

RESEARCH / INVESTIGACIÓN

Phytochemical screening and *in vitro* anti-inflammatory activity of ethanolic extract of *Epidendrum coryophorum* leavesIrina Francesca González Mera¹, Orestes Darío López Hernández² and Vivian Morera Córdova*

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Abstract: *Epidendrum coryophorum* belongs to the Orchidaceae family. Traditional uses of some species for this genus include infusions of the leaves used for kidney problems, treat influenza, conjunctivitis, liver pain, relieve kidney symptoms, and hypoglycemic effect. This work's objective was to determine the phytochemical profile of the ethanolic extract of *Epidendrum coryophorum* leaves and to evaluate the potential anti-inflammatory activity *in vitro* of the extract employing the erythrocyte membrane stabilization method. The phytochemical screening carried out in this work suggested phenols, coumarins, flavonoids, tannins, steroids, and sterols in the ethanolic extract of *Epidendrum coryophorum* leaves. Cardiotoxic glycosides and carbohydrates were also found. The ethanolic extract's UV-Vis spectrum showed absorption maxima at 268 nm and 332 nm, which could correspond to flavonoids of the flavonoid classes, 3-OH substituted flavonols, or isoflavones. The quantitative determination of total phenols of the ethanolic extract was carried out using the Folin-Ciocalteu method. The total phenolic content expressed as mg Gallic acid equivalent (G.A.E.) per gram of extract was found to be 19,96 mgGAE/g of *Epidendrum coryophorum*. The ethanolic extract of *Epidendrum coryophorum* leaves showed hemolysis inhibition values of 18,19% at 1,0 mg/mL, 38,98% at 1,5 mg/mL and 40,94% at 2,5 mg/mL compared with aspirin (positive control) giving values of 65,33% at 1,0 mg/mL, 72,26% at 1,5 mg/mL and 73,75% at 2,5 mg/mL. The values obtained for inhibition of hemolysis with ethanolic extract, compared with the values obtained with a pure anti-inflammatory, are significant and demonstrate anti-inflammatory activity in *Epidendrum coryophorum*.

Key words: *Epidendrum coryophorum*, total phenolic content, microencapsulation, anti-inflammatory activity.

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Introduction

Inflammation is a body's normal physiological response when it tries to protect itself from cellular damage caused by pathogens, irritants, or physical damage. The inflammation function is to remove damaged and necrotic tissues generated by the causative agent and initiate tissue repair. A not adequately cured inflammation is the basis of hypersensitivity reactions and chronic diseases¹. Current treatments to alleviate the symptoms of acute and chronic inflammation cause adverse effects, and in many cases, the treatments have low effectiveness². An alternative may be to develop drugs based on plants that have been used in traditional medicine for many years. The analysis of natural products through biochemical, phytochemical, and pharmacological studies has allowed the identification and characterization of various active compounds capable of counteracting the effects of inflammation, including phenolic compounds³⁻⁵.

The orchids, besides being used as ornamentals and food, are also used for medicinal purposes. The orchid's extracts are prepared from different parts of the plant. It could be tubers, leaves, rhizomes, stems, pseudobulbs, roots, flowers, sheaths, bulbs, and in some cases, the whole plant. They are prepared as an infusion, decoction, dried and ground, paste, Yin tonic, tincture, and juice^{6,7}. These extracts are used as diuretics, anti-rheumatics, anticancer, hypoglycemic, anti-aging, antimicrobial, anticonvulsants, antivirals, and anti-inflammatories⁸⁻¹³. The genus *Epidendrum* (Orchidaceae) is found in the Andes, represented by 360 species, of which 178 are native species to the Andean forest^{14,15}. Traditional uses of some species for this genus include infusions of the leaves for kidney problems, treat influenza, conjunctivitis, and hepatic pain; other authors

report the use of orchids of this genus with hypoglycemic effect and to alleviate renal symptoms^{16,17}. Despite the broad representation of genus *Epidendrum* in the Ecuadorian Andes and the wide use of orchids for medicinal purposes in many regions of the world, there are only a few scientific publications concerning the chemical composition and pharmacological properties of this genus. Regarding *Epidendrum coryophorum*, there are no scientific reports at all on this species.

There are different and varied methods to evaluate anti-inflammatory activity. These methods are classified as *in vivo* (if using animal models) or *in vitro* (when performed in a controlled environment outside of a living organism). Each of these methods is associated with some peculiarities, advantages, and disadvantages¹⁸. Although the *in vivo* models correlate well with the clinical picture and help understand the mechanisms of inflammation better, they are more complex and expensive. Among the *in vitro* methods, the method based on the stabilization of the erythrocyte membrane has gained wide popularity among researchers since it is based on the similarity of the erythrocyte membrane with the lysosomal membrane and its stabilization by the secondary metabolites of the plant extract is considered a good experimental indicator of the anti-inflammatory activity of secondary metabolites; additionally, it is a relatively simple method and is inexpensive.

This work's objective was to determine the phytochemical profile of the ethanolic extract of *Epidendrum coryophorum* leaves and to evaluate the potential anti-inflammatory activity *in vitro* of the extract employing the erythrocyte membrane stabilization method.

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Materials and methods

Leaves preparation

The *Epidendrum coryophorum* specimens were acquired at Ecuadorian company Ecuagenera, Guayaquil (<http://www.ecuagenera.com>). The plants were kept in pots until the leaves were separated for processing. Using a scalpel, leaves were cut from the base of the plant. Then, leaves were washed separately with common water followed with distilled water to remove dust and particles. Subsequently, the leaf was drained and dried in the shade at 25°C. The dried tissues were pulverized using an electric grinder and stored at -20°C until they were used¹⁹.

Organic extraction

For the extraction of *Epidendrum coryophorum* leaves' phytochemicals, the solid-liquid extraction process was used with 70% ethanol as solvent²⁰. The ratio of dry tissue powder and the organic solvent was 1:10 (w:v). Extraction was allowed to proceed for 48 hours with occasional manual stirring. Finally, the amber bottle with ground tissue, and the solvent was ultrasound for 1 hour at 25 °C. The solvent was filtered through filter paper with the vacuum's help, and the extract was stored in a new clean amber bottle, protected from light at 4 °C until use.

Phytochemical screening

Once the ethanolic extract from dried leaves was obtained, the preliminary phytochemical analysis applied simple, rapid, and selective phytochemical screening assays targeting the qualitative detection of secondary metabolites^{21,22}.

UV-Vis analysis

The ethanolic extract was diluted to 1:20 (v:v) with the same solvent. The spectrum was registered from 200 nm to 800 nm using a UV-VIS NIR Lambda 1050 spectrophotometer.

Total phenolic content

To determine the total phenolic content (TPC) in ethanolic extract of *Epidendrum coryophorum* leaves, a dilution of 1:10 (v:v) in distilled water was made. TPC was determined by Folin-Ciocalteu spectrophotometric method²³. Gallic acid was used as reference material in concentrations of 0,02 mg/mL, 0,04 mg/mL, 0,06 mg/mL, 0,08 mg/mL, 0,10 mg/mL, 0,12 mg/mL, 0,14 mg/mL, 0,20 mg/mL and 0,30 mg/mL. The solutions were kept protected from light. For standard solutions and ethanolic extract, to 300 µL of the solution was added 150 µL of 10% Folin-Ciocalteu reagent, then the mixture was let to react for 3 minutes at room temperature. Next to each tube, 120 µL of 7,5% sodium carbonate was added and let them rest for 30 minutes in a dark place. At the end of the reaction, the absorbance was measured at 765 nm using a UV-VIS NIR Lambda 1050 spectrophotometer. The absorbance's values were extrapolated in the standard calibration curve. The results were expressed as mg in equivalents of Gallic acid (G.A.E.) per grams of dry material. The determination was performed in triplicate, and the results were expressed as mean ± S.D.

Microencapsulation

The extract was concentrated in a rotary evaporator at 30 °C, which allowed the ethanol's distillation from the mixture. Then, the concentrated extract was weighed, labeled, and sto-

red in an Eppendorf tube at 4 °C, avoiding the metabolites' degradation. Then, the extract was first dissolved in Sacha inchi oil until most of the extract homogenize in the oil. So, for the microencapsulated extract matrix, it was used maltodextrin, Arabic gum, and water in a proportion of 30:30:30 (v:v:v). The mixture was homogenized using an Ultra turrax Pt25 I.K.A. at 25,000 rpm for 1 minute. For the microencapsulation, the Mini Spray Dryer Buchi B290 was used, which was fed with the respective combination, once the equipment conditions were verified to be adequate (-50 mbar, 150 °C inlet and 90 °C outlet)²⁴. Microencapsulated extract was dissolved in PBS to reach concentrations of 1,0 mg/mL, 1,5 mg/mL, and 2,5 mg/mL.

Anti-inflammatory activity

Preparation of blood samples

The human red blood cell membrane stabilization method was used to study *in vitro* anti-inflammatory activity²⁵. The human blood was obtained from two healthy volunteers who did not have used anti-inflammatory medications for at least a week and counted with a medical certificate to corroborate the health status. 3 mL of human blood was transferred to a conical tube and centrifuged for 10 min at 3000 rpm. The supernatant was taken out, and an equivalent volume of PBS was placed on the test tube to realize three consecutive washes to the erythrocyte suspension. After each extraction of plasma and the addition of PBS, the sample was centrifuged. 800 µL of the erythrocyte suspension was mixed with 1200 µL of PBS to form a suspension of 40% v/v in PBS.

Inhibition of heat-induced hemolysis

In 25 mL conical tubes were added 3 mL of the evaluated samples and 30 µL of 40% erythrocyte's suspension. The negative control was prepared using the same quantity of PBS instead of the sample. The microencapsulated extract and the commercial aspirin (positive control, from Sigma-Aldrich, USA) were evaluated at concentrations of 1,0 mg/mL, 1,5 mg/mL, and 2,5 mg/mL. All reaction mixtures were prepared in triplicates except the negative control. One conical tube of each reaction mixture was incubated for 20 min at 54 °C, and the other was left at room temperature. The negative control was incubated too. After 20 min of incubation, all test tubes were centrifuged at 3000 rpm for 10 min. The absorbance of supernatants was measured at $\lambda = 560$ nm²⁵. The percentage of hemolysis inhibition was calculated according to the following expression:

$$\% \text{ hemolysis inhibition} = \left[1 - \frac{A_I - A_{WI}}{A_{NC} - A_{WI}} \right] \times 100$$

Where: A_I represents the absorbance of the sample with incubation, A_{WI} represents absorbance of the sample without incubation, and A_{NC} represents the absorbance of the negative control.

Statistical analysis

Statistical analysis was performed by calculating the mean and standard deviation of the percentages of hemolysis inhibition for the extract's three concentrations. The used program was STATGRAPHICS Centurion XV, Version 15.2.05. The data were processed through a one-way analysis of variance (ANOVA), and subsequently, Duncan's test was performed, considering significant differences of at least $p < 0.05$.

Results and Discussion

The phytochemicals produced by plants have useful pharmacological activities for treating various diseases, including those that occur with inflammation. There are numerous studies and reviews on plants containing phytochemicals, specifically flavonoids, triterpenes, tannins, phenolic compounds, coumarins, and steroids; anti-inflammatory activity has been attributed²⁶⁻³³. However, very little has been investigated about these phytochemicals and their possible anti-inflammatory activity, especially in *Epidendrum* (Orchidaceae). In the ethanolic extract of *Epidendrum coryophorum* leaves, we found that phenols, coumarins, flavonoids, tannins, and anti-inflammatory activity were demonstrated. This work constitutes the first report on the phytochemical screening and anti-inflammatory activity of *Epidendrum coryophorum*.

Phytochemical screening

Phytochemical screening is one of the initial stages of phytochemical research, which allows the to determining qualitatively the main chemical groups present in the plant and guide the extraction and/or fractionation of the extracts, isolation and characterization of specific metabolites³⁴. The phytochemical screening carried out in this work suggested phenols, coumarins, flavonoids, tannins, steroids and sterols in the ethanolic extract of *Epidendrum coryophorum* leaves. There were also found cardiogenic glycosides and carbohydrates. Anthocyanins, anthraquinones, flobatanins, saponins, proteins and free amino acids were not detected (Table 1). The presence of secondary metabolites in a given extract depends on the used solvent. For a solute (secondary metabolite) to be dissolved in a solvent, it is necessary that the solvent disassociate the molecules and thus facilitate their solvation; this process depends on both the dielectric constant of the solvent and the solute's (secondary metabolites) and solvent's polari-

ty. Generally, in ethanolic extracts, tannins, phenols, flavanols, terpenoids, sterols and alkaloids have been detected³⁵, which coincides with the type of metabolites detected in the ethanolic extract of *Epidendrum coryophorum* leaves (Table 1).

The phytochemical screening of ethanolic extract of *Epidendrum coryophorum* leaves constitutes the first report on the presence of specific secondary metabolites in this species. However, phytochemical screening of other species belonging to the genus *Epidendrum* has been performed before. Cerna *et al.* evaluated the phytochemical content of ethanolic extracts of the species *Epidendrum blepharoclinium*, *Epidendrum blepharoclinium*, *Epidendrum cochlidium sp1*, *Epidendrum cochlidium sp2*, *Epidendrum jamiesonis*, *Epidendrum medusae*, *Epidendrum nocturnum*, *Epidendrum paniculatum*, *Epidendrum porphyreum*, *Epidendrum secundum*, *Epidendrum secundum*. Even though these authors' extraction conditions are not identical to those used in our work, they also found flavonoids in 5 species, saponins in 11 species, and tannins in 7 of the 11 studied³⁶.

UV-Vis analysis

UV-Vis spectroscopy is a technique that allows the detection of compounds in which can occur electronic transitions of the type $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ as a result of interaction with light in the ultraviolet and visible regions of the electromagnetic spectrum. The absorption maxima can be correlated with compounds containing π bonds and lone pairs of electrons³⁷. In the UV-Vis spectrum of ethanolic extract of *Epidendrum coryophorum* leaves, the absorption maxima were recorded at 268 nm, 332 nm, 410 nm, 468 nm, 504 nm, 536 nm, 606 nm, and 664 nm. Of these maxima, the most intensive peaks (Peak 1-268 nm and Peak 2-332 nm, Figure 1A) could correspond to flavonoids of flavone, flavonols 3-OH substituted, or isoflavones classes. Flavonoids are natural pigments, especially abundant in plants' leaves, and their primary function is

	Secondary metabolites	Reaction	Results
Phenols	Phenols	Ferric chloride test	+
	Anthocyanin	a)	-
	Coumarins	b)	+
	Flavonoids	Ammonium test	+
	Tannins	Modified Braemer's test	+
	Anthraquinones	Bornstrager's test	-
	Flobatanins	Hydrochloric acid test	-
Terpenes	Terpenoids	Salkowski test	-
	Saponins	Froth test	-
	Steroids and sterols	Modified Lieberman – Burchardt test	+
	Cardiotonic glycosides	Modified Kellar – Kiliani test	+
Others	Proteins and amino acids	Ninhydrin test	-
	Carbohydrates	Benedict's test	+
	Reducing sugars	Fehling's test	-

a) 1,0 mL of ethanolic extract was mixed with 3,0 mL of water. Subsequently, 1,0 mL of 2 mol/L HCl and 1,0 mL of 0,5 mol/L ammonia were added. The appearance of red-pink color that turns blue-violet could indicate the presence of anthocyanins.

b) 1,0 mL of ethanolic extract was mixed with 1,0 mL of 10,0% NaOH. The formation of a yellow coloration could indicate the presence of coumarins in the extract.

(+) indicates the presence of the evaluated secondary metabolite

(-) indicates its absence.

Table 1. Secondary metabolites tested and detected by chemical reactions in the ethanolic extract of *Epidendrum coryophorum* leaves.

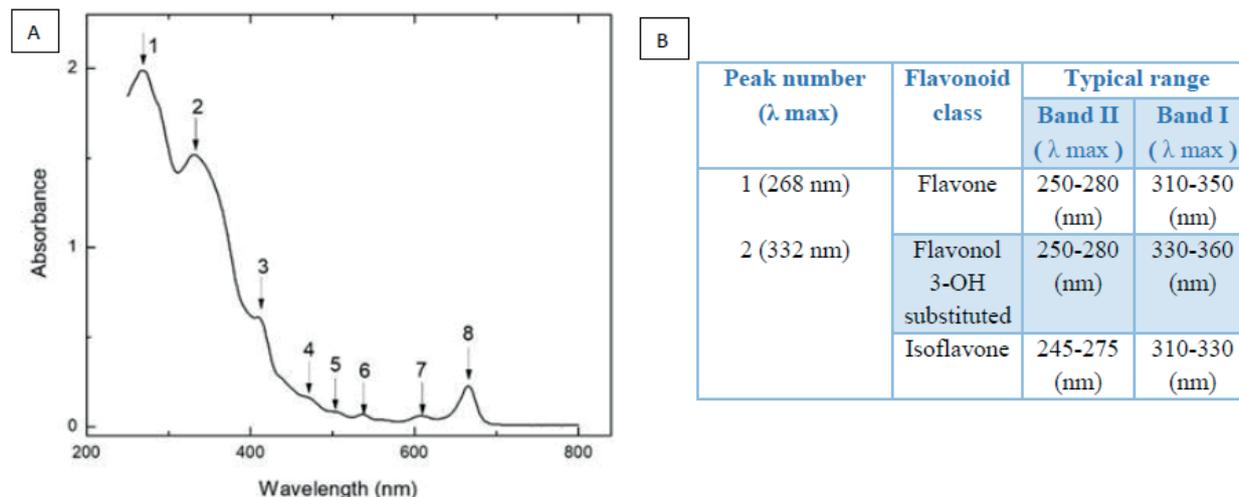


Figure 1. UV-Vis analysis of an ethanolic extract of *Epidendrum coryophorum* leaves. A: in the spectrum, the observed absorption maxima are indicated with arrows and numbers. B: correlation between the wavelengths of observed absorption maxima in the spectrum and typical ranges of absorption maxima wavelengths for some flavonoid classes.

to protect them from ultraviolet rays. The flavonoids have in their structure a standard diphenylpyran skeleton (C6-C3-C6), composed of two phenyl rings (A and B) linked through a pyran C ring (heterocyclic). This basic structure allows for a multitude of substitution patterns and variations in the C ring³⁸. UV-Vis spectroscopy is a useful technique in the differentiation of flavonoids since the bands' position in the UV-Vis spectrum depends on the extent of conjugation and, therefore, on the nature of the C ring and the position of the phenyl group called ring B (C-2, C-3 or C-4 substituent). For these compounds, the UV-Vis spectrum presents two absorption maxima in the ranges from 310 to 360 nm and from 245 to 280 nm corresponding to bands I and II of rings B and A, respectively, according to the class of flavonoids (Figure 1B)³⁹.

Total phenolic content

Considering that several secondary metabolites were detected in the extract of *Epidendrum coryophorum* leaves classified as phenolic compounds⁴⁰, the TPC was determined using the Folin-Ciocalteu method as previously described. The value was calculated using a calibration curve with Gallic acid, whose equation resulted in $y = 8.8282x + 0.0328$ with $R^2 = 0.9987$. The TPC in the ethanolic extract was 19.96 mgGAE/g of *Epidendrum coryophorum* (Table 2). Values of TPC of *Epidendrum coryophorum* have not been reported before in the literature or other genus *Epidendrum* species.

Anti-inflammatory activity

The erythrocyte membrane stabilization test in the presence of microencapsulated ethanolic extract in an isotonic medium was positive and showed concentration-dependent anti-inflammatory activity. An increase in the percentage of hemolysis inhibition was observed as the amount of microencapsulated extract increased. The microcapsules of ethanolic extract of *Epidendrum coryophorum* leaves gave values of he-

molysis inhibition of 18,19% at 1,0 mg/mL, 38,98% at 1,5 mg/mL, and 40,94% at 2,5 mg/mL in comparison with aspirin (positive control) giving values of 65,33% at 1,0 mg/mL, 72,26% at 1,5 mg/mL, and 73,75% at 2,5 mg/mL (Table 3). The results were statistically evaluated using the statistical method of ANOVA and presented statistical significance; that is, the treatments used were significantly different ($p < 0.05$). Therefore, the data were analyzed using Duncan's Test, with which we were able to corroborate the obtained results. The concentration of 1,00 mg/mL of the extract showed a significant difference concerning the concentrations of 1,5 mg/mL and 2,5 mg/mL of the extract itself and a lower percentage of hemolysis inhibition. However, the concentrations of 1,5 and 2,5 mg/mL of extract did not present significant differences between them and presented similar values of percentage of hemolysis inhibition. Similar behavior occurred in the case of aspirin (Figure 2). The erythrocyte membrane stabilization test is considered as an *in vitro* measure of the anti-inflammatory activity of drugs or extracts of plants⁴¹. The erythrocyte membrane is very similar to the membrane lysosomal so the effect of drugs on the stabilization of the erythrocyte membrane could be extrapolated to the stabilization of the lysosomal membrane; its stability is crucial since it can limit the inflammatory response, inhibiting the release of inflammation mediators as proteases and bactericidal enzymes⁴².

Additionally, this test is a convenient, rapid, and inexpensive method to be applied for preliminary investigations on the anti-inflammatory activity of crude extracts of plants. Numerous investigations associate the presence of phenolic compounds in plants with the anti-inflammatory activity evaluated by this method⁴³. Thus, we could assume that the observed anti-inflammatory activity can result from certain phenols' individual action in the extract or of synergy between them⁴⁴. Considering the coarse texture of the ethanolic extract after drying and to ensure the bioavailability of its active principles

Absorbance 1	mgGAE/gEc	Absorbance 2	mgGAE/gEc	Absorbance 3	mgGAE/gEc	Average	SD
0,620	22,17	0,525	18,58	0,539	19,11	19,96	1,94

The extract was analyzed by triplicate

GAE: Gallic acid equivalent

Ec: *Epidendrum coryophorum*

SD: standard deviation

Table 2. Total phenolic content of ethanolic extract from *Epidendrum coryophorum* leaves.

Concentrations of microencapsulated ethanolic extract	% of hemolysis inhibition	% of hemolysis inhibition	% of hemolysis inhibition	Average	SD
1,0 mg/mL	20,92	17,86	15,79	18,19	2,58
1,5 mg/mL	40,45	37,78	38,70	38,98	1,36
2,5 mg/mL	35,17	44,09	43,56	40,94	5,00
Concentrations of commercial aspirin					
1,0 mg/mL	65,70	66,50	63,78	65,33	1,40
1,5 mg/mL	73,76	73,40	69,61	72,26	2,30
2,5 mg/mL	75,13	73,42	72,71	73,75	1,24

SD: standard deviation

Table 3. Percentage of inhibition of hemolysis of the erythrocyte membrane for the three tested extract and control drug concentrations.

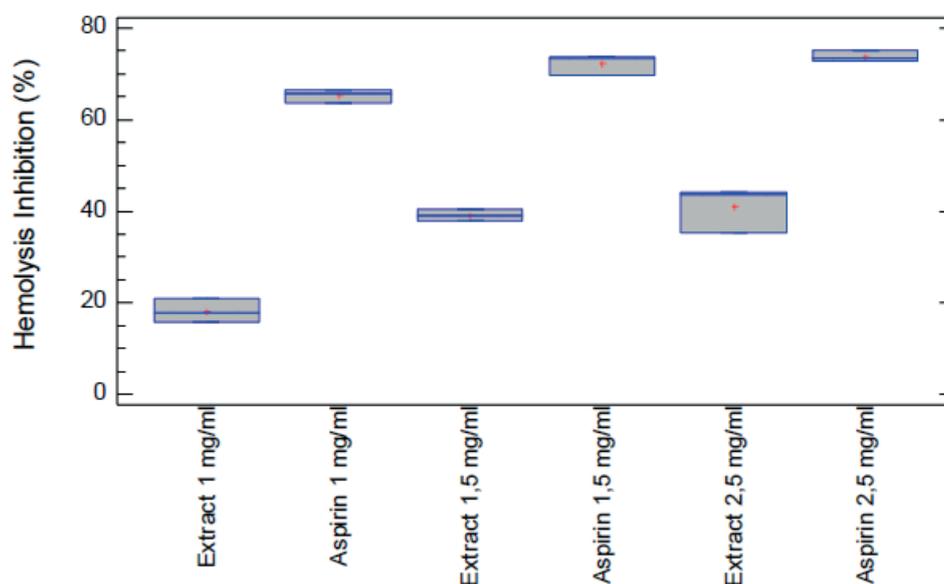


Figure 2. Percentages of hemolysis inhibition of the ethanolic extract of *Epidendrum coryophorum* leaves. Commercial aspirin was used as a positive control.

$p < 0.05$ was considered statistically significant in all analyses.

during the hemolysis inhibition test, it was microencapsulated using natural water-soluble polymers. During the microencapsulation procedure of the ethanolic extract of *Epidendrum coryophorum* leaves, the homogenization step is essential for forming the emulsion. This guarantees the formation of microcapsules with a polynuclear internal structure, which allows the dispersion of the microcapsules in water, forming a nanoemulsion on contact with the environment in which the cells are found and better dispersion of the active principles in the medium⁴⁵.

Conclusions

The phytochemical screening carried out in this investigation suggested the presence of phenols, coumarins, flavonoids, and tannins in the ethanolic extract of *Epidendrum coryophorum* leaves. The ethanolic extract's UV-Vis spectrum showed absorption maxima at 268 nm and 332 nm, which could correspond to flavonoids of the flavonoid classes, 3-OH substituted flavonols, or isoflavones. The ethanolic extract of *Epi-*

dendrum coryophorum leaves contains phenolic compounds. The total phenolic content was found to be 19.96 mgGAE/g of *Epidendrum coryophorum*. The extract showed an erythrocyte membrane stabilization effect. The values obtained of hemolysis inhibition with ethanolic extract (crude extract) in comparison with those values obtained with a pure anti-inflammatory drug are significant and demonstrate anti-inflammatory activity in *Epidendrum coryophorum*. However, it should be mentioned that this study constitutes a preliminary study on the anti-inflammatory effect of this extract; therefore, it is suggested to expand the study of the anti-inflammatory effect using other methods and to consider the use of experimental animal models.

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Competing interest

The authors have no competing interests.

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