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Article

Autophagy induction using Resveratrol enhances the anti-cancer efficacy of Doxazosin in breast cancer cells.

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ABSTRACT

The combination of anti-cancer drugs improves effectiveness compared to the mono-therapy scenario by targeting key pathways synergistically or in an additive way. Doxazosin (DOX) and Resveratrol (RES) are reported to have an anti-cancer impact against different cancer cells. Aim: To evaluate the anti-cancer properties of Doxazosin and Resveratrol, each alone or in combination, in inhibiting breast cancer cell proliferation. Methods: MCF-7 cancer cells were seeded to a confluent monolayer and treated with 100, 50, 25, 12.5, 6.25, and 3.12 µM of each drug alone and as a combination. Cytotoxicity was evaluated using dimethyl thiazolyl diphenyl tetrazolium salt (MTT) assay and colony formation assay. The immunocytochemistry technique was conducted to evaluate caspase-3 and GABARAP expression. Results: All the drugs have a significant effect on cancer cell proliferation. The combination treatment of Doxazosin-Resveratrol has a more cytotoxic effect than each drug alone, as well as in colony formation of MCF-7 cancer cells. In combination treatment, caspase-3 had a higher expression pattern than other treatments. Resveratrol treatment elevated GABARAP expression in cancer cells, indicating the induction of the autophagy process. Conclusion: Although each drug has its characteristic result of a cytotoxic effect, the combination of Doxazosin and Resveratrol synergizes the inhibition of MCF-7 cancer cell proliferation.

Keywords: Resveratrol; Doxazosin; combination; MCF-7; Autophagy.

INTRODUCTION

Cancer is a group of diseases that starts when uncontrollable cell division occurs due to genetic and environmental disturbances. Usually, the cell division is controlled by checkpoints localized after each primary stage to ensure the integrity of the coping process and generate two identical cells without any errors. Cancer cells lose their checkpoint advantage due to several oncogenic factors, and abnormal cells are continuously generated [1]. Globally, according to the World Health Organization (WHO) final report, breast cancer is the most frequent cancer occurrence, estimated at 11.7% of all kinds of cancer in both males and females. In females

alone, it counts for about 24.5% of all types of cancer, with a mortality rate of approximately 15.5%. In Iraq, the total cancer cases showed that lung cancer occupies the higher records, which counts for 12.2%, followed by breast cancer, 11.4% in both genders. In contrast, in females, breast cancer was the primary type of cancer, with a 24% count for 3.3 million cases and a mortality rate of 15% for about one million women. Despite these horrible numbers, the average of the last five years was 30.1% for females, showing positive feedback for the rights of patients [2]. Doxazosin drug, an antagonist of the alfa-1 Adreno receptor, is key to cancer suppression and chemotherapy resistance [3]. It downregulates autophagy by activating the Phosphoinositide 3-kinases (PI3K)-Akt-mTOR signaling pathway in the human hepatic stellate cell line (LX-2) [4]. Doxazosin upregulates the autophagy process in a hypotriploid alveolar basal epithelial cell line (A549) and human lung cancer cell line (PC-9-NSCLS) through inhibiting methyladenine, which suppresses the autophagy process by inducing cytotoxic autophagy [5]. Resveratrol is a natural product that induces autophagy by suppressing the p52-mTOR- nuclear factor erythroid 2–related factor (2Nrf2) antioxidant pathway [6]. It also induces autophagy leading to apoptosis by activating the liver kinase B1 (LKB1)- AMP-activated protein kinase (AMPK) pathway and inhibiting the Phosphoinositide 3-kinases (PI3K)-AKT pathway, which results in the suppression of mTOR pathways in a human promyelocytic leukemia cell line (HL-60) [7]. It increases autophagic proteins like light chain protein (LC-II) and the Protein 62 (P62) sequestosome-1 (SQSTM1). Additionally, it plays other roles like mitochondrial degradation and Deoxyribonucleic acid (DNA) fragmentation in human leukemia cell lines (MOLT-4 and HL-60) [8]. The current study aimed to evaluate the cytotoxic effect of Doxazosin and Resveratrol as single pure drugs and as a combination of two drugs on breast cancer cell line (MCF-7). The study also aimed to find the doses that inhibit the proliferation to 50% (IC_{50}) of the MCF-7 cell line and find the best treatment with an anti-clonogenic effect compared with all treatments.

MATERIALS AND METHODS

Sterilization of instruments was performed by autoclaving, while other reagents, buffers, and biological materials were sterilized according to the Freshney tissue culture manual [9].

Preparation of MCF-7 Cell Line

Cell line (MCF-7) was delivered from ICCMGR and cultured in MEM; when the subconfluent monolayer was observed, the cells detached from the cultivation flask using 3ml of Trypsin-Versene® solution. New culture media (5ml) were added, including 10% serum in Falcone tissue culture. Cells and media are mixed by pipetting. The plastic lid was applied, and the plate was shaken gently for further mixing. Tissue culture cell plates were incubated at 37°C and 5% Co2 for 24 with continuous observation for standard growth characteristics, including cellular attachment, proliferation, and confluent monolayer. The plate which had abnormal characteristics was discarded. The cellular culture with positive growth features was maintained by pouring off the included media and adding new ones [9].

Preparing of Treatment (DOX, RES) Solution for Exposure

Stock solution for each drug was prepared according to its molecular weight as flow DOX (molecular weight 547.58). 1 μM stock was prepared by dissolving 4mg in 7.30ml. RES (molecular weight 228.24) stock solution 70 was prepared by dissolving 150mg in 6.26 ml. The Final work solutions (100, 50, 25, 12.5, 6.25, and 3.12 μM) were prepared by diluting the stock solutions by distilling water (D.W.)

Exposure of Treatment to MCF-7 Cell Line and Determination of Cytotoxicity

The cell line of MCF-7 was seeded in a 96-well plate at a concentration of 5 × 103 cells/well and incubated for 24h. After the confluent monolayer formed, the cells

were treated with components. The viability of cells was evaluated after 72h. The evaluation was performed by removing the media and adding 28µL of 2 mg/mL MTT solution. After incubation at 37°C for 1.5h, MTT solution was removed. The remaining crystals were solubilized by adding 130 µL of DMSO (Dimethyl Sulphoxide), followed by further incubation at 37 °C for 15 min with shaking. Using a microplate reader at a wavelength of 492 nm, the samples' absorbance was recorded, and for accuracy, the test was performed in triplicate. The cellular growth inhibition rate (cytotoxicity percentage) was calculated using the following equation[10]:

$$\text{Inhibition rate (I.R.)} = \frac{\text{O.D.Mean of control} - \text{O.D.Mean of Sample}}{\text{O.D.Mean of control}} \times 100 \quad (1)$$

Evaluating The Synergistic Effect of The Treatment Combination

The synergistic effect of the treatment combination was evaluated using Chou–Talalay analysis methods by CompuSyn software. Using doses and responses for single drugs treatments with two combination doses and responses at the same ratio 1:1 for the six doses (100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM and 3.12 µM) points. Depending on the normalized Isobologram, if CI < 1, combination treatment is recorded as a synergism effect; if CI =1, treatment was recorded as additive effects, and treatment was recorded as having an antagonism effect if CI >1 [11].

Colony Formation Assay

Colony formation inhibition determined in 6 well plates included 3ml of MCF-7 cell line at a concentration of 5000 cells/3ml. Plates were incubated at 37°C for 24h. The cells were treated with different agents at selected IC50 values (7.67 and 10.68) for Doxazosin, Resveratrol, and Doxazosin-Resveratrol combination. Media was discarded after 72h of treatment, and PBS was used for rinsing the cells. The cells were then fixed and stained with crystal violet previously prepared, followed by the final washing step to remove the excessive amount of the stain before photographing [12,13]. Colonies included more than 50% of cells alive were counted, and the survival percentage was calculated [at 14].

Immunocytochemistry Assay (ICC)

Apoptosis level was evaluated using an Immunocytochemistry assay staining kit (plus Poly-HRP Anti Rabbit IgG Detection System®. Elabscience-USA) to determine the surface antigen apoptosis markers CASP3 and Gamma-Aminobutyric Acid Receptor Associated Protein (GABARAP). The procedure was performed according to the manufacturer's instructions. Summarily, the monolayer culture of the MCF-7 cell line was seeded to confluence in an 8-well Cell Culture Slide. Then, the cells were fixed for 10 minutes using 4% neutral buffer formalin. Cells were then washed with PBS to remove any further amount of the fixative. Cells were then incubated under humidity twice for 10 min with a blocking reagent and for 60 min with different marker antibodies. Finally, the staining was performed using a horseradish peroxidase (HRP) Immunostaining kit. Photographs were taken and analyzed.

RESULTS

Cytotoxicity Assay of Treatment to MCF-7 Cell Line

Microscopically with the aid of MTT assay. It was clear that the MCF-7 cell line exposed to a treatment combination of Doxazosin-Resveratrol had the highest level of growth inhibition, while the Resveratrol treatment alone showed the lowest inhibitory effect on the cancer cell line at the lowest concentration of treatment. The cytotoxicity percentage (Figure 1, Figure 2) for different treatment concentrations on the MCF-7 cell line was evaluated using an MTT assay. Findings showed that

the cytotoxicity of all drugs was dose-dependent, and increased concentration of all treatments resulted in an increased percentage of cytotoxicity after incubation of the MCF-7 cell line with all different treatments. Results also showed that Resveratrol had the lowest percentage of cytotoxicity (28.6%) when the cancer cells were incubated with the lowest concentration (3.12µM) of all treatments. The combination of Doxazosin-Resveratrol treatment showed the highest percentage of cytotoxicity (96.6%) at a high treatment dosage (100µM). The Doxazosin-Resveratrol treatment combination also had a cytotoxicity percentage (95.3%) larger than all other treatments, even in lower concentrations (50µM). Results showed that the MCF-7 cell line is more resistant to low concentration (3.12µM) of all treatments as a single drug, especially Resveratrol, and lower resistance to the treatments as a combination, especially to the combination of Doxazosin-Resveratrol. The combination treatment (Figure 2, Table 1) results for six points (doses) showed that the Doxazosin-Resveratrol combination index (CI) was below 1, which means all the points had a synergistic effect. This result makes the Doxazosin-Resveratrol treatment work as an anti-proliferative at lower doses than other treatments.

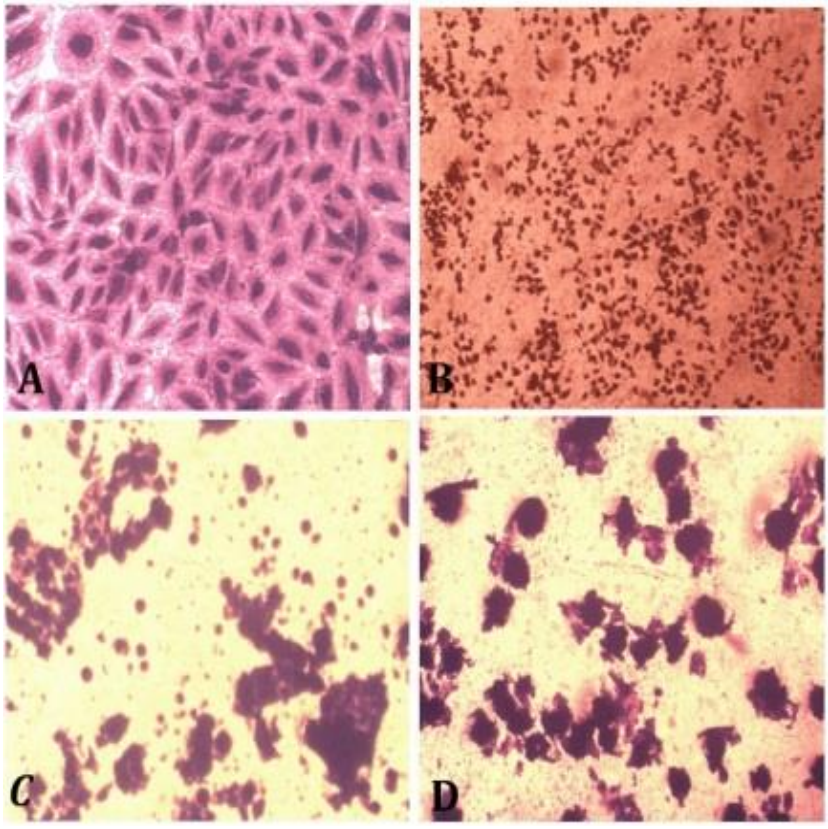


Figure 1. Microscopic images for MCF-7 cell line using MTT assay showing the cytotoxic effect of different treatments: A) Control, B) Doxazosin, C) Resveratrol, D) Doxazosin-Resveratrol combination.

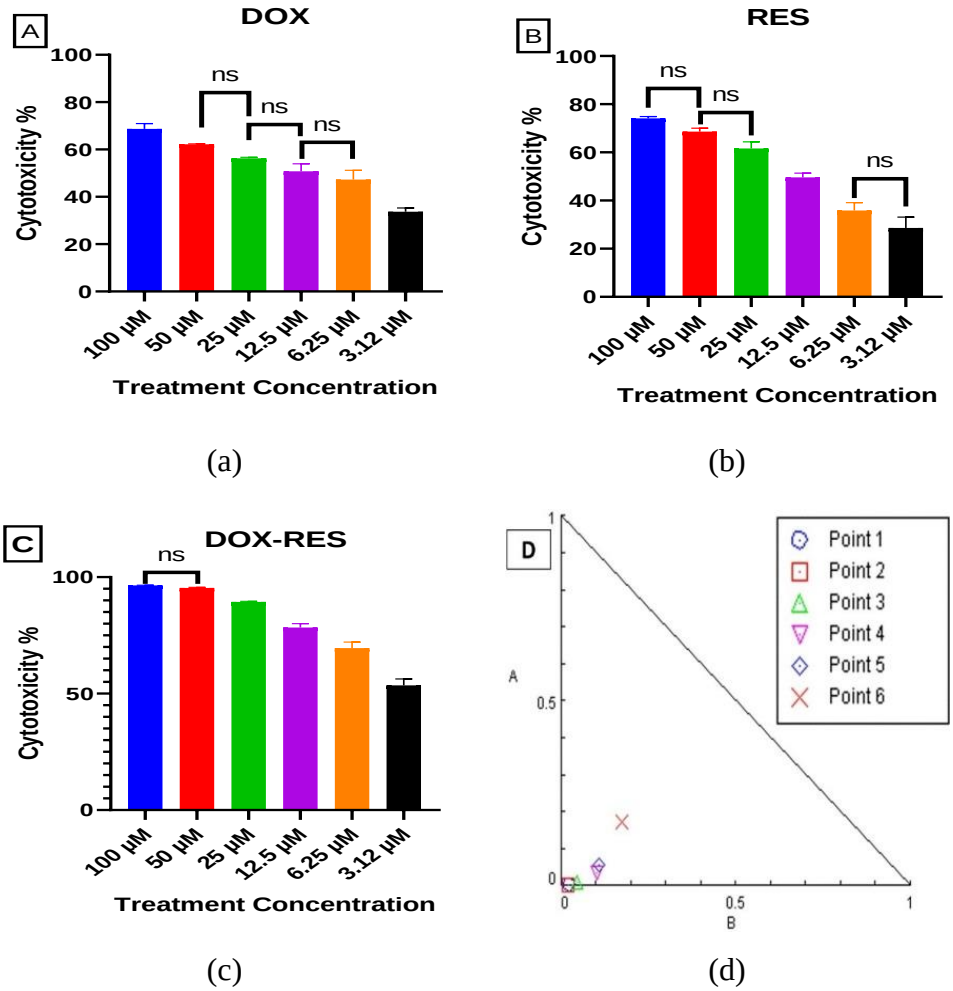


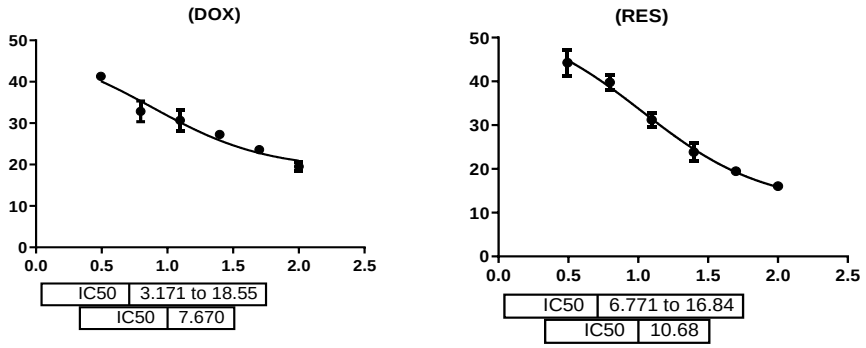
Figure 2. Cytotoxicity percentage and synergistic isobolograms for drug combination treatments and concentration on MCF-7 cell line using MTT assay and graph prism software A) Doxazosin B) Resveratrol C) Doxazosin-Resveratrol combination. D)Normalized Isobologram for D+R drugs combination. ns= non-significant p<0.05.

Point	DOX	RES	CI	Effect
1	100	100	0.02677	Synergism
2	50	50	0.02403	Synergism
3	25	25	0.05688	Synergism
4	12.5	12.5	0.13773	Synergism
5	6.25	6.25	0.16759	Synergism
6	3.12	3.12	0.34605	Synergism

Table 1. Effect of drug combinations with CI value for each dosage point

Median Inhibitory Concentration (IC50) of Treatment to MCF-7 Cell Line

The median inhibitory concentration (IC_{50}) is essential to evaluate only single doses of treatment that inhibit 50% of a cell line from proliferation. Results showed that in a single treatment (Figure 3), Doxazosin had the lowest IC_{50} (7.67 µM). In comparison, Resveratrol showed larger IC_{50} (10.68 µM) than Doxazosin, while the results showed that both drug combinations had an effective dose curve larger than the single drug treatment (Figure 4); this makes the drug combination work more effectively at the same IC_{50} of every single drug due to the synergism effect of the combination.



(a) (b)

Figure 3. IC50 of treatments to MCF-7 cell line presented using MTT assay and GraphPad Prism software; RES) Resveratrol DOX) Doxazosin.

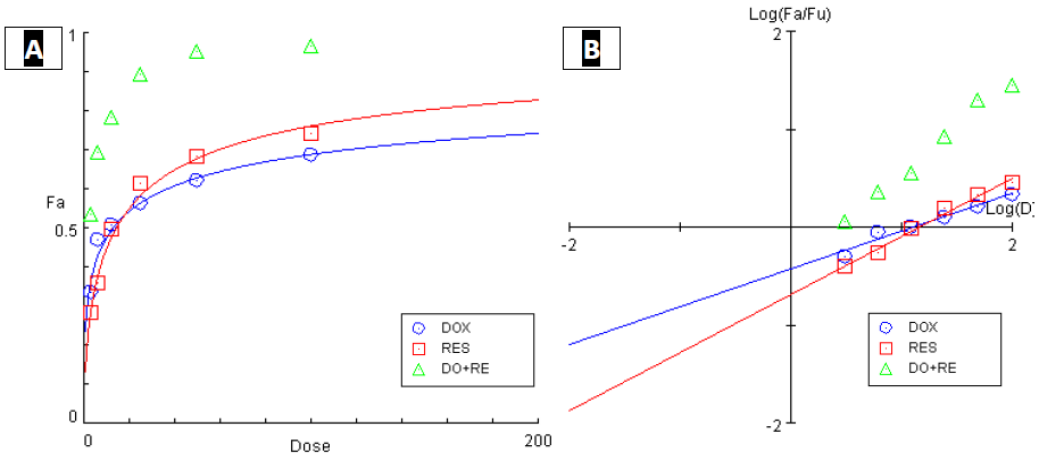
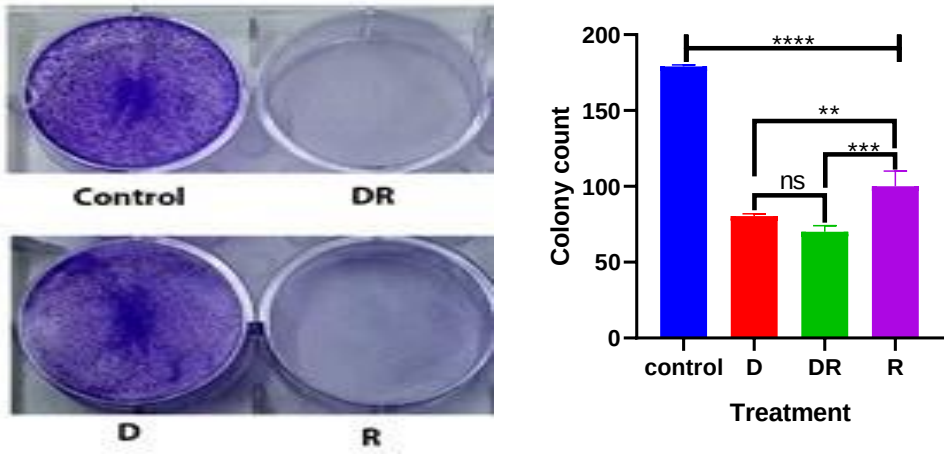


Figure 4. Dose effective curve and median effect plot. (A) Dose effective curve of Doxazosin, Resveratrol combination. B) Median effect plot of A. The fraction affected (fa) vs. the fraction unaffected (fu) is equal to the dose (D) vs. the median-effect dose.

Treatment Exposure Results to MCF-7 Cell Line Using Clonogenic Assay

The effect of treatment on proliferation and colonization after 72h of incubation with MCF-7 was determined using a clonogenicity survival assay. The results (Figure 5) showed that all the treatments had a significant anti-clonogenic effect on the MCF-7 cell line when compared with the control group $P<0.05$. Results also showed that some treatments had more potent effects than others. Doxazosin-resveratrol significantly induces a more significant ani-clonogenic effect than Resveratrol treatment alone. Results showed no significant differences between the Doxazosin-Resveratrol combination and Doxazosin treatment alone $P<0.05$, While Doxazosin is significantly more potent than Resveratrol treatment alone anti-clonogenic treatment $P<0.05$.



(a) (b)

Figure 5. Clonogenic effect of treatments on MCF-7 cell line using crystal violet stain assay and GraphPad prism software. D) Doxazosin, R)Resveratrol, and DR) Doxazosin-Resveratrol combination. *=p<0.05, **=P<0.01, ***P<0.001, ****P<0.0001

Treatment Exposure Results to MCF-7 Cell Line Using Immunocytochemistry Assay

Investigation of two protein receptors associated with apoptosis was performed: the CASP3 selective apoptosis signal-mediated receptor and the GABARAP receptor associated with autophagosome-mediated selective autophagy. Results (Figure 6) of the CASP3 receptor showed that the Resveratrol had a lower expression effect, followed by Doxazosin and Doxazosin-Resveratrol combination, which had a higher (darker) color intensity. The expression of the GABARAP receptor was lowest in the Doxazosin-Resveratrol combination, followed by Doxazosin and Resveratrol, which had the higher color intensity (expression).

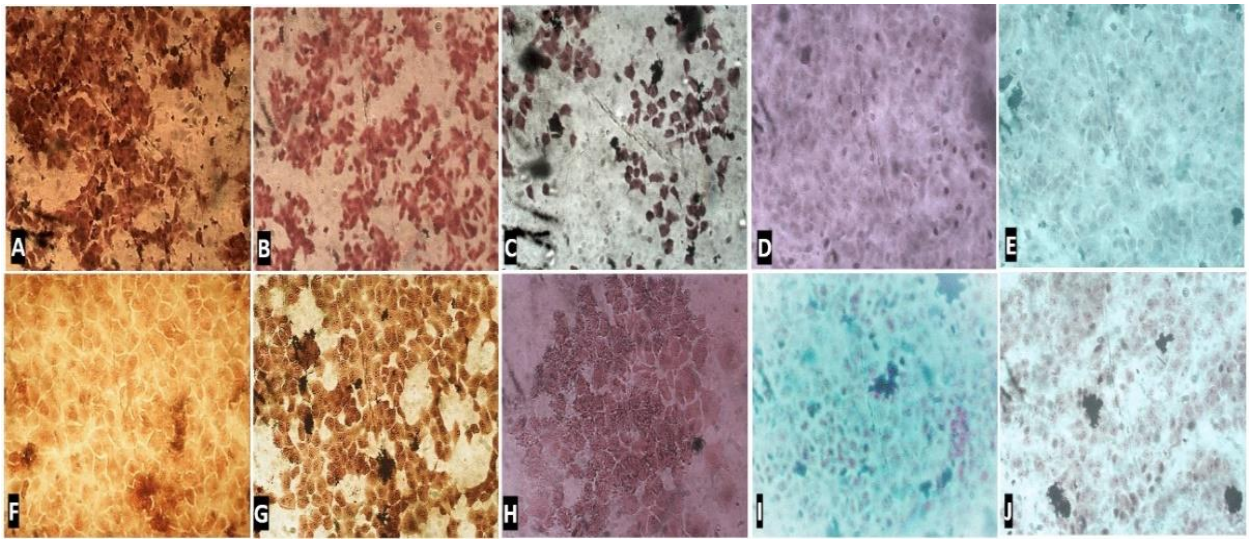


Figure 6. Immunocytochemistry to MCF-7 cell line after treatment and labeling using apoptosis antigen markers CASP3 and GABARAP: A, B, C, D, E are CASP3, Resveratrol, Doxazosin, Resveratrol-Doxazosin combination, control and Negative control respectively, While F, G, H, I, J are GABARAP, Resveratrol, Doxazosin, Resveratrol-Doxazosin combination, Negative control and control.

DISCUSSION

All treatments used in this study induced an anti-proliferative effect compared to the control group. This effect appears due to different direct or indirect autophagic pathways involved in each drug. Doxazosin activates the PI3K-Akt-mTOR and inhibits methyladenine, so it controls the autophagy process in different ways [4,5]. Resveratrol is also involved in autophagy regulatory pathways by suppressing the p52-mTOR-Nrf2, activating the LKB1-AMPK, and inhibiting the PI3K-AKT pathways [6–8]. The microenvironment of cancer plays a major role in cancer progression and suppression. Different degrees of cancer development appear depending on how drugs affect the microenvironment. Some drugs directly induce changes in the microenvironment of cancer cells, surviving variable signaling pathways to result in cancer development [15]. Other microenvironmental drug changes the cancer-associated cells, inducing indirect progression or suppression of cancer [16]. For example, drugs with anti-inflammatory properties and lower adipose tissue mass are more potent in inducing anti-proliferative effects than other drugs [17]. Some drugs are more effective than others because they work on other vital cellular hemostatic regulators like ion transporters protein (TRPM7), reducing the chances of chemotherapy resistance [18]. Doxazosin had a lower IC_{50} probably because it is strongly associated with three main strategies that affect cancer cells. It lowers lipids [19], mediating the sodium and glucose contransportive-2 protein (SGLT2) [20], works as an extracellular kinase regulator (ERK) [21], and induces apoptosis with a less toxic effect[22]. MCF-7 cell line's main feature is its relation to estrogen receptors [23]. The binding ability of RES to estrogen receptors and mediating cell growth is an exciting point in RES anti-cancer therapy [24,25]. Resveratrol also has two other beneficial points: it has a prophylactic effect on non-small cancer cells and reduces mitochondrial energy metabolism [26–28].

CONCLUSION

Combination treatment of Doxazosin-Resveratrol induces a higher caspase-3 expression pattern than other treatments. Resveratrol treatment elevated GABARAP expression in cancer cells, indicating the induction of the autophagy process.

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