se estableció como técnica estándar el uso de la prueba de Reacción en Cadena de la Polimerasa en tiempo real (RT-PCR) para la detección del SARS-CoV-2 con muestras de hisopado nasal y faringeo recolectadas mediante técnica estándar en medio de cultivo viral y transportadas al centro de referencia, en cadena de frío. Siguiendo las recomendaciones de la OPS, Paraguay optó por la detección de dos genes: el gen E (cuya presencia indica que pertenece a la familia de beta coronavirus) y RdRp (cuya presencia es específica para el SARS-CoV-2 y sirve como confirmación)^{32,33}.

Conclusiones

Las pruebas moleculares están basadas en la técnica de transcripción reversa seguida de la reacción en cadena de polimerasa cuantitativa (qRT-PCR) denominada como el Gold estándar para detección de SARS-CoV-2. Esta prueba detecta genes conservados del virus como el gen RNA dependiente de RNA polimerasa (RdRp), el gen de la cápside (E) y el gen de la proteína Spike (S); actualmente existen kits moleculares en los que se identifica más de un gen, mejorando así la especificidad y eficiencia de la prueba. Para la detección de genes virales se usan muestras de hisopado nasofaríngeo, esputo y lavado bronquio-alveolar de una persona infectada, siendo estos dos últimos los de mejor sensibilidad; esta técnica, en algunos casos, permite la detección incluso hasta una semana antes del inicio de los síntomas. Además del qRT-PCR, existe otra técnica molecular siendo implementada recientemente para la detección de SARS-CoV-2, la transcripción reversa seguida de una amplificación isotérmica mediada por lazo (RT-LAMP) desarrollada por Baek y colaboradores a inicios de este año. En este ensayo se utilizan *primers* diseñados para amplificar genes del virus SARS-CoV-2, como por ejemplo el gen de la nucleocápside del RNA viral, como es mostrado en otros estudios, los genes ORF1ab y Spike. RT-LAMP genera resultados en aproximadamente 30 minutos y es interpretado por una evaluación colorimétrica que no tiene reacción cruzada aparente con otros coronavirus, virus de influenza u otros virus respiratorios. Tanto qRT-PCR como RT-LAMP tienen alta especificidad en casos de bajo o nulo sesgo operacional, sin embargo, la existencia de un error en la toma de muestra puede dar como resultado un falso negativo siendo este uno de los problemas más frecuentes. Eventualmente la falta de experiencia del personal de colecta, dan como resultado una amplificación insatisfactoria o un resultado falso negativo. Un paciente falso negativo es un riesgo para la salud pública por la capacidad de diseminación, obstaculizando así el objetivo de contener la propagación del virus.

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Conflictos de interés

Los autores declaran no tener conflicto de interés.

Declaración de ética

Los autores confirman que están de acuerdo con las políticas éticas de la revista. No se requirió aprobación ética ya que esta es un artículo de revisión sin datos de investigación originales.

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NEWS AND VIEWS / NOTICIAS Y OPINIONES

A scientific note of pest-insects associated with stingless bee hive *Melipona eburnea* in the Ecuadorian Amazon Region

Una nota científica de insectos-plaga asociados con las colmenas de abejas sin aguijón *Melipona eburnea* en la región amazónica ecuatoriana

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2348

Resumen: Bees are the primary pollinators in nature. However, climate change, excessive use of fertilizers and invasive species have caused the decline of bee colonies. Therefore, this study aimed to analyze the presence of pests in colonies of *Melipona eburnea*. For this, the colonies of *M. eburnea* were examined during the honey extraction process. We found 4 different pests associated with the physical conditions of colonies and the fragile defense of the bees against the invaders. In conclusion, this report of the presence of pests is to alert researchers and meliponicultures to prevent the decline of stingless bees.

Palabras clave: Honey production, meliponiculture, propolis, stingless bees.

Abstract: Las abejas son los principales polinizadores en la naturaleza. Sin embargo, la influencia del cambio climático, el uso excesivo de fertilizantes y especies invasoras han provocado la disminución de sus colonias. El objetivo del presente estudio fue analizar la presencia de plagas en colonias de *Melipona eburnea*. Se examinaron las colonias de *M. eburnea* durante el proceso de extracción de miel. Cuatro plagas diferentes fueron encontradas, asociadas con las condiciones físicas de las colonias y la frágil defensa de las abejas contra los invasores. En conclusión, este informe de plagas es para alertar a los investigadores y meliponicultores y evitar el declive de las abejas sin aguijón.

Key words: Abejas sin aguijón, meliponicultura, producción de miel, propóleos.

Introduction

Bees are a group of efficient pollinating insects since they visit the flowers constantly; appreciating this food habit, they have come to offer a significant ecological and economic importance¹. In this context, the interaction between plant-pollinator ensures the survival and reproduction of plants². However, interactions that depend highly on a specific type of pollinator, there will be a high vulnerability of rupture and subsequent collapse when it is absent. An example of pollinators are bees of the Meliponini tribe that are widely distributed in the Neotropics and one of Melipona genus; it has been reported 16 species in Central America and 60 in South America³. In Ecuador, Colombia, Venezuela, and Brazil, some species of this genus have been documented⁴.

In Ecuador, the use of these bees for the production of honey is not widespread, and it has been recorded that in the provinces of Zamora, Morona Santiago and Loja are dedicated to meliponiculture with six species; *Melipona eburnea* (Friese, 1900); *M. cf. indecisa*, *M. mimetica* (Cockerell, 1919); *M. rufiventris* (Lepeletier, 1836) and two unidentified species⁵. Unfortunately, cases have been reported in which the colonies disappear, but in most of these, the reason is unknown⁵.

One of the reasons for the decline in bees is viruses that are transmitted by mites⁶⁻⁹. There are few studies on parasites (virus vectors) and pests that can affect stingless bees compared to *A. mellifera*¹⁰, even though the oldest bee fossil that has been found corresponds to a stingless one^{2,11}. In this study, we analyzed the presence of pests and parasites in *M. eburnea* from meliponaries in Porotoyacu community of the Ecuadorian Amazon Region.

M. eburnea was recollected manually of 11 boxes in the Porotoyacu community ($0^{\circ}52'31.25''$ S and $77^{\circ}44'54.37''$ O, 811 masl), Napo province, Ecuador. The community is responsible for extracting honey in an artisanal way, so the hives are built by hand. These are placed in wooden boxes on top of their roofs so that the bees can take advantage of the grape plantations near their homes. We collected workers of stingless bees, pollen and propolis, which were placed in plastic jars and airtight covers Ziploc®, respectively. These were taken to the laboratory and stored in a 96% alcohol solution. The Motic® optical microscope model BA210 and a Motic® stereoscope model SMZ168 were used to analyze the samples. Keys corroborated the bee's taxonomic identification following^{12,13}.

After analyzing and recognizing the organisms present in the samples taken, four pests of the stingless bee colony were identified. One of the specimens collected in the field was identified as part of the insect order Psocoptera (without wings; apterous) (Figure 1, A). All the species described to date (5000) are free-living and do not have parasitic characteristics^{14,15}. According to several studies, Psocoptera feeds on microorganisms, mainly fungal organisms, which are agents in the process of organic matter degradation; in other words, the presence of these insects can be considered as an indicator of some decomposition process¹⁶. probably due to the disproportionate dimensions of the box that contained the colony of bees and the intervention of some abiotic factors, there was a process of decomposition of organic matter by fungi and other microorganisms, causing Psocoptera to enter into the hive. This decom-

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position could spread to the bee-hive, where the stability can be compromised, generating that these insects at this point might be considered to be pests. The microenvironments present in the Amazon forests, characterized by the density of forests, high temperatures, humidity, and shaded areas, are ideal for developing microorganisms that serve as a source of food for the Psocoptera. On the other hand, the principal predators of Psocoptera are spiders, Coleoptera and/or Hemiptera¹⁴, we observed spiders around the box (bee-hive), so it can be interpreted that maybe Psocoptera was escaping from them.

The colony of *M. eburnea* was also infested with adult hoverflies (Diptera, Syrphidae) (Figure 1, B), that were found in the propolis, and according to Moquet L 2018¹⁷, these invertebrates depend on pollen (a substance rich in proteins consumed by females for reproduction) and nectar (a substance rich in sugars consumed by females and males for obtaining energy). In this context, we can suggest that the hoverflies were stealing the floral resources of the colony, and the stingless bees were defending themselves, killing them.

Another organism found in the colony was a Lepidoptera: Noctuidae (larva) (Figure 1, C), with almost 300 species around the world being considered as pests to crop in the majority of cases¹⁸. However, there is no information about Lepidoptera found in the colony of stingless bees or honeybee of the *Apis* genus. Furthermore, Lepidoptera feed with herbaceous plants and in the colony, no remains of herbs that could serve as food were observed, so it is inferred that perhaps some Lepidoptera left eggs inside the colony due to the ample space in the hive bees.

Finally, an ant (Hymenoptera: Formicidae), an adult insect (Figure 1, D) was observed in the hive of bees *M. eburnea*. The presence of these in-

sects has been detailed in the literature,

such as *Camponotus pennsylvanicus* Degeer¹⁹ and *Solenopsis invicta* Buren^{20,21}. It has been described that these can be considered as a pest because they can exterminate whole colonies of bees. Experts and beekeepers recommend the use of extra protection in places where ants can climb and stay. Also, it is helpful to use materials such as slaked lime, ash, and oil with water. It's important to remember not to use any chemicals because they are toxic to these insects²².

Herein we provide the first report of four possible pests of meliponaries in the Amazon Region Of Ecuador to alert meliponicultures and researchers to be vigilant to prevent the diffusion of these pests throughout Ecuador and Amazon Region.

2349

Conclusions

Specimens of the orders Psocoptera, Diptera, Lepidoptera and Hymenoptera were founded, which according to several studies, can be considered as pests for the bee colony, mainly involved in processes of decomposition of organic matter and competition for resources. However, future work will investigate all impacts in lifestyle and /or diseases in bees due to these invasive species. Furthermore, we hope that our research will be helpful to motivate similar studies in Melipona bees that are used for the production and commercialization of honey in the Amazon region.

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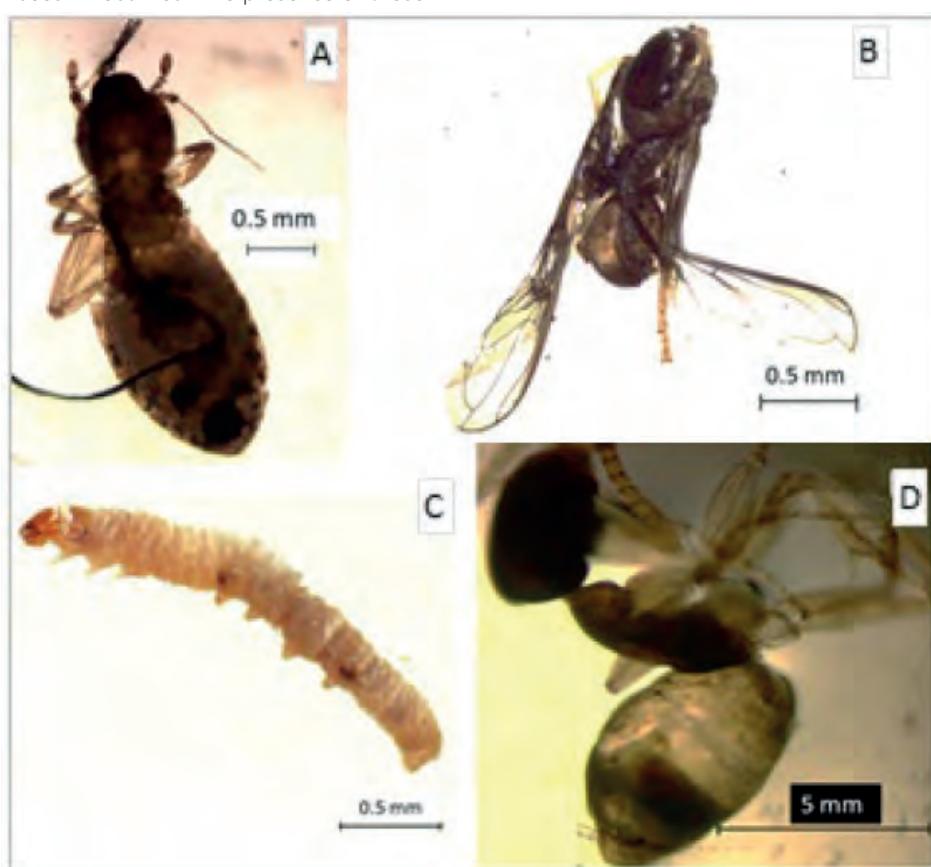


Figure 1. Pests in the colonies of *Melipona eburnea*. A: Psocoptera, B: Diptera, Syrphidae, C: Lepidoptera, Noctuidae (larvae), D: Hymenoptera, Formicidae, an adult.

Author Contribution Statement

All authors conceived, designed research, conducted experiments, and wrote the manuscript. All authors read and approved the manuscript.

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NEWS AND VIEWS / NOTICIAS Y OPINIONES

Molecular Photoacoustic Imaging

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Abstract: Medicine has gone through several challenges to make it much more accurate and thus prolong the human being's life. A large part of this challenge is diseased, so early detection can help carry out treatment on time. There is a technology that allows detecting an abnormality within the body without using an invasive method. Ultrasound is a diagnostic test used to scan organs and tissues through sound waves. Although this technique has been widely used, the results are not desired because the images generated are not high resolution. On the other hand, X-rays are used because it presents an image with a much higher resolution than other techniques based on light waves or ultrasound; despite this, they are harmful to cells. In consequence of this problem, another method called molecular photoacoustic imaging has been implemented. This technique bridges the traditional depth limits of ballistic optical imaging and diffuse optical imaging's resolution limits, using the acoustic waves generated in response to laser light absorption, which has now shown potential for molecular imaging, allowing the visualization of biological processes in a non-invasive way. The purpose of this article is to give a critically scoped review of the physical, chemical, and biochemical characteristics of existing photoacoustic contrast agents, highlighting the pivotal applications and current challenges for molecular photoacoustic imaging.

Key words: Photoacoustic Image, Biomedicine, Clinical Imaging, Optical Imaging.

Introducción

Optical imaging plays a crucial role in biomedicine because it provides a convenient way to visualize and understand biological events¹, verifying the body's proper functioning and early detection of diseases². Conventional images are known to have features that limit their ability to obtain *in vivo* images of tissues. Several widely used optical images are used, such as intrinsic signals³, magnetic resonance imaging (MRI)⁴, Spectroscopy⁵, laser staining contrast images⁶, and used. Optical images that use light to visualize cells have the characteristic that light undergoes a significant dispersion in biological tissue, which requires additional effort, so this method is relatively fast but limited⁷. Microscopy and other photon-using methods can provide high-resolution images. They achieve noise-free detection of individual absorbent nanoparticle shots, giving the technique a high potential for tissue cell applications, but only up to a depth of ~1mm in most of these biological tissues⁸.

MRI has been one of the highest resolution methods applied so far⁹. This technique manages to emit a 3D image¹⁰. It employs a mechanism in which the patient undergoes a magnetic field, so the protons, leading hydrogen, are aligned to this field, after which an electromagnetic pulse of radiofrequency is applied. This disturbs the balance of these atoms and introduces a transient phase of magnetization, which is perceived as a radio wave and transformed into an image¹¹. One of its most significant limitations is that at the microscopic level is a deficiency in its detection sensitivity¹².

Ultrasound images such as ultrasound, because they do not use radiation, are widely used in pregnant women¹³, which has had a high impact on producing two-dimensional up to three-dimensions ultrasound images¹⁴; this diagnostic test is based on emitting high-frequency sound waves. These waves travel through the body and bounce when colliding with density changes, allowing you to create an image¹⁵. Because they emit a longer wavelength and go further into the tissues without dispersing in large magnitude, although the ultrasounds do not have a high resolution¹⁶. Computed tomography (CT) and fluorescence have always-on signals, and it is often challenging to design them to have biomarker-induced changes¹⁷.

Also, they can provide depths of penetration by sacrificing spatial resolution¹⁸. The X-ray technique has been used to provide a clear image for diagnosing a fracture and detecting pneumonia¹⁹. This method consists of influencing a beam of X-rays into the body's tissues; this attenuation creates an overlapping shadow of the body region's internal structure to be studied. Thus a detector sensitive to X-rays transforms this transmitted fraction and converts it into an image¹⁶. Despite having a reasonably high resolution, the incidence of X-rays can cause mutations in fetuses, increasing the likelihood of developing cancer and cataracts, among other problems; that is why their exposure should be limited to the maximum¹⁸.

This method has different applications; it has been used to obtain preclinic images *in vivo* in small animals for various disease indicators. On the other hand, it has been applied significantly in cancer research: detection of primary tumors and molecular Characterization, therapeutic monitoring, identification, and evaluation of metastatic lymph nodes. This review focuses on molecular photoacoustic imaging (MPI), which has attracted increasing interest due to its specific advantages. This method allows to visualize and quantify biological processes at the molecular and cellular level in a non-invasive way, providing an opportunity to detect, stage, predict, and monitor diseases' development.

Methods for MPI development

MPI is a pop-up method that combines the optical image's high contrast with the ultrasound's high spatial resolution. The success of the MPI is based on the intrinsic properties of this physical process²⁰. During this procedure, the photo energy influenced by short laser pulses is absorbed by contrast agents, either exogenous or endogenous, partially converted into heat, resulting in increased broadband acoustic waves at MHz frequencies²¹. These waves can be detected by an ultrasound transducer on the tissue surface and reconstructed to form an image of the absorbed optical energy distribution and, therefore, the formation of acoustic photo imaging²².

Obtaining MPI relies on exogenous or endogenous contrast

agents to transform absorbed photons into heat. It is not invasive for the *in vivo* organism. Various inorganic nanomaterials have been shown, such as quantum dots (QD), carbon nanotubes, gold nanoparticles, and silver nanoplates²³. They are promising as contrast agents in MPI. Also, as sound waves are less dispersed in tissue than photons, MPI can overcome the limitations of traditional optical images. Some MPI methods have been developed to obtain images of biological tissues such as MPI using endogenous chromophores like hemoglobin, melanin, water or lipids²⁴, MPI using dyes²⁵, MPI with nanostructures like silver nanoplates²³, and molecular photoacoustic contrast agents (MPCA)²⁶. The use of the different contrast agents will depend on the depth you want to reach at the study time. MPI can reach a penetration depth of several cm with an order resolution of about 100μm²⁷. The perspective provides an overview of each method mentioned to help and provide valuable and current MPI information for future applications such as cancer.

Contrast agents

As mentioned above, a throbbing laser is used, and this range's depth will depend on its wavelength. Contrast agents absorb the laser to generate MPI, which can be endogenous or exogenous process²⁸. These agents must possess three physical photo properties: Their maximum absorbance wavelength must be between 680-950nm. To obtain a higher photoacoustic signal, Quantum fluorescence performance should be low to maximize energy dissipated through non-radioactive pathways. Another important property is that its extinction coefficient is higher than $10^4 \text{ M}^{-1}\text{cm}^{-1}$ to maximize the amount of light absorbed²⁹.

Endogenous

There are endogenous contrast agents, i.e., produced by

the body, including water and lipids, which are weak chromophores compared to hemoglobin, a protein of 64kDa, absorbs much more than the chromophores present in other tissues, has a wavelength range between 950nm-1400nm oxygenated and deoxygenated²⁸. Water has absorption bands of 970nm, 1200nm, and <1400nm, while lipids are at wavelengths of 930nm, 1040nm, 1210nm, and 1390nm. Although endogenous chromophores have long wavelengths, they need exogenous contrast agents for MPI to have a high resolution³⁰.

Exogenous

Exogenous contrast agents must comply with specific properties. Physical photo properties: high molar coefficient of extinction to maximize the amount of light absorbed; characteristic absorption spectrum to avoid confusion, even at low concentrations; have a wavelength between 650nm-950nm, among others³¹. Biological properties: orientation and biocompatibility must overcome cellular barriers, the size of the targeting molecules must be small to cross physiological barriers³². There are different types of exogenous chromophores, such as:

Nanostructures

Nanostructures have also been a method used as contrast agents in MPI. Combining optical images such as MPI is very useful for therapeutic tracking, thus having the potential to propel the nanomedicine field towards authentic personalized medicine³³. There are two main classifications according to their physical properties. The first group is based on surface plasmon resonance (SPR), specific property of certain metals such as gold^{34,35}. This property occurs when the surface-free loads of these nanoparticles oscillate with an electromagne-

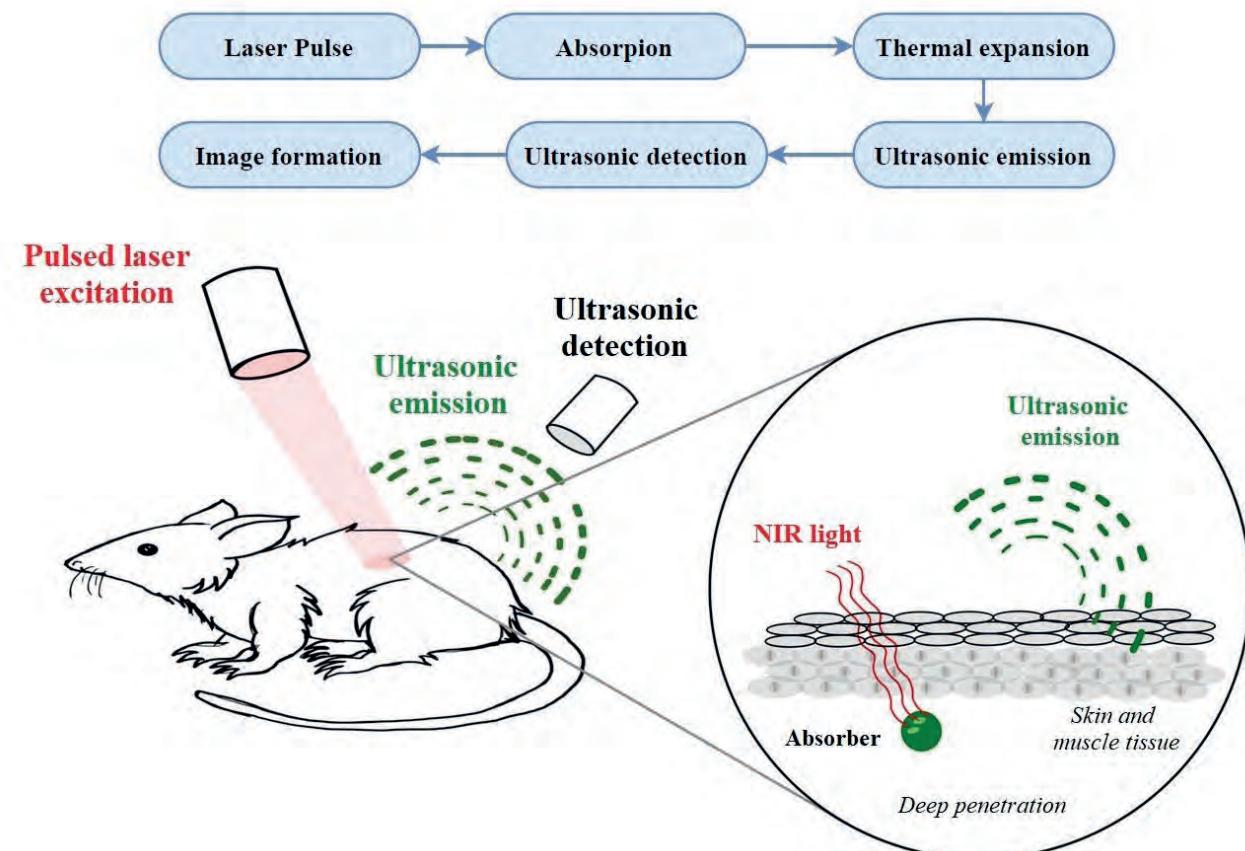


Figure 1. Schematic illustration, which shows the process of MPI.

tic field, leading to optical absorption³⁶. The second group is dyed-containing nanoparticles.

An example is a water-soluble indocyanine. Most of these contrast agents are encapsulated in layers of nanoparticles³⁶. The nanoparticles used have an absorption peak in the NIR region because tissue attenuation is lower at³⁷.

Molecular Photoacoustic Contrast Agents

MPCA can be classified into three types: Linear absorption (LA)⁴¹: In this case, the dye has a shorter excitation state than the laser pulse. There is no absorption in that state, and a linear dependence on the amplitude of the PA signal is observed. Saturable absorber (SA)⁴²: Its absorption in a state of excitation is negligible, but its lifespan is much longer than the laser pulse. Reverse-saturable absorber (RSA)⁴¹: In this type of canning agent, a nonlinear increase in absorption and PA response is observed by increasing laser creep⁴³.

Cyanine dyes

Cyanine dyes are an exogenous contrast agent. Two halves of nitrogen, indolin heterocycles, thiazole, or quinoline joined by a linear polymethine chain, may be composed of 1, 3, 5, or 7 carbons⁴⁴. Water-soluble indocyanine (ICG) has been extensively studied for in vivo fluorescence imaging due to its low toxicity⁴³. FDA approved the use of this contrast agent with nanoparticle encapsulation. Cyanine dyes have a molar extinction coefficient in a range between a Furthermore, quantum fluorescence performance (proportion of photons emitted relative to the number of photons absorbed) in a range of 11.30% to 4.39%⁴⁵.

Curcumin dyes

This dye is found in nature in the rhizomes of Curcuma plants⁴⁶. This plant is known to possess anti-inflammatory, purifying, antifungal, antibacterial properties⁴⁷. The boron difluoride derivative of natural curcumin (curcumin BF₂) is a compound with a high quantum fluorescence performance, which may exhibit an amplified photoacoustic contrast to the standard crystal violet compound has been widely used in molecular photoacoustic images⁴⁸. Curcumin BF₂ has a maximum wavelength of 498nm. Because this wavelength is less than recommended, a 4-dimethylaminophenyl group is introduced at the end ends of the main chain to increase its wavelength to 684nm⁴⁹.

BODIPY

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BODIPY contrast agents have been widely used as signaling molecules and in imaging⁵⁰. Despite this, nude BODIPY chromophores cannot be used in MPI because they have very high fluorescence. In this way, they have been combined with 1H-pyrrole (PyBODIPY) and PEG-400 to improve aqueous solubility to apply this compound in vivo imaging. These contrast agents' most important properties are: wavelength greater than 800nm, are non-photo-toxic, photo-stable, and have a high extinction coefficient⁵¹.

Biomedical applications

Molecular photoacoustic imaging is a technique that has various biomedical applications thanks to its advantages over other imaging methods. This technique is safe and effective in diagnosing diseases by providing images of different tissues' morphological structure and physiological characteristics. It has also been proposed as a tool to guide in vivo therapies^{52,53}.

Cancer Imaging

MPI is a non-ionizing technique that can be captured in real-time⁵⁴. For cancer screening, it is necessary to locate which regions of the body are infected with tumors. Because a tumor is a buildup of tissue, which has cells that undergo abnormal growth, this formation also needs nutrients that will be transported by blood; As mentioned above, hemoglobin is a dominant endogenous contrast agent in the optical window so, high contrast images of the microvasculature can be obtained around the tumor⁵⁴. Although hemoglobin is a potent contrast agent, it is necessary to use various exogenous chromophores; because tumors have leaky vascular systems, a low lymphatic drainage system, nanometer-sized contrast agents conjugated with targeted ligands such as peptides, antibodies have been used to bind to receptors that are in an over-exposed form in tumor tissue; with it, you get an image⁵⁵.

Imaging of Atherosclerosis Plaques

Atheroma plaques are an injury that affects the cardiovas-

Nanostructures	Similarities	Differences	Important Features
Gold	Link sources that allow covalent surface modifications to optimize biocompatibility. GNP's key advantages are to generate MPI signals that generally have orders of magnitude higher than small molecule dyes. ³⁸	The longer the time limit for establishing the impact of non-biodegradable MPI images is the longer ³⁵ .	It is based on surface plasmon resonance (SPR) ³⁹ .
Carbon		It is highly flexible ³⁸	There is a risk of cytotoxicity and inflammatory potential ³⁶ .
Organic		They are formed from small GNPs trapped within synthetic or natural amphiliids ⁴⁰ .	FDA approval of individual elements would not expedite approval of the synthesized product ³⁷ .

Table 1. Essential features of the most commonly used nanostructures for MPI applications.

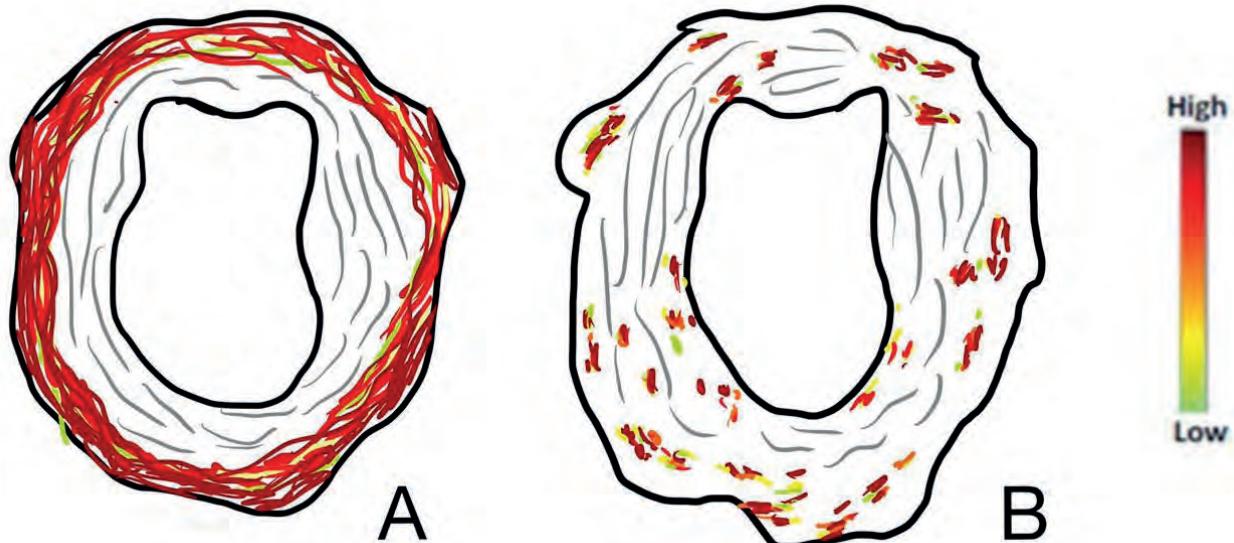


Figure 2. Ultrasound and photoacoustic imaging of an atherosclerotic rabbit aorta.

cular system, and these appear in the body due to the accumulation of low-density cholesterol (LDL), which causes the radius of the arteries. In some cases, the artery is so affected that it can explode from a lack of diagnosis. This has been one of the biggest causes of death in industrialized countries⁵⁴, in which the population tends to eat junk food. There are other methods for detecting atheroma plaques, such as angiography or ultrasound. However, they do not provide sufficient information. Also, angiography uses x-rays, so this study should be minimized to the maximum because it can be harmful to the patient. Intravascular and extravascular molecular photoacoustic imaging (IVPA) and extravascular (EVPA) have been shown to detect atheroma plaques thanks to their composition⁵⁴. Endogenous chromophores have been shown to contrast specific components present in this atheroma plaque, such as lipids, calcium deposits, macrophage content, and fibrous material; However, it is necessary to use exogenous contrast agents directed with biomarkers to intensify differentiation and improve image quality⁵⁴.

Similarly, MPI has been used to obtain stent images of the coronary arteries. This technique applies to arteries that have a severe case of atherosclerosis. Although stents are successful, they can bring restenosis and hyperplasia, so to treat it, you need imaging. In these cases, IVPA is used because it shows good penetration into the tissue and a high resolution. These images are taken *ex vivo*. Other methods are used to obtain *in vivo* images, but the stent is made of metal, which is very susceptible and challenging to obtain the image⁵³.

Conclusions

MPI is a modality with increasing interest that allows simultaneous imaging through endogenous chromophores present in exogenous tissues and contrast agents that visualize biological processes *in vivo*. These chromophores are of paramount importance in applying molecular photoacoustic images because they are responsible for absorbing energy. Compared to traditional optical images, molecular acoustic photo images have advantages because they detect acoustic signals that are much less attenuated than photons in tissues. In addition to using non-ionizing radiation and real-time images with high spatial resolution, technology also can perform a relatively inexpensive system. Due to these advantages, this

molecular imaging modality has allowed the detection of biological and pathological events *in vivo* at unprecedented tissue depths with enhanced fluorescence images that traditional optical images expose us to. For this reason, molecular acoustic photo images are in high demand due to their relatively high advantage and biosecurity in living organisms.

This method is expected to significantly improve deep tissue images of anatomical structures, disease-related biomarkers, or physiological processes to improve the diagnosis of life-threatening diseases. In addition to obtaining deep tissue images, the flexibility of this method opens up doors of integration with other functional remains for multiple purposes, such as multifunctional theragnostic platforms, studies in oncology, neuroscience, and cardiovascular diseases; preliminary clinical trials have focused mainly today on the detection, staging and therapeutic follow-up of cancer.

Competing interest

The authors declare that they have no competing interests.

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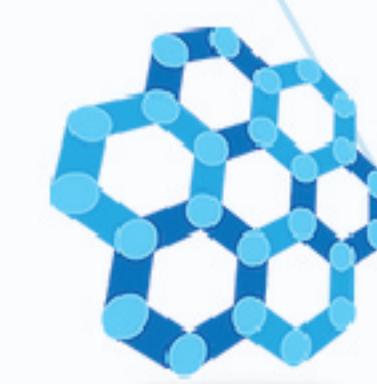


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