UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Químicas e Ingeniería

TÍTULO: Preliminary phytochemical characterization of extracts of the orchid *Epidendrum coryophorum*

Trabajo de integración curricular presentado como requisito para la obtención del título de Químico (a)

Autor:

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A mis seres amados,

Por el apoyo incondicional y su amor infinito.

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Irina Francesca González Mera

RESUMEN

Las plantas son de gran importancia para los seres humanos ya que son fuente de alimentos, materiales combustibles, fibras, y sustancias que mitigan el dolor, la fatiga, y alivian los síntomas de muchas enfermedades. Orchidaceae es la segunda familia más grande en el reino de las plantas con flores. En esta familia existen alrededor de 850 géneros y más de 20 000 especies. A lo largo de la historia las orquidáceas, distinguidas por sus flores atractivas y complejas, han sido utilizadas como ornamento, aromatizantes, saborizantes, y alimento, así como en la medicina tradicional y celebraciones religiosas. El objetivo de este trabajo fue realizar el análisis fitoquímico preliminar de extractos de *Epidendrum coryophorum*. Los especímenes usados en este estudio fueron adquiridos de Ecuagenera (http://www.ecuagenera.com). El tratamiento de las hojas consistió en limpiarlas con agua destilada y luego secarlas a la sombra a una temperatura de 30°C. Posterior a la trituración mecánica del tejido, se realizaron extracciones solido-líquidas para obtener extractos en metanol, etanol y cloroformo. Todos los extractos de hojas se sometieron a un análisis fitoquímico cualitativo. Se detectaron fenoles, flavonoides, cumarinas, taninos, terpenoides, esteroides, esteroles, glucósidos cardiotónicos, carbohidratos, y azúcares reductores. Se determinó el contenido fenólico total de cada extracto mediante el método de Folin-Ciocalteu. Los valores más altos se encontraron en los extractos metanólicos y etanólicos. Adicionalmente se evaluaron la actividad antimicrobiana y la actividad antiinflamatoria. En los extractos metanólicos y etanólicos se obtuvieron resultados positivos.

PALABRAS CLAVE: Orquidaceae, *Epidendrum coryophorum*, marcha fitoquímica, análisis UV-VIS, contenido fenólico total, actividad antimicrobiana, actividad antiinflamatoria.

ABSTRACT

Plants are of great importance to human beings as they are sources of food, combustible materials, fibers, and substances that mitigate pain, fatigue, and relieve the symptoms of many diseases. Orchidaceae is the second largest family in the kingdom of flowering plants. In this family, there are about 850 genera and more than 20,000 species. Throughout history, orchids, distinguished by their attractive and sophisticated flowers, have been used as ornaments, flavoring, and food, as well as in traditional medicine and religious celebrations. The objective of this work was to perform the preliminary phytochemical analysis of extracts of *Epidendrum coryophorum*. The specimens used in this study were purchased from Ecuagenera (http://www.ecuagenera.com). The treatment of the leaves consisted of cleaning them with distilled water and then drying them in the shade at a temperature of 30°C. After the mechanical shredding of the tissue, solid-liquid extractions were performed to obtain extracts in methanol, ethanol, and chloroform. All leaves extracts were subjected to a qualitative phytochemical analysis. Phenols, flavonoids, coumarins, tannins, terpenoids, steroids, sterols, cardiotonic glycosides, carbohydrates, and reducing sugars were detected. The total phenolic content of each extract was determined by the Folin-Ciocalteu method. The highest values were found in the methanolic and ethanolic extracts. Additionally, antimicrobial activity and anti-inflammatory activity were evaluated. In the methanolic and ethanolic extracts, positive results were obtained.

KEYWORDS: Orchidaceae, *Epidendrum coryophorum*, phytochemical screening, UV-VIS analysis, total phenolic content, antimicrobial activity, anti-inflammatory activity.

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1. TITTLE

Preliminary phytochemical characterization of extracts of the orchid *Epidendrum coryophorum*.

2. INTRODUCTION-JUSTIFICATION

Orchidaceae is the second largest family in the flowering plant kingdom. There are almost 850 genera and more than 20 000 species.[1] Orchid flowers are very exotic and striking. They exist in a variety of colors and shapes that make them very attractive. Species of the gender *Vanilla*, are frequently used in the food and perfumery industries due to their distinguished fragrance, another attribute of Orchidaceae flowers.[2] Currently, orchids' cultivation and commercialization are an important part of the cosmetic, food, and ornamental plants industry. They are frequently used in cultural rituals and the ancestral medicine of many countries.[2,3]

The use of orchids extracts is a common practice around the world. They are consumed for several health-promoting benefits. These extracts are made from different parts of the plants; it could be tubers, leaves, rhizome, stems, pseudo bulbs, roots, flowers, sheaths, bulbs, and in some cases, the whole plant.[1] The preparation of the medicine is very variable, depending on the orchid species. They are prepared as an infusion, decoction, dried and ground, paste, Yin tonic, tincture, and juice. Anticancer and antitumor activity have been found in these species, also anti-rheumatic, anti-inflammatory, anticarcinogenic, antimicrobial, anticonvulsive, neuroprotective, and antiviral activities.[1]

In China, about 365 species of medicinal plants are used, including several species of orchids. Among the traditional medicinal orchids, rhizomes of *Bletilla striata* are employed for different purposes since cracked skin to inflammation and gastrointestinal disorders. Also, its leaves are used to cure lung disease. Additionally, species of the *Gastrodia* gender were used for food, and as medicine to treat headaches, dizziness, blackouts, numbness of the limbs, hemiplegia, epilepsy, limb cramps, spasms, migraine, expulsion of poisonous effluvia, rheumatism, vertigo, neuralgia, facial paralysis, dysphasia, infantile convulsions, lumbago, fever, and nervous afflictions.[4]

Beltrán and Martinez[3] interviewed people in the urban areas of Cuba to know what species do they use and what are the affections that are treated with these species. They found that eight species of orchids were used in their traditional medicine: *Bletia purpurea* for stomach disorders, *Cyrtopodium punctatum* for cough, diseases of the chest, asthma, alopecia, dislocations, falls, blows, inflammations, *Ecyclia cochleata* for expel phlegm and asthma, *Encyclia phoenicia* for catarrh and menstrual flow, *Oncidium luridum* for asthma, *Vanilla dilloniana* for intestinal worms and psychoastenia, *Vanilla phaeantha* for parasites and psychoastenia, and *Vanilla planifolia* for ulcerations, fever, parasites, anuria, amenorrhea, and neuralgia.

Ecuador is the wealthiest country in Orchidaceae species; for that reason, it was declared "The country of Orchids" in the year 2013. In Ecuador, it can be found four of the five subfamilies of orchids existing worldwide. From 4,032 species of orchids that have been found in Ecuador, 1,714 species are endemic. All of them are classified.[5] The flora of endemic orchids in Ecuador is represented principally by the genus *Pleurothallidinae* (65%), *Laeliinae* (12%), and *Oncidiinae* (9%).[6]

*Epidendrum coryophoru*m is a species belonging to the genus *Epidendrum*, which contains more than 1000 species[7] (**Figure 1**). This species is an epiphytic plant that usually grows in cold climates, at elevations of 1400-3800 (masl). Its habitat is found in the mountains of Ecuador, Colombia, and Venezuela. It blooms in winter and late autumn.[8] The average growth sizes are from 15 cm to 25 cm. There are researches on some species of the genus *Epidendrum* that include phytochemical detection and antioxidant activity, but the pharmacological properties of these species have not been studied.[9,10] In the scientific literature, there is no research on the phytochemical characterization of the species *Epidendrum coryophorum*.

Figure 1. *Epidendrum coryophorum* obtained from Ecuagenera.

2.1 Secondary metabolites

Secondary metabolites (SM) are organic compounds produced by plants as a by-product of the primary metabolism, and their principal functions are not directly related to allowing the healthy growth of plants as is that of primary metabolites. These metabolites have been present along the plant's evolution process as the first mechanism of adaptation. For many centuries secondary metabolites have been used for medicinal and aesthetic purposes. For that reason, in the middle of the previous century, scientists began to investigate the nature of these compounds.[11] Since then, many techniques have been improved and also created many methods for identification, isolation, purification, and characterization. As an effect, these metabolites have gain industrial importance.[12]

2.2 Classification of secondary metabolites

Several criteria have been considered for the classification of SM: chemical structure (presence of rings or sugars), composition (containing nitrogen or not), their solubility in organic solvents or water, and the biosynthetic pathway. Of them, the most common criterion used for grouping the SM in plants has been the biosynthetic pathway. According to this, the SM in plants can be divided into three broad groups: terpenes, phenolic compounds, and alkaloids.[13]

2.2.1 Terpenes

They constitute the largest group of SM in plants to which more than 40,000 different molecules are allocated.[14] They are also known as isoprenoids since the basic structural unit that forms them is the isoprene molecule[15] (**Figure 2**). The terpenes are classified in monoterpenes, with three units in sesquiterpenes, with four diterpenes, with six in triterpenes, with eight in tetraterpenes, and with more than 10 in polyterpenes.[16,17]

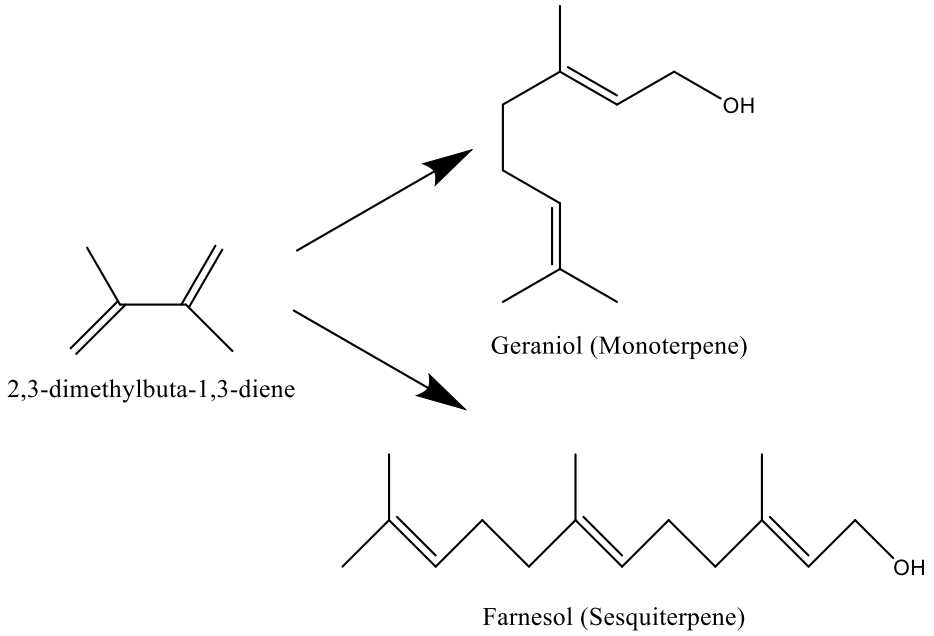


Figure 2. Isoprene, geraniol, and farnesol structures.

Terpenes have several biological functions and participate in both the primary metabolism and the secondary metabolism of plants. In the central metabolism, they are photosynthetic pigments, electron carriers (ubiquinone and plastoquinone) regulators of plant growth and development, are part of the cell membranes (phytosterols) and participate in protein glycosylation. In secondary metabolism, they participate as defense molecules, toxic compounds, and food deterrents for insects. In some plants, they are responsible for molecules for attracting pollinators, or they function as dispersers.[18,19,20,21]

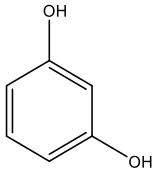
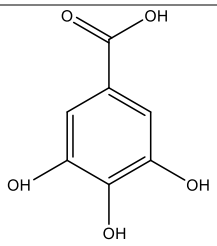
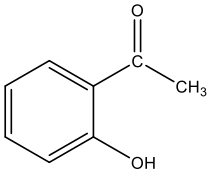
They are synthesized from primary metabolites by two pathways: that of mevalonic acid, active in the cytosol, in which three molecules of acetyl-CoA condense to form mevalonic

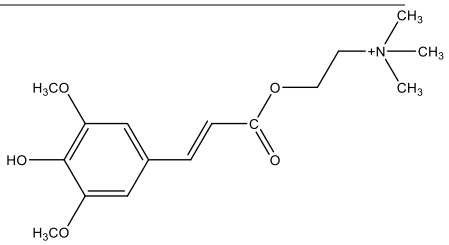
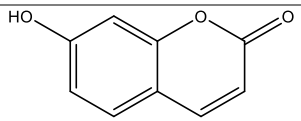
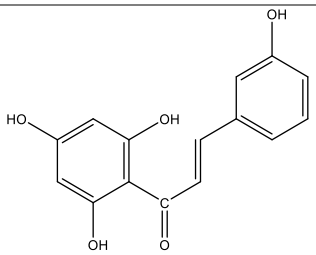
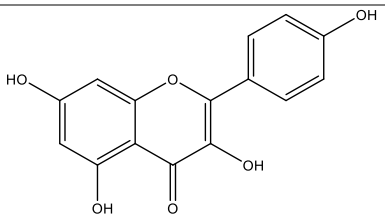
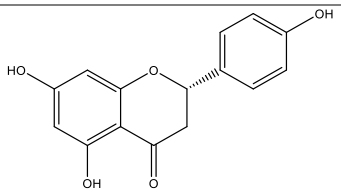
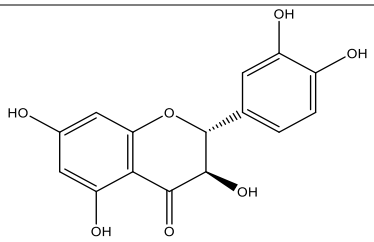
acid that reacts to form isopentenyl diphosphate (IPP) or the pathway of methylerythritol phosphate (MEP) that functions in chloroplasts and also generates IPP.[22]

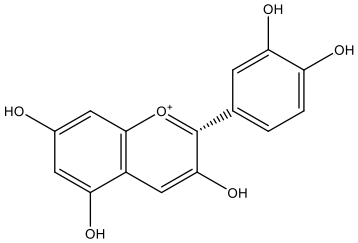
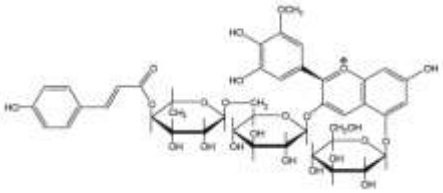
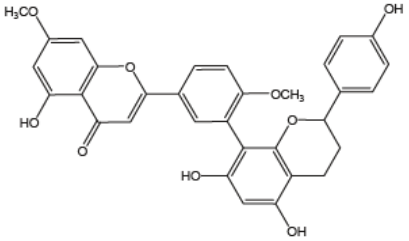
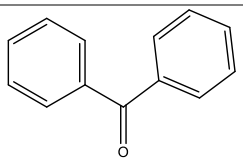
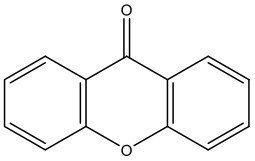
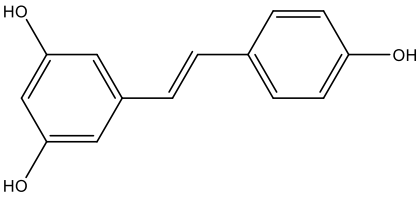
2.2.2 Phenolic compounds

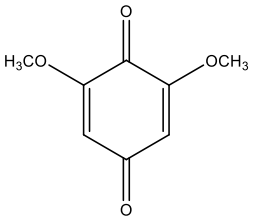
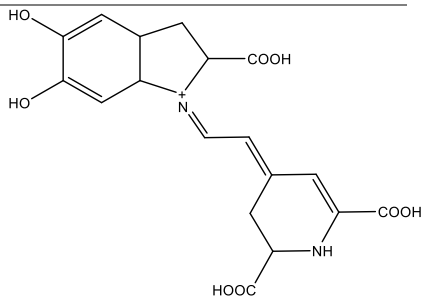
They are chemical compounds containing a hydroxyl group directly attached to an aromatic hydrocarbon. Chemically, phenolic compounds are a very diverse group of SM. The simplest representative of this class is phenol.[23,24] The most important criterion for classifying them is the number of carbons present in the molecule. According to this criterion, the phenolic compounds are classified into simple phenols, acidic phenols, acetophenones, and phenylacetic acids, hydroxycinnamic acids, coumarins, flavonoids, biflavonyls, benzophenones, xanthones, stilbenes, quinones, and betacyanins (**Table 1**). Lignans, neolignans, tannins, and phlobaphenes also belong to this group. The latter are polymers and have more complex structures.[25,26]

Table 1. Classification of phenolic compounds according to the number of carbons in the structure.

Skeleton structure	Class	Characteristics	Examples	Structure
C6	Simple phenolics	Substituted phenols	Resorcinol	
C6 - C1	Phenolic acids and related compounds	A carboxyl group substituted on a phenol	Gallic acid	
C6 - C2	Acetophenones and phenylacetic acids	Are rarely found in nature	2-hydroxyacetophenone	

C6 - C3	Cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols	Are commonly found in plants as esters of quinic acid, shikimic acid, and tartaric acid or as sugar esters	Sinapoyl choline	
C6 - C3	Coumarins, isocoumarins, and chromones	They possess an oxygen heterocycle as part of the C3-unit	Umbelliferone	
C6-C3-C6 (C15) Flavo-noids	Chalcones, aurones, dihydrochal-cones	Two benzene rings are linked together by a group of three carbons	Butein	
	Flavones	Contains a ketone group, and an unsaturated C-C bond	Kaemferol	
	Flavanones	Contains a ketone group	Naringenin	
	Flavanonols	Occur in association with tannins	Taxifolin	

	Anthocyani-dins	The heterocycle is a pyrilium kation	Cyanidin	
	Anthocyanins	Are water-soluble glycosides of anthocyanidins	Pentanin	
C30	Biflavonyls	Are dimers of flavones or methylated derivatives	Ginkgetin	
C6-C1-C6	Benzophenon es	Are aromatic ketones	Benzophenone	
	Xanthones	Are yellow pigments in flowers	Xanthone	
C6-C2-C6	Stilbenes	Two benzene rings are linked together by a group of two carbons	Resveratrol	

C6, C10, C14	Quinones	Oxidizing agents	2,6-dimethoxybenzoquinone	
C18	Betacyanins	Are red pigments and contain nitrogen	Betanidin	

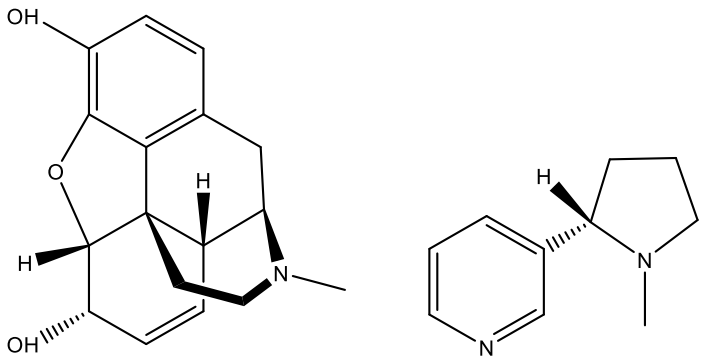
Phenolic compounds are synthesized in plant cells by the shikimic acid pathway or the malonate/acetate pathway (or both, for example, flavonoids).[25] The shikimic acid pathway provides the synthesis of phenylalanine and cinnamic acids and their derivatives (simple phenols, phenolic acids, coumarins, lignans, and phenyl propane derivatives).[27,28] The polyacetate pathways combine precursors of both the shikimic acid pathway and the polyacetate pathway. This is the case of flavonoids.[29]

Phenolic compounds fulfill various functions in plants: they oxidize quickly and act as antioxidants[30], they act as plant growth inhibitions[31], seeds accumulate significant amounts of phenols that act as a filter so that oxygen does not reach the embryo and inhibit its germinations.[32] Phenols are accumulated on surfaces of leaves, capturing up to 90% of UV radiation.[33] Phenols confer aromas and colors to the fruits, making them appetizing for herbivores, which favors the dispersion of seeds through feces.[34] Plants compete with each other to preserve their territories, and this process (allelopathy) the phenols participate.[35] Plants also defended themselves against the attack of pathogens by synthesizing phytoalexins that are toxic to microorganisms, and their presence prevents infections.[36] Phenols also protect plants by generating bitter flavors or textures that are unpleasant for herbivores.[37]

2.2.3 Alkaloids

Alkaloids constitute another large and diverse group of SM that includes molecules isolated primarily from vascular plants.[38] Plants generally produce a complex mixture of alkaloids, in which a significant constituent dominates. In a given plant, the biosynthetic origin of the alkaloids present is common, even if their structures are slightly different.[38] Another interesting observation is that the concentration of alkaloids varies considerably from one part to another of the same plant, and even in some parts, it may not contain those at all.[38] Alkaloids are also found in fungi, bacteria, and animals.[39] They include an atom of nitrogen in their structure, are toxic compounds and respond to common precipitation reactions.[40]

Even when there is no uniform classification of alkaloids, several criteria have been used in order to classify them: biosynthetic origin, presence of a basic heterocyclic nucleus in the structure, pharmacological properties, and distribution in plant families.[41] Among these criteria, the biosynthetic origin of the alkaloids has been used quite frequently. According to this criterion, the alkaloids are classified as true alkaloids, protoalkaloids, and pseudoalkaloids. Pure alkaloids strictly comply with the fundamental characteristics of the alkaloids. Most alkaloids found in plants belong to this group. They contain an intracyclic nitrogen, have basic character and are compounds of high reactivity, even in small quantities. In plants, they can be found free, although they predominate as salts. The precursor compounds of the true alkaloids are amino acids (L-ornithine, L-lysine, L-tyrosine, L-tryptophan, L-histidine, and L-arginine). Some pure alkaloids have been derived from anthranilic and nicotinic acids.[42,43] The protoalkaloids constitute a smaller class in number. In this group, the nitrogen atom is not part of the heterocycle, and they derive from L-thyroid, L-tryptophan, and L-ornithine. They can also be considered aromatic amines.[40] The pseudoalkaloid contains heterocyclic rings with nitrogen but is not derived from amino acids. They are formed by subsequent incorporation of nitrogen into compounds initially free of this element. To this group belong terpenic alkaloids.[43]



Morphine (True alkaloid) Nicotine (True alkaloid)

Figure 3. Examples of true alkaloids.

Although the presence of alkaloids is not vital for the plant, there is evidence that indicates the roles that these substances play in vegetables. As for the functions they fulfill, at first, they were considered waste products of nitrogen metabolism, nitrogen reservoirs in the plant, and were even mentioned as growth regulators. Today it is accepted that the role they play is to defend the plant against insects and herbivores due to its toxicity and deterrent capacity. While some serve to protect the plant from predators or microorganisms (toxic or repellent substances), others do so to compete with other plant species in a given habitat (allelopathic substances).[44,45]

2.3 Phytochemical analysis

Phytochemical studies generally are based on previous ethnobotanical and ethnopharmacological knowledge about plants and often constitute hypothesis-driven studies. The general methodology for studying SM from plants comprises several stages: extraction from natural sources, the phytochemical screening of extracts to determine qualitatively the main chemical classes of SM present in the plant, the purification of individual components and elucidation of their chemical structures, the biological activity studies through *in vitro*/*in vivo* assays and the toxicity-cytotoxicity studies on organisms or cells. The methodology involves a combination of different analytical techniques. In this methodology, the method of extracting secondary metabolites, and their identification in phytochemical gait is crucial.[46] Phytochemical research is aimed at finding out compounds synthesized by plants with pharmacological activities of importance for the treatment of

human diseases. The development of efficient methods of extraction and the battery of methods that exist for the scrutiny of the extracts of these medicinal plants allow more profound studies on the pharmacological activities of metabolites and their potential application in human health.[47]

2.4 Extraction methods

Extraction is an exercise where different solvents are used to extract interest compounds depending on their polarity. Conditions of extraction as temperature, time, and equipment are important factors to achieve high-quality extracts.[48] The most common extraction methods are maceration, infusion, percolation, decoction, Soxhlet or continuous extraction, microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), accelerated solvent extraction (ASE), and supercritical fluid extraction (SFE).

2.4.1 Maceration

Maceration is a solid-liquid extraction technique. For this process, the vegetal material has to be in small pieces to increase the contact surface between the solvent and the solid.[49] The method consists of using a solvent or a mixture of solvents that have an affinity with compounds that are going to be extracted. The mixture (plant-solvent) is put in a bottle or a container with lid and left for some days until the interest compounds could be transferred from vegetal tissues to the extraction solvent. This method is widely used with soft vegetal material.[46]

2.4.2 Soxhlet extraction

In this method, a Soxhlet extractor, a condenser, and a round bottom flask are used. The vegetal material is loaded into the thimble of a strong paper of cellulose and then placed in the Soxhlet extractor. The solvent goes in the round bottom flask, and it needs to be heated. The vaporized solvent goes into the thimble and then returns to the flask after being condensed in the condenser. The system is left for some hours, at least sixteen.[50]

2.4.3 Microwave-assisted extraction (MAE)

The sample (plant-solvent) is heated using electromagnetic radiation. This method improves the extraction of intracellular compounds due to the rupture of the cellular wall. Increasing temperature, the humidity inside the cell is transformed into vapor. So, it increases the intracellular pressure, and the lysis is provoked. This factor comes together with other effects in the solution that benefit the interaction of the compounds to be extracted with the solvent.[51]

2.4.4 Ultrasound-assisted extraction (UAE)

Many mechanisms occur in ultrasound-assisted extraction. One of the most important or relevant is the fragmentation provoked by the collisions between the ultrasonic waves and particles. The other effects as erosion and shear stress increase the interaction between solvent and particles. There are two forms of applying it, the direct form and indirect form. The direct form is a relatively new technique where the ultrasound is in direct contact with the sample, and its efficacy is 100 times better than the indirect form. The second form is carried out using an ultrasound bath, where the contact is given through the walls of the bottle.[52]

2.5 Phytochemical screening

Phytochemical screening is a qualitative analysis to obtain information on the main secondary metabolites that a plant contains since each class or group of SM is related to specific biological activities. Depending on the results obtained in the preliminary phytochemical examination, it is possible to guide further investigations to characterize or determine the biological activity of the species in question and the active principles involved.[53] The phytochemical examination consists of executing standard chemical reactions in aliquots of plant extracts. The reactions produce colorimetric changes, and precipitates, of specific colors, depending on the metabolite to be evaluated (**Table 2**).

Table 2. Summary of phytochemical screening methods.

Secondary metabolite	Name of test	Reactants	The expected result if positive
Alkaloid	Dragendorff's test	A solution of potassium bismuth iodide	A red precipitate
	Wagner´s test	Iodine in potassium iodide	A brown/reddish precipitate
	Mayer's test	Potassium mercuric iodide	A yellow colored precipitate
	Hager ´s test	Saturated picric acid solution	A yellow colored precipitate
Saponins	Froth test	Water	Formation of 1 cm layer of foam
	Foam test	Water	Produced foam persists for ten minutes
Phytosterols	Salkowski's test	Chloroform, concentrated sulphuric acid,	The appearance of a golden yellow color
	Libermann Burchard's test	Chloroform, acetic anhydride, concentrated sulphuric acid	Formation of the brown ring at the junction
Phenols	Ferric chloride test	Ferric chloride solution	Formation of bluish-black color
Tannins	Gelatin test	1% gelatin solution, sodium chloride	Formation of white precipitate
Flavonoids	Alkaline reagent test	Sodium hydroxide solution	Formation of intense yellow color, which becomes colorless on the addition of dilute acid
	Lead acetate test	Lead acetate solution	Formation of a yellow color precipitate
Glycosides	Modified Borntrager's test	Ferric chloride, benzene, ammonia solution	Formation of rose-pink color in the ammonical layer
Cardiac glycosides	Legal's test	Sodium nitroprusside in pyridine, sodium hydroxide	Formation of pink to a blood-red color
Carbohydrates	Molisch's test	Alcoholic α-naphthol solution	Formation of the violet ring at the junction
	Benedict's test	Benedict's reagent	Orange-red precipitate
	Fehling's test	Fehling's A & B solutions	Formation of red precipitate
Proteins and amino acids	Xanthoproteic test	Concentrated nitric acid	Formation of yellow color
	Ninhydrin test	Ninhydrin reagent	Formation of blue color

Colorimetric reaction for the detection of phenolic compounds

The reaction between phenolic compounds and ferric chloride results in the formation of a colored complex (**Equation 1**). The color varies depending on the substitutions in the phenolic compound. In the case of substitutions in the meta position, $–CH_3$, $–OH^-$, $–NH_2$, or a halogen, the result is a compound of color from blue to violet. In the case that the phenolic compounds are substituted with –COOH, –CHO, –COOR, $–NO_2$ or $–SO_3H$, colored complexes are produced ranging from violet to red.[54]

$$6ArOH + FeCl_3 \longrightarrow [Fe(OAr)6]^{3-} + 3H+ + 3HCl$$

Equation 1. Reaction between phenolic compounds and ferric chloride.

Precipitate reaction for the detection of tannins

In this reaction interact sodium chloride, tannins from the extract, and gelatin in a liquid medium. If the test is positive, the reaction results in a precipitate. Sodium chloride causes the collagen from gelatin to denature and precipitate[55]. Denatured collagen can interact with tannic compounds through hydrogen bonds. The -OH groups of tannic compounds interact with the N atoms of the collagen amino acid residues through hydrogen bonds. Furthermore, there are hydrophobic interactions between some amino acid residues and the carbon atoms of tannins. The mixture of collagen and tannins precipitates in the solution as an agglomerate[56] (**Figure 4**).

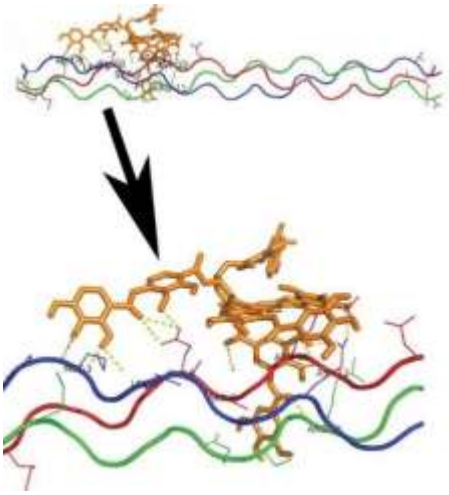


Figure 4. Computational docking of interactions between tannic acid (tannin) and collagen-like peptide.[56]

Colorimetric reaction for the detection of coumarins

The coumarins found in the extracts react with the solution of sodium hydroxide. This compound acts as nucleophile and opens the ring of the coumarins. The resultant compound is the coumaric acid. This compound has a yellow color that can be observed in the solution[57] (**Figure 5**).

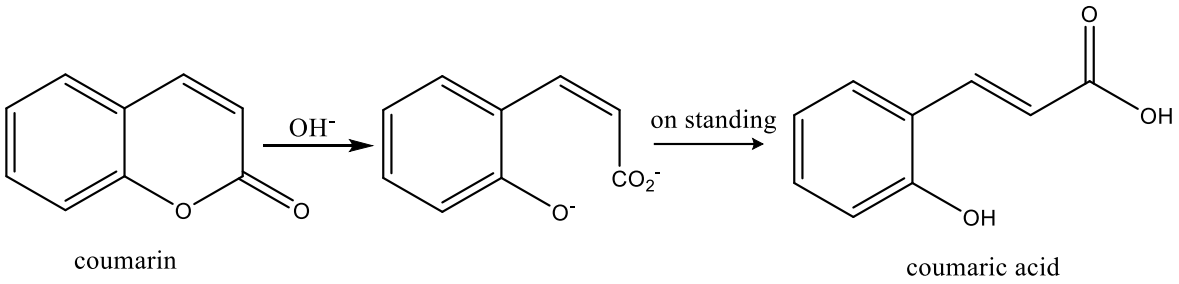


Figure 5. Reaction between coumarins and sodium hydroxide to form coumaric acid.

Precipitate reaction for the detection of carbohydrates

Carbohydrates found in the extracts react with the Benedict's reagent. The free aldehydes groups or α-hydroxymethyl ketone can react with the cupper ion of Benedict´s reagent in an oxide-reduction reaction. The copper is reduced from Cu^{2+} to Cu^{+}, and the carbonyl group is converted to a carboxyl group. Cu^{2+} has a blue coloration, while the Cu^{+} changes in alkaline medium precipitating as Cu_2O with red-orange coloration[58] (**Figure 6**).

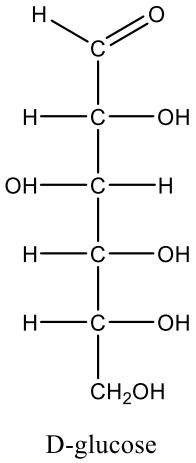


Figure 6. D-glucose, reducing sugar.

2.6 Total phenolic content

The methods to calculate the phenolic content are based on colorimetric reactions whose final product can be measured spectroscopically. The quantification of phenols is realized using a calibration curve with Gallic acid. As a reagent, it is possible to use compounds that interact with phenols, such as ammonium sulfate. It should be noted that this compound is not efficient for this test since the wavelength used to measure the complex formed is also the maximum absorbance of some compounds that have color.[59]

The most commonly used compound to evaluate the total phenolic content is the Folin-Ciocalteu reagent. The reaction of the Folin-Ciocalteu reagent with phenolic compounds is performed in basic media. The resultant product is a complex with blue coloration and can be measured at 765 nm in a spectrophotometer. Folin-Ciocalteu reagent consists of a mixture of disodium dioxido(dioxo)tungsten; dihydrate, disodium dioxido(dioxo)molybdenum; dihydrate, and phosphoric acid. This molecule reacts with the phenolic compounds in the media. The F-C reagent has a yellow color, and after being reduced by phenolic compounds, it results in an intense blue-colored complex.[60]

2.7 UV-VIS analysis

Ultraviolet-visible spectroscopy is a useful technique to determine the presence of secondary metabolites. This technique uses light in the visible and ultraviolet regions. The wavelengths in these regions range from 190 nm to 800 nm.[61] In these regions of the electromagnetic spectrum organic compounds absorb the radiation, and as a consequence, they have electronic transitions between their different electronic levels.[62] The transitions that are analyzed in this technique are $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$. Despite of the transition of the type $n \rightarrow \pi^*$ is forbidden it is commonly observed in the spectra with lower intensity than for allowed transitions. The wavelength of the absorption maximum of chromophores from some of the basic structures of secondary metabolites can be predicted with the Woodward-Fieser rules.[63] Chromophores are a group of atoms that have the property of being excited by absorbing radiation from this range of the electromagnetic spectrum. As an effect, they have electronic transitions that can be quantified[62] (**Figure 7-11**).

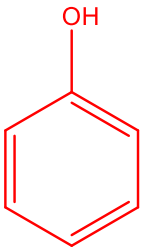


Figure 7. Benzene has a maximum absorption at $\lambda_{max} = 203.5$ nm in the primary band and $\lambda_{max} = 254$ nm in the secondary band. In the case of phenol, the benzene ring has an electron-releasing substituent (-OH). This substitution provokes a shift from 203.5 nm to 210.5 nm in the primary band, a shift from 254 nm to 270 nm in the secondary band, and also an intensity increase of the secondary band.[63]

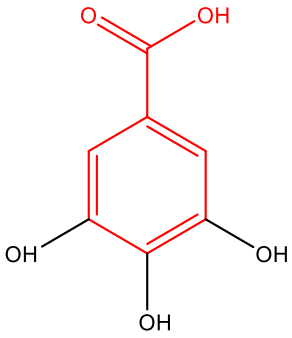


Figure 8. The basic structure of hydrolyzable tannins, Gallic acid. The highlighted chromophore (benzoic acid) has a maximum absorption at λ_{max}=230 nm. Benzoic acid has three electron-releasing substituents (-OH). Each one of the substitutions in the meta position provokes an increase of 7 nm, and the one in the para position provokes an increase of 25 nm. Resulting in a total shift from 230 nm to 269 nm.[63]

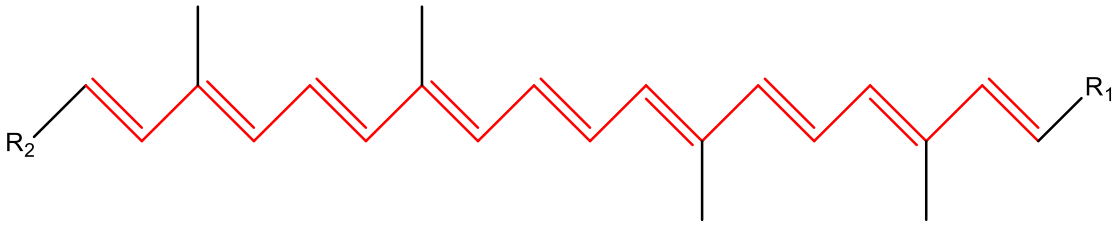


Figure 9. Basic structure of some carotenoids (terpenes). The general formula for determining the theoretical wavelength at the maximum absorption is: $\lambda_{max} = 114 + 5M + n(48.0 - 1.7n) - 16.5$ Rendo $-$ 10 Rexo, where n= number of conjugated double bonds, M= number of alkyl or alkyl-like substituents in the conjugated system, Rendo= number of rings with endocyclic double bond in the conjugated system, and Rexo= number of rings with exocyclic double bond in the conjugated system. This structure has n= 9 and M= 5, so its $\lambda_{max} = 493.7$nm.[64]

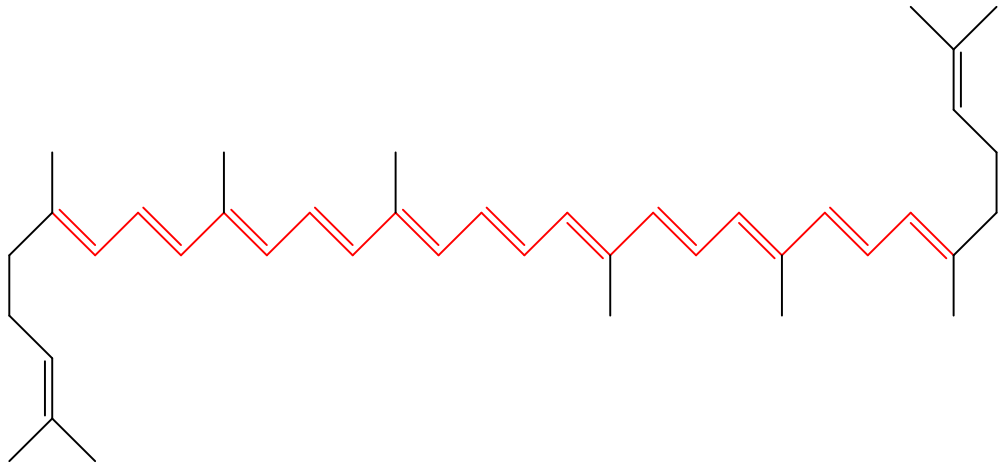


Figure 10. Structure of lycopene. This structure has n=11 and M= 8, so its $\lambda_{max} = 476nm$.

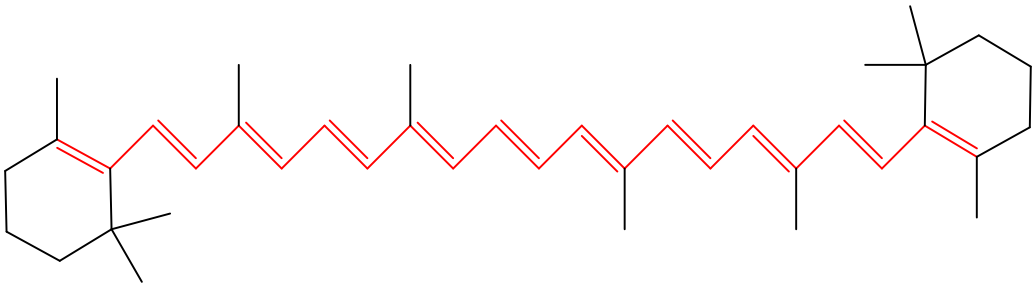


Figure 11. Structure of beta-carotene. This structure has n= 9, M= 5, and Rendo= 2 so its $\lambda_{max} = 453nm$.

Flavonoids have been studied using various techniques such as NMR, MS, and UV-VIS spectrophotometry. In UV-VIS spectra of flavonoids there are two characteristic bands. Band I for B ring, and band II for the A ring. The band I is in the range from 300-550nm, and the band II is in the range from 240-285nm (**Figure 12**). The ranges of wavelengths of maximum absorption change depending on the type of flavonoids because of differences in the substitutions in the rings. Flavones and flavonols have the band I in the range from 240-285nm, and the band II in the range from 303-304nm.[65]

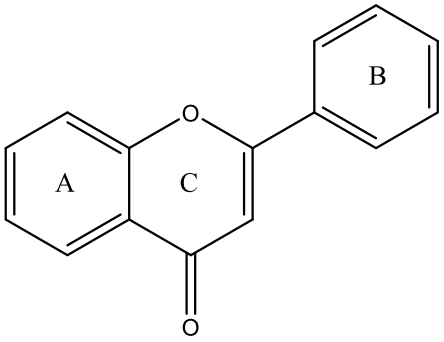


Figure 12. Basic structure of flavonoids.

2.8 Biological activity

Plants have the ability to synthesize a vast and diverse group of secondary metabolites. Many of them constitute bioactive substances that plants use as defense molecules. These molecules interact with specific targets in microorganisms or animal cells to exert some biological activity that neutralizes them. On the other hand, the diversity of metabolic pathways that plants use in the production of SM guarantees the existence in these defense molecules of specific structures useful to develop new drugs and medicinal products. That is why plants constitute a valuable source of substances that can be used for improving health and/or curing diseases.[66,67] Currently, the phytochemical composition and the functional activity of many plants are investigated, to find out what are the compounds that provoke their biological effects.[68] Plant extracts could show biological activities such as anti-inflammatory, antimicrobial, antifungal, antiviral, among others, and these responses are directly related to their phytochemical composition.[51] Finding new products or compounds that could make a better response than actual drugs could be achieved with investigations of secondary metabolites that are contained in plants.[51]

The biological effect depends on the type of secondary metabolite; it means the nature of the organic compound. SM can have toxicological, insecticidal, microbiological, fungicidal effects, or another.[69] The antimicrobial activity of many plant extracts has demonstrated to be effective against Gram-positive and Gram-negative bacteria.[70] Besides, several authors have pointed out the possible synergy between antibiotics and plant extracts.[71] In the case of polyphenols, the antibacterial activity is based on the ability of these compounds to inhibit growth, reproduction, respiration, and any other vital function of microorganisms. This

action is performed by the oxidation of specific enzymes, which inhibit some critical functions, such as breathing. It is also reported that polyphenols bind to DNA chains disrupting protein synthesis in microorganisms. Other authors suggest that some polyphenols can break the cell membranes of some microorganisms like bacteria or virus, producing cell apoptosis.[72,73] It is also known that monoterpenes can interact with the phospholipids of cell membranes of many microorganisms due to their lipophilic nature. As a result, the ordered structure of the membranes is interrupted, thus causing cell lysis.[74]

The antioxidant activity has also been studied from plant extracts. It is mainly related to the presence of polyphenols or phenolic compounds. Flavonoids act primarily as buffers and capture free radicals to generate the flavinic radical, much less reactive since, in their structure, the missing electrons are more delocalized. Also, flavonols such as quercetin can chelate transition metal ions such as iron or copper, preventing the formation of reactive oxygen species.[75,76]

$$FlOH + R^{\cdot} \rightarrow FlO^{\cdot} + RH$$

Equation 2. Oxidation of flavonoid compound to form a less reactive radical.[77]

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + OH^- + OH^{\cdot}$$

Equation 3. Formation of flavonoid metal complexes to prevent the Fenton reaction (univalent reduction of hydrogen peroxide).[77]

The anti-inflammatory effect of plant extracts is mainly due to the presence of phenolic compounds, alkaloids, saponins, sterols, and essential oils. Flavonoids, condensed tannins, coumarins, and gallotannins are some secondary metabolites that have anti-inflammatory activity and are phenolic compounds. proanthocyanidins are condensed tannins that are present in many species. They exhibit anti-inflammatory activity, and have anti-cancer, antimicrobial, antihypertensive, and cardioprotective activity. Similarly, flavonoids have anti-inflammatory activity in vivo/ in vitro, and gallotannins have other pharmacological effects such as anti-cancer and antimicrobial activity.[78]

2.8.1 Antibacterial activity

There are many methods to screen the *in vitro* antibacterial activity. The most used are the disk-diffusion and broth or agar dilution. To make an extensive analysis of antibacterial activity, it is necessary to use methods like time-kill test and flow cytofluorometric that include information about the type of inhibition. These can be bactericidal, bacteriostatic, time-dependent, or concentration-dependent.[78] These essays are made with microorganisms that are resistant to almost all types of antibiotics, using the approved techniques. To find new substances that could solve this resistance problem of many pathogens.[79] There are drugs to control the inflammatory process. These can be steroids or non-steroids. Many of the medications currently used to curb the inflammatory process come from compounds synthesized by plants.[78]

2.8.1.1 Agar diffusion and well diffusion methods

The most commonly used methods are Agar disk-diffusion and Agar well diffusion. Both methods consist of inoculating the standard microorganism in an immaculate agar and dried for a few minutes. However, the forms that extract interact with the microorganism are different. In the first method, 6mm diameter filter paper discs are used. These papers are soaked in different plant extracts of known concentrations. Then the filter papers are placed on the top of the agar plate. In the second method, a well of 6-8mm is made in the agar. Then a sample of the extract is placed in the well. In both methods, there must be controlled temperature and time of incubation. Also, there must be positive and negative control samples in the Petri dishes, to make the comparisons with the growing inhibition diameter of the extracts.[80,81]

2.8.1.2 Minimum inhibitory concentration (MIC)

In the case that extract has a positive effect inhibiting the bacterial growth, the minimum inhibitory concentration can be determined. This MIC is the lowest concentration at which the extract still has an inhibitory effect on bacterial growth after 24 hours of incubation. To determine the MIC are used agar well diffusion, the experiment for antibacterial activity previously mentioned, among other methods. The aim is to prove different low concentrations of the extract and verify what the lowest concentration that has an antibacterial

effect is. Using the disk diffusion method, the species *P.granatum* has a MIC of 2.5 mg/ml against *S.aureus* and *P.aeruginosa*.[80,81]

2.8.1.3 Growth inhibition assay

The bacterial concentration in a solution can be calculated using UV-VIS spectroscopy. With this technique, a graph of the growth of the bacteria regarding time can be made. The bacteria are inoculated in a culture medium, and the optical density is taken for 12 h – 24 h with intervals of 30 min - 60 min. As a result, the plot of absorbances versus time shows the three stages of bacterial growth. The growth of the bacteria is compared with the growth of the bacteria in the presence of the compound to be evaluated. The difference in absorbance values is the effect of changes in the concentration of bacteria. The drop in values means that the concentration of bacteria is decreasing, and the compound has antibacterial activity.[82]

2.8.2 Anti-inflammatory activity

Inflammation is a complex process whose remarkable response is tissue growth, redness, a sensation of heat, burning, pain, among others. The inflammation is the result of high concentrations of leukocytes and the leakage of liquids and proteins.[78] It usually occurs as an effect of some diseases or injuries, which causes medical treatment to be difficult. Many factors trigger the inflammatory response. The answer is characteristic and goes according to the stimulus. The magnitude of the response of the inflammatory process is relevant because of a response greater or less than that required causes immunodeficiency or morbidity and mortality in certain diseases. Inflammatory processes have been studied in recent years to know and understand the mechanism and molecules that are involved.[83]

2.8.2.1 Inhibition of albumin protein denaturation and inhibition of heat-induced hemolysis

The percentage inhibition of albumin protein denaturation is based on the capacity of the plant extracts to inhibit the albumin protein denaturation at high temperatures. On the other hand, the method was erythrocytes are used instead of the albumin protein, measures the capability of the extract to protect their membrane when they are exposed to high temperatures. In both experiments, the capacity of the extract to inhibit inflammatory response is measured. The erythrocytes are used because their membrane is similar to the

lysosome membrane. The lysosomes are involved in the inflammatory responses, and the stabilization of their membrane does not allow the release of lysosomal constituents of activated neutrophils.[84,85]

3. PROBLEM STATEMENT

Medicinal plants have been traditionally used to this day. The compounds that confer them the pharmacological effects are the secondary metabolites. Just as new compounds have been obtained that have soon become synthesized for commercialization, it has also happened that the use of synthetic compounds has been changed for compounds from natural sources. The variety and complexity of the molecules found in nature make this an immeasurable source of compounds that could have pharmacological effects. Today the pharmacological activity of a large number of SM is known, and new compounds are still being studied.[86] Currently, there are no studies on the phytochemical of the Ecuadorian species *Epidendrum coryophorum*. Since the results of the phytochemical characterization of other species belonging to the same genus have given promising results, it is important to study the phytochemical of this species.[87,10] Some species of the same family are used as medicinal plants, so active ingredients may be found when studying this species.[88]

4. GENERAL AND SPECIFIC OBJECTIVES

4.1 General objective

Perform the preliminary phytochemical analysis of the species *Epidendrum coryophorum.*

4.2 Specific objectives

- To determine the phytochemicals contained in the species *Epidendrum coryophorum* qualitatively.
- To corroborate the presence of secondary metabolites by UV-VIS analysis.
- To determine the total phenolic content of extracts of *Epidendrum coryophorum.*
- To evaluate the antibacterial and anti-inflammatory *in vitro* activities of extracts of *Epidendrum coryophorum.*

5. METHODOLOGY

5.1 Reagents

Methanol 99% (Alpha Chemika), ethanol 96% (NOVACHEM DEL ECUADOR), and chloroform 99% (NOVACHEM DEL ECUADOR), ferric chloride (Sigma-Aldrich), distilled water (NOVA Laboratorios), Hydrochloric acid (SCIENCE Company), ammonia (NOVACHEM DEL ECUADOR), sodium hydroxide (Sigma-Aldrich), sulfuric acid (LabChem), acetic acid (Sigma-Aldrich), ninhydrin (Sigma-Aldrich), Benedict's reagent (SCIENCE Company), Fehling´s solution A and B (SCIENCE Company), Folin-Ciocalteu reagent (Thomas Scientific), sodium carbonate (Thomas Scientific), Gallic acid (Fisher Scientific), dimethyl sulfoxide (Sigma-Aldrich), Luria-Bertani agar (PROBIOTEK), kanamycin (Sigma-Aldrich), Sacha Inchi oil, maltodextrin (Roig Pharma), gum Arabic (Roig Pharma), Phosphate buffered saline tablet (PBS) (Fisher Scientific), Aspirin (Bayer).

5.2 Equipment

Zuzi spectrophotometer model 4211/50, PerkinElmer UV/VIS/NIR spectrometer lambda 1050, Balance Cobos precision HR-150A, drying stove SLW 115 POL-EKO-APARATURA, Bante MS 300 hot plate magnetic stirrer, P/SELECTA Ultrasound HD, BUCHI Rotavapor R-210, Thermo Scientific NanoDrop 2000 Spectrophotometer, Thermo Scientific MaxQ 4450 orbital shaker, Thermo Scientific Heratherm Refrigerated incubator IMP -180, Teslar AV-30/70 vertical laminar flow bench, mini spray dryer BUCHI-B 290, UV-VIS Microplate Spectrophotometer Fisherbrand™ accuSkan™ GO, centrifuge Rotina 380, Grinder moler acero 200w 50g.

5.3 Plant material and its preparation

The source of obtaining phytochemicals was the orchid *Epidendrum coryophorum*. The *Epidendrum coryophorum* specimens were acquired in Ecuadorian company Ecuagenera, Guayaquil (http://www.ecuagenera.com), in June 2019. The plants were kept in pots until the leaves were separated from the plant for processing.

Using a scalpel, leaves and stems were cut from the base of the plant. Then, leaves were washed separately with plain water followed with distilled water to remove dust and particles.

Subsequently, the tissues were drained and dried in the shade at 25°C in the drying stove. The dried tissues were pulverized by using an electric grinder, labeled and stored at room temperature.

The yield of dried plant material was calculated as follow:

$$Dried\ plant\ material\ \% = \frac{Dried\ plant\ material}{Plant\ material} \times 100 \quad \text{(Eq1)}$$

5.4 Obtaining plant extracts

The extraction method used was maceration[89]. Leaves were extracted successively with methanol, ethanol, and chloroform in a glass container with protection from light, for 45 h using a ratio powder: solvent of 1:10 (w:v). The extracts were obtained by vacuum filtration on filter paper, and between each extraction step, the powder was allowed to aerate to remove the solvent residues. After that, each extract was taken into an ultrasound bath for 1 h at 25 ^{0}C (**Diagram 1**).

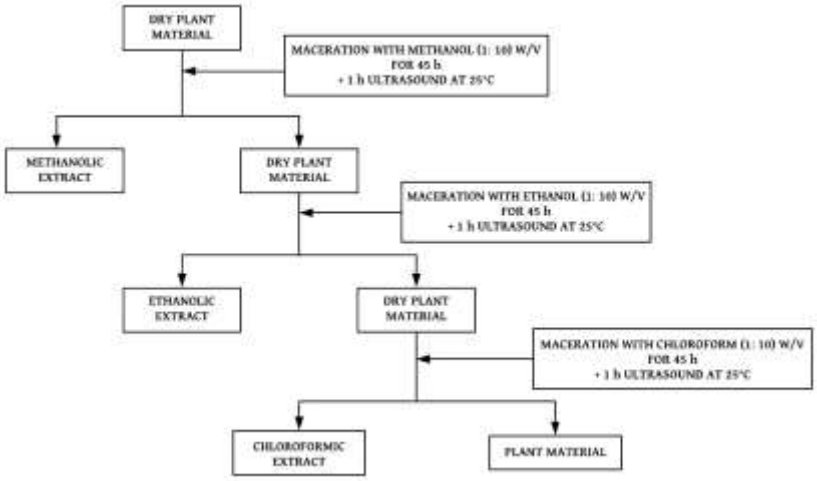


Diagram 1. Successive extraction of phytochemicals from powdered dry tissues of orchid *Epidendrum coryophorum* using methanol, ethanol, and chloroform.

5.5 Preliminary qualitative phytochemical analysis

Once the extracts from leaves of orchid *Epidendrum coryophorum* were obtained, the preliminary phytochemical analysis was carried out according to the methods described in

the scientific literature for the identification of different classes of phytochemicals. A brief description of each determination follows:

Test for phenols[90]: a few drops of 1.5% ferric chloride were added to 1 mL of each extract. The test could be considered positive if a red, blue, green, or purple coloration appears.

Test for anthocyanins[91]: 1.0 mL of each 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.

Test for coumarins[91]: 1.0 mL of each 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.

Test for flavonoids[92]: 1.0 mL of 1.0 % dilute ammonia solution was added to 1.0 mL of each extract. The formation of yellow color in the solution could indicate the presence of flavonoids.

Tests for tannins[91]: 1.0 mL of each extract was mixed with 2.0 mL of distilled water, and the mixture was heated in a thermostated bath. It was subsequently filtered with filter paper, and two drops of 1.0 % ferric chloride solution in methanol were added to the supernatant. The presence of tannins could be identified by the formation of a dark green or dark blue color in the solution.

Test for anthraquinones[93]: 1.0 mL of extract was heated with 1.0 mL of 10% ferric chloride solution and 1.0 mL of concentrated hydrochloric acid. The cooled extract was filtered. The filtrate was shacked with an equal amount of diethyl ether. Further, the ether extract was extracted with strong ammonia. The formation of a rose pink color could indicate the presence of anthraquinones.

Test for flobatanins[91]: 1.0 mL of each extract was mixed with a 2.0% hydrochloric acid solution and heated at 100°C. The presence of flobatanins could be determined by the formation of a red precipitate.

Test for terpenoids[91]: 1.0 mL of each extract was mixed with 1.0 mL of chloroform, and then 2.0 mL of concentrated sulfuric acid was added. The red-brown coloration at the interface could indicate the presence of terpenoids.

Test for saponins[91]: 1.0 mL of each extract was mixed with 3.0 mL of distilled water, it was stirred with vigor, and subsequently, the mixture was heated to 100°C. Foaming with small bubbles could show the presence of saponins.

Test for steroids[91]: 1.0 mL of each extract was mixed with 3.0 mL of chloroform, and then the mixture was stirred. Subsequently, they were added carefully to 2.0 mL of concentrated sulfuric acid on the sides of the test tube. The formation of red color in the upper layer and green coloration in the sulfuric acid layer could indicate the presence of steroids in the extract.

Test for cardiotonic glycosides[91]: 2.0 ml of each extract was mixed with 2.0 mL of glacial acetic acid containing a drop of 1.0% ferric chloride solution. 1.0 mL of concentrated sulfuric acid was carefully added to the mixture by the walls of the test tube. The presence of characteristic deoxysugars of the cardiotonic compounds could be observed by the formation of a brown ring at the interface next to a purple ring below.

Test for proteins and amino acids[94]: 2.0 mL of extract in a test tube was mixed with 3-4 drops of 2.5% ninhydrin solution, and the mixture was heated. The appearance of purple or violet color could indicate the presence of protein or free amino acids.

Test for carbohydrates[94]: 1.0 mL of Benedict's reagent was placed in a test tube containing 1.0 mL of extract. The mixture was heated for 2 min in a water bath until boiling. The appearance of an orange-red could indicate the presence of carbohydrates.

Test for reducing sugars[95]: 5.0 mL of a mixture (1:1) of Fehling´s solution A and B were added to 2ml of the extract. The reaction mixture was boiled in a water bath for five minutes. A brick-red precipitated indicates the presence of reducing sugars.

5.6 Determination of total phenolic content

The determination of total phenolic content (TPC) was performed according to the protocol reported by Cheok et al[96]. To determine the TPC in methanolic and ethanolic extracts of leaves, a dilution of 1:10 (v:v) in distilled water was made.

The TPC assay was based on a colorimetric reaction with Folin-Ciocalteu reagent. The extracts were diluted to concentrations measurable in the UV-VIS spectrophotometer previous to the addition of the reagent. 300 μL of each extract was placed in a test tube using a micropipette and then mixed comprehensively with 1.5 mL of Folin-Ciocalteu reagent. The mixture was shaken 3 min, and then 1.2 mL of 7.5% sodium carbonate was added to de solution. The final solutions were incubated in the dark 30 min. After 30 min, the absorbance was measured in the UV-VIS spectrophotometer at λ= 765 nm. The blank for the measurements was pure solvents instead of the extract, following the same procedure. The resultant absorbance values were referred to as the standard calibration curve that was performed with Gallic acid, using concentrations from 0.02 to 0.3 mg/mL under the same conditions as with the extracts. The results were expressed as mg of GAE/g of dry plant material.

5.7 UV-VIS analysis

The ethanolic, methanolic, and chloroformic extracts were diluted by 1:20 using the same solvent and scanned using a spectrophotometer in the UV-VIS range from 250 nm to 800 nm. The blank for the measurements was pure solvents instead of the extract, following the same procedure. The characteristic peak´s values were recorded.

5.8 Antibacterial activity

20 mL aliquots of each extract were dried in vacuum using a rotary evaporator at 30ºC to yield greenish residues that were used for determining antibacterial activity. The dried material of leaves obtained from ethanolic, methanolic and chloroformic extracts was dissolved in dimethyl sulfoxide (DMSO) to concentrations of 10.0 mg/mL and 25.0 mg/mL for the antibiogram, and the concentration of 1 mg/mL for the growth inhibition assay.

5.8.1 Agar diffusion test or Kirby-Bauer test

The agar diffusion method was developed as reported by Tresina[97], with some modifications. The antibacterial activity of extracts was determined against the Gram-negative bacteria *Escherichia coli TG1* (ATCC).

The inoculation of *Escherichia. coli* (*E. coli)* was performed in Luria-Bertani agar (LB agar). The back of the Petri dish was divided into quadrants and labeled with a marker, then approximately 25 mL of LB agar was dispersed in the Petri dish and allowed to solidify. The process was executed inside a laminar flow hood. 300 μL of the strain of *E. coli* in Luria-Bertani broth (LB broth) was placed on the surface of the agar using a micropipette, and a sweep was made with a Drigalski spatula until the surface was coated uniformly. After a few minutes, the LB broth dried, and 3 μL of each sample was placed in each quadrant using a micropipette. The Petri dish was then placed in an incubator for 24 h at 37°C. The evaluated samples were the ethanolic, methanolic, and chloroformic dried extracts of leaves dissolved in DMSO at specified concentrations, as positive and negative controls Kanamycin and pure DMSO were used, respectively.

5.8.2 Growth inhibition assay

The growth inhibition assay was done as reported by Dávila[98], with some modifications. *E. coli* was seeded in LB broth 18 h before the experiment and incubated at 37°C in a shaker. The strain was reseeded in LB broth after the 18 h of incubation. LB broth was added to the bacteria solution until reaching an absorbance value of 0.02. The process was executed inside a laminar flow hood. 2 mL of bacteria solution was transferred to a conical tube for each sample. 100 μL of each sample was added to a conical tube using a micropipette. Then, all tubes were placed in the shaker at 37°C. The absorbance of each sample was measured for 7 hours at λ = 620nm in a Nano Drop with an aliquot of each sample.

The evaluated samples were the ethanolic, methanolic, and chloroformic dried extracts of leaves dissolved in DMSO at specified concentration, and as a negative control, DMSO pure was used. The calculation of the inhibition percentages was made by comparing the proportionality constants. The proportionality constant that describes the behavior of

bacterial exponential growth was calculated using the equation that describes the exponential growth of bacteria.[98]

$$\mu = \frac{\ln(N) - \ln(N_0)}{t - t_0} \qquad \text{(Eq2)}$$

Where:

μ: proportionality constant

N: number of bacteria at the beginning of a time interval

N_0: number of bacteria at the end of the time interval

$t - t_0$: time interval in hours or minutes

5.9 Anti-inflammatory activity

20 mL aliquots of each extract were dried in vacuum using a rotary evaporator at 30ºC to yield greenish residues that were used for determining anti-inflammatory activity. The paste obtained by drying the ethanol extract was homogenized in Sacha Inchi oil. To evaluate the anti-inflammatory activity of the homogenate, it was microencapsulated in a mixture of maltodextrin and gum Arabic.[99]

5.9.1 Microencapsulation of extracts

The microencapsulation was applied only to the dried ethanolic extract of leaves. 171 mg of dried ethanolic extract of leaves were dissolved in 4.595 g Sacha Inchi oil. The mixture was heated on a water bath in order to homogenize.

In order to prepare the microencapsulated extract, 5 g of maltodextrin were mixed with 5 g of gum Arabic and water. This polymeric mixture was incorporate into the extract solution. Then the mixture was homogenized using a homogenizer-disperser at 19700 rpm for 1 min. Subsequently, the mixture entered the Spray dryer to start the microencapsulation process in its hot chamber at 150°C, allowing the encapsulating material to mix with the particles of the compound and its subsequent solidification.[100]

5.9.2 Collection and preparation of erythrocyte suspension

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 3 consecutive washes to the erythrocytes 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.[85]

5.9.3 Preparation of microencapsulated extract´s solutions

Microencapsulated extract was dissolved in PBS to reach concentrations of 1.0 mg/mL, 1.5 mg/mL, and 2.5 mg/mL.

5.9.4 Inhibition of heat-induced hemolysis

In 25 mL conical tubes were added 3 mL of the evaluated samples at a concentration of 1.0, 1.5, and 2.5 mg/mL 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) were evaluated at concentrations of 1.0 mg/mL, 1.5 mg/mL, and 2.5 mg/mL. All reaction mixtures were prepared in duplicate except for 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 the supernatants was measured at λ = 560nm in triplicated.[85]

The percentage of hemolysis inhibition was calculated according to the following expression:

$$\%\ hemolysis\ inhibition = \left[1 - \frac{A_I - A_{WI}}{A_{NC} - A_{WI}}\right] \times 100 \qquad \text{(Eq3)}$$

Where:

$A_{I:}$ Absorbance of the sample with incubation

$A_{WI:}$ Absorbance of the sample without incubation

$A_{NC:}$ Absorbance of the negative control

6. RESULTS, INTERPRETATION, AND DISCUSSION

6.1 Plant material

In this work, plants acquired in Ecuagenera (http://www.ecuagenera.com) were used. Ecuagenera is a company specialized in the production and conservation of species and hybrids of orchids. By analyzing the morphological characters, they identify this species as *Epidendrum coryophorum*.

125.95 g of wet leaves were used. The resulting weight of leaves after the drying process was 21.00 g. The moisture percentage resulted in 16.67%. As a result of the maceration with methanol, ethanol, and chloroform, three extracts were obtained. A nomenclature adapted to this work was used to facilitate reading and understanding of it (**Table 3**).

Table 3. Nomenclature of the extracts obtained from the leaves of *Epidendrum coryophorum*.

	Solvents used for extraction by maceration		
Tissue	**Methanol**	**Ethanol**	**Chloroform**
Leaves	EHM	EHE	EHC

6.2 Qualitative phytochemical screening of leaves of the species *Epidendrum coryophorum*

The evaluated metabolites in leaves of *Epidendrum coryophorum* were phenols, anthocyanins, coumarins, flavonoids, tannins, anthraquinones, flobatanins, terpenoids, saponins, steroids, sterols, cardiotonic glycosides, carbohydrates, proteins and amino acids, and reducing sugars.

In the methanolic extract of leaves of *Epidendrum coryophorum* were detected, phenolic compounds such as coumarins, simple phenols, and tannins. Terpenic compounds such as terpenoids, sterols, and steroids were also detected in the methanolic extract. Other secondary metabolites as carbohydrates and reducing sugars were also detected.

In the ethanolic extract of leaves of *Epidendrum coryophorum*, coumarins, simple phenols, and flavonoids, sterols, steroids were detected. Also, there were found nitrogen-containing

compounds such as cardiotonic glycosides, and other secondary metabolites as carbohydrates and reducing sugars.

In the chloroformic extract of leaves of *Epidendrum coryophorum*, coumarins, simple phenols, flavonoids, sterols, steroids, cardiotonic glycosides, carbohydrates, and reducing sugars were detected (**Table 4**).

Table 4. Secondary metabolites detected in leaves of *Epidendrum coryophorum*.

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

a) 1.0 mL of each 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 [91]

b) 1.0 mL of each 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 [91]
*(+) indicates the presence of the evaluated secondary metabolite while (-) indicates its absence (**Annex1**)*

The presence of secondary metabolites in a given extract depends on their affinity with the solvent. Another fact that influences the extraction of secondary metabolites is the ability of the solvent to extract and preserve such compounds. Generally in the ethanolic extracts are founded secondary metabolites such as tannins, polyphenols, polyacetylenes, flavonols, terpenoids, sterols, and alkaloids.[101] In the ethanolic extract of leaves of *Epidendrum coryophorum*, the aforementioned metabolites were evaluated except for polyacetylenes and alkaloids, obtaining positive results for tannins, flavonoids, sterols, and polyphenols.

Methanol is a solvent used to extract anthocyanins, terpenoids, saponins, tannins, lactones, xanthoxylines, totarol, quassinoids, flavones, phenones, and polyphenols.[101] Some of these secondary metabolites were evaluated in the methanolic extract of leaves of *Epidendrum coryophorum*: anthocyanins, terpenoids, saponins, tannins, and polyphenols. Positive results were obtained for terpenoids, tannins, and polyphenols.

Chloroform is used for the extraction of terpenoids and flavonoids.[101] When evaluating such metabolites in the chloroformic extract of Epidendrum coryophorum, positive results were obtained for flavonoids.

This is the first research that evaluates the phytochemical content of the species *Epidendrum coryophorum*. However, phytochemical screening of other species belonging to the same genus has been performed. Cerna et al[10] evaluated the phytochemical content of ethanolic extracts of the species *Epidendrum blepharoclinium, Epidendrum blepharoclinium blanco*, *Epidendrum cochlidium* sp1, *Epidendrum cochlidium* sp2, *Epidendrum jamiesonis, Epidendrum medusae, Epidendrum nocturnum, Epidendrum paniculatum, Epidendrum porphyreum, Epidendrum secundum, Epidendrum secundum blanco*. The secondary metabolites found in these species that coincide with the results obtained from the ethanolic extract of leaves of *Epidendrum coryophorum* are tannins and flavonoids. Besides, the author also included several species of the genus *Caucaea* and *Oncidium*. The secondary metabolites

that coincide with those present in the ethanolic extract of *Epidendrum coryophorum* leaves are flavonoids and tannins (**Table 5**).

Table 5. Secondary metabolites in other *Epidendrum* species.

Solvent	*Epidendrum* Species	Phenols	Coumarins	Flavonoids	Tannins	Terpenoids	Triterpenes	Saponins	Alkaloids	Glycosides
Ethanol	*E. blepharoclinium*			-	+		+	+	-	
	E. blepharoclinium blanco			-	-		+	+	-	
	E. cochlidium sp1			-	+		+	+	-	
	E. cochlidium sp2			+	+		+	+	-	
	E. jamiesonis			-	+		+	+	-	
	E. medusae			-	-		+	+	-	
	E. nocturnum			+	+		-	+	-	
	E. paniculatum			+	-		+	+	-	
	E. porphyreum			-	+		+	+	-	
	E. secundum			+	-		+	+	-	
	E. secundum blanco			+	+		+	+	-	
	E. coryophorum	+	+	+	+	-		-		+
Methanol	*E. radicans*	-	-	+	-	+		-	-	+
	E. coryophorum	+	+	-	+	+		-		-
Chloroform	*E. radicans*	-	-	+	-	-		-	-	+
	E. coryophorum	+	+	+	+	-		-		+

(+) indicates the presence of the evaluated secondary metabolite while (-) indicates its absence
Blank spaces indicate that these secondary metabolites were not evaluated

Tipaz and Benavides[87] performed phytochemical screening of extracts in methanol, ethanol, chloroform, and hexane from leaves of the species *Epidendrum radicans*. The secondary metabolites resulting in the methanolic extract in both the *Epidendrum radicans* species and the *Epidendrum coryophorum* species are terpenoids and flavonoids. The secondary metabolites found in the ethanolic extract in both the *Epidendrum radicans* species and the *Epidendrum coryophorum* species are glycosides, flavonoids, and coumarins. The secondary

metabolites that chloroform extract have in common in both the *Epidendrum radicans* species and the *Epidendrum coryophorum* species are flavonoids (**Table 5**).

The secondary metabolites that the studied species of the genus *Epidendrum* have in common are flavonoids, terpenoids, tannins, coumarins, and glycosides. Additionally, in the extract of the leaves of *Epidendrum coryophorum* were evaluated metabolites that have not been evaluated in the other *Epidendrum* species. Positive results were obtained for carbohydrates, steroids, sterols, and reducing sugars.

Five species of orchids that do not belong to the genus *Epidendrum* were phytochemically characterized by Parveen et al. (2018)[102]. The extracts were made with different tissues and, in some cases, the whole plant in 70% ethanol. The species evaluated were *Aerides maculosum*, *Coelogyne breviscapa*, *Dendrobium macrostachyum*, *Pholidota pallida*, and *Vanda testaceae*. The secondary metabolites found in these species were polyphenols, alkaloids, flavonoids, terpenoids, steroids, glycosides, and saponins. The results of the metabolic content of these species agree with the phytochemical content of the species *Epidendrum coryophorum* in the presence of polyphenols, flavonoids, steroids, and glycosides.

In the phytochemical content of *Epidendrum coryophorum*, there are several secondary metabolites with pharmacological properties. A clear example of this is that phenolic compounds have pharmacological effects of great importance, such as anticancer, antibacterial, and anti-inflammatory effects. Among the main phenolic compounds are flavonoids and tannins.[103]. Flavonoids have antiviral, anti-inflammatory, anti-cancer, anti-allergic, and antioxidant effects. Tannins have antidiarrheal, antibacterial, antitumor and anti-inflammatory effects.[103] On the other side, terpenic compounds have pharmacological properties such as anticancer, anti-inflammatory, anti-irritant, and analgesic.[104] The therapeutic effects of cardiotonic glycosides include antiseptic, diuretic, and digestive properties.[105] The presence of such metabolites in the species *Epidendrum coryophorum* could grant it medicinal properties.

The phytochemical composition of plants depends on several environmental factors, both biotic and abiotic. The stress level at which the plant is subjected determines what type of

adaptation mechanisms it develops. These mechanisms involve molecular responses that alter the metabolism of plants, either by the synthesis, storage, or degradation of secondary metabolites. The tendency to increase enzymatic activity in stressful situations causes plants to synthesize higher amounts of secondary metabolites. Therefore, the results of the phytochemical content of species will depend directly on the environment and the conditions in which they developed.[106]

6.3 Total phenolic content determination of leaves of *Epidendrum coryophorum*

Considering that several of the secondary metabolites present in the extracts are classified as phenols[107], it was determined the phenolic content in the extracts of leaves of *Epidendrum coryophorum.* The values of the TPC of methanolic, ethanolic, and chloroformic extracts of leaves were obtained using the Folin-Ciocalteu method.[96] Folin-Ciocalteu reagent consists of a mixture of phosphomolybdic acid and phosphotungstic acid. In an alkaline medium, phenols can reduce these compounds to form complexes that give blue coloration (**Figure 13**). These complexes can be detected spectroscopically at approximately 760 nm.[108] TPC was calculated using a calibration curve with Gallic acid whose equation resulted in $y = 8.8282x + 0.0328$ with $R^2 = 0.9987$ where x is the absorbance and y is the concentration of Gallic acid (**Figure 14**).

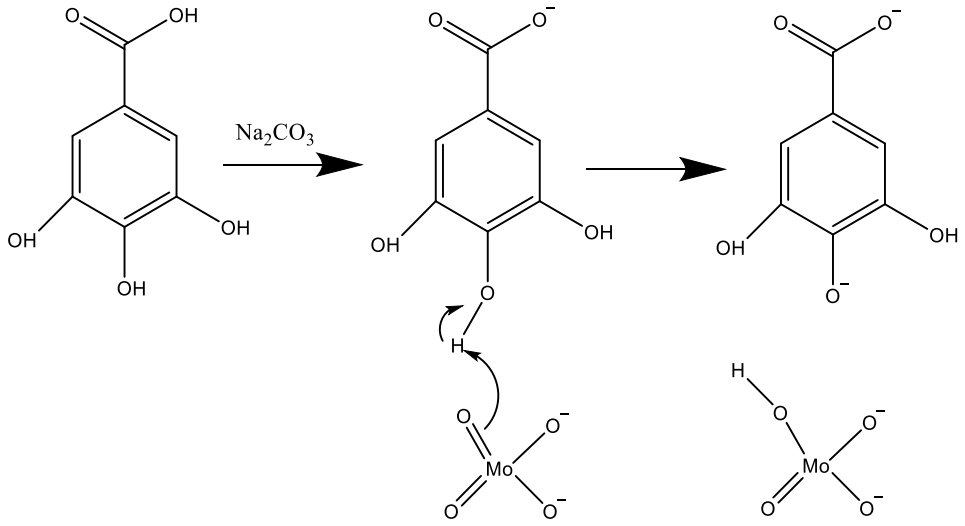


Figure 13. The reaction between Gallic acid and Folin-Ciocalteu reagent.[109]

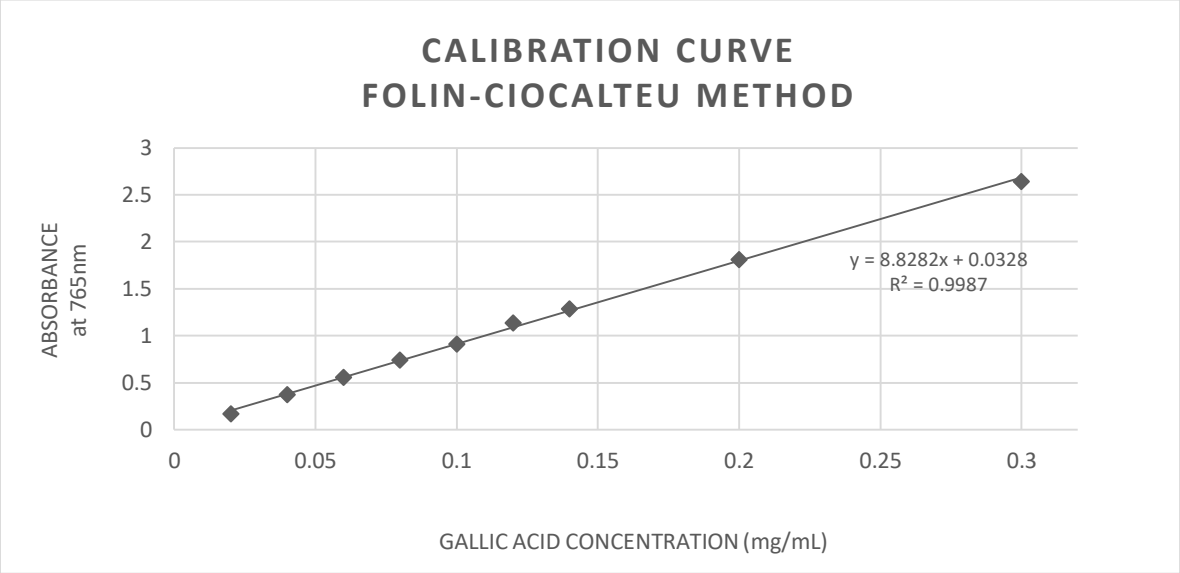


Figure 14. Calibration curve employed to calculate the TPC values of the methanolic, ethanolic and chloroformic extracts of *Epidendrum coryophorum* (**Annex 2**).

The total phenolic content in the methanol extract of *Epidendrum coryophorum* leaves was 43.57 mg GAE / g of *Epidendrum coryophorum*. In the ethanol extract, the value found was 19.96 mg GAE / g of *Epidendrum coryophorum*, and in the chloroform extract was 3.10 mg GAE / g of *Epidendrum coryophorum* (**Table 6**).

The dielectric constant of the solvents and the TPC values are directly proportional.[96] This is reflected in the results of the TPC of the chloroformic extract since it has the lowest dielectric constant and, in turn, the lowest TPC values. On the other hand, the methanolic and ethanolic extracts have the highest dielectric constant and also have higher TPC values than in the case of chloroformic extract (**Table 6**). This relationship between the dielectric constant and the TPC is because the dielectric constant is the measure of the ability of a solvent to separate opposite charges. Therefore, the higher the dielectric constant, the greater the solvent's ability to extract phenolic compounds from the tissues of the species in question. It was important to extract phenolic compounds because these types of compounds have a lot of biological activities, so the methanolic extraction results in a good option for that purpose.

Table 6. Total phenolic content of methanolic, ethanolic, and chloroformic extracts of leaves of *Epidendrum coryophorum*.

Extract	Solvent	Dielectric constant (ε, at 25 °C)	mg GAE/ g *Epidendrum coryophorum*
EHM	Methanol	32.7	43,57±2,282
EHE	Ethanol	24.5	19,96±1,936
EHC	Chloroform	4.8	3.10±0.21

Data of TPC is in ***Annex 3***

Values of TPC of *Epidendrum coryophorum* have not been reported in the literature, nor of other species of the same genus. However, Bhattacharyya et al[110] analyzed the TPC of a medicinal Orchidaceae species from *Dendrobium* gender, whose values were also expressed in mg GAE / g of dried tissue weight. They found in the chloroformic extract of leaves of *Dendrobium nobile* a TPC value of 3.25 mg GAE / g *D. nobile*. Besides, the TPC value for the methanolic extract of leaves of *Dendrobium nobile* was 20.53 mg GAE /g *D. nobile*. TPC values obtained for *Epidendrum coryophorum* are similar to TPC values obtained from extracts of leaves of *Dendrobium nobile*. In the chloroformic extract of leaves of *Epidendrum coryophorum,* the obtained TPC value was 3.10 mg GAE / g of *E. coryophorum* and 43.57 mg GAE / g of *Epidendrum coryophorum* for the methanolic extract of leaves.

The differences between the TPC values from one species to another may be due to differences in the metabolic composition of plants[106], or to the extraction method used.[111] Several factors influence the performance of the extraction of secondary metabolites, such as used tissue, solvent, type of extraction, extraction time, and temperature.[101] Unconventional techniques, such as that used in the extraction of SM in *Epidendrum coryophorum*, have several positive effects in the extraction.[112] Specifically, ultrasound-assisted extraction benefits the extraction process since it increases the net movement of mass and capillary effects.[113] Factors such as time and intensity influence the quality of extraction.[111]

In the extraction made in *Epidendrum coryophorum*, the same plant material was used to make all the extracts. First, the extraction was made in methanol, then in ethanol, and finally in chloroform. Phenolic compounds are present in the extracts of the three solvents.[101] Each

extraction affects the phenolic concentration of the next solvent. For this reason, consecutive extractions are usually done using the most non-polar solvent first and, subsequently, the polar ones.[114] Since our interest was to obtain the highest amount of phenols in a single extract; we opted to perform the extraction in methanol first.[101] The results for the methanol extract gave the highest TPC values. The extraction was carried out in this way due to the interest in phenolic compounds since phenolic compounds have an essential role in the allelopathic effects of the plant. Phenols allelochemicals are involved in cell division, root growth, changes in cell structure, and, therefore, in plant growth and development.[35] Phenols are antioxidant compounds *in vitro*, with antioxidant capacities higher than the compounds currently used as vitamin C, vitamin E, and carotenoids. Antioxidant activity is important since it reduces or prevents the oxidation of oxidizable materials such as proteins, enzymes, and DNA by eliminating free radicals and reducing oxidative stress.[26]

6.4 UV-VIS analysis

Ultraviolet-visible spectroscopy is a technique that allows the detection of compounds that have electronic transition of the type $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$. The wavelengths of the absorption maximums can be correlated with the bond types contained in molecules since for a given substance there is a wavelength in which a maximum absorbance is observed in the UV-VIS spectrum[115]. This technique is very useful in phytochemistry because SM usually have aromatic compounds, carbonyl compounds, or other structures that are chromophores.[116]

The maximum absorbance values were registered between 268 nm and 668 nm. In this range, in addition to chlorophylls, were observed absorption maxima that correspond to phenolic compounds such as simple phenols and mostly flavonoids (**Table 7**). All the extracts of the leaves of *Epidendrum coryophorum* showed maximum absorbance at 664 nm that could correspond to the maximum absorption of chlorophyll at 663 nm.[117] In the methanolic extract of leaves of *Epidendrum coryophorum,* the maximum absorbance values were obtained at 270 nm, 340 nm, 414 nm, 436nm, 468 nm, 536 nm, 608 nm, and 664 nm. These maximum absorptions are in the absorption range of various compounds of the flavonoid, carotenoid, and simple phenol group. In the ethanolic extract of leaves of *Epidendrum coryophorum* maximum absorbance values were obtained at 268 nm, 332 nm, 410 nm, 468 nm, 504 nm, 536 nm, 606 nm, and 664 nm. These maximum absorptions are in the absorption range of

several compounds belonging to the group of flavonoids, carotenoids, and simple phenols. Finally, in the chloroformic extract of leaves of *Epidendrum coryophorum* absorption maxima were obtained at 288 nm, 414 nm, 612 nm, and 664 nm. These maximum absorptions are in the range of simple phenols, and flavonoids. Some absorption maxima were overlapped in several ranges. Through this technique, it is not possible to make a direct correlation between the maximum absorbance and a specific metabolite.

Table 7. Correlation between the wavelengths of maximum absorption observed in the spectra, and typical ranges of the wavelengths of maximum absorption for the most common secondary metabolites.[46,118]

Range (nm)	Evaluated secondary metabolite	EHM	EHE	EHC
200-290	Chalcones and aurones (flavonoids)	270[1]	268[1]	288[1]
230-290	Simple phenols	270[1]	268[1]	288[1]
240-280	Flavonoids (Phenols)	270[1]	268[1]	-
250-270	Flavonols (flavonoids) and betacyanins (phenols)	270[1]	268[1]	-
300-560	Flavonoids derivatives	340[2]	332[2]	-
315-330	Coumarins (Phenols)	-	-	-
330-350	Flavones (Flavonoids)	340[2]	332[2]	-
370-410	Chalconas (Flavonoids)	-	410[3]	-
400-430	Naphthoquinones (Phenols)	414[3]	410[3]	414[2]
420-470	Carotenoids [117]	436[4]-468[5]	468[4]	-
430-470	Chlorophylls	436[4]-468[5]	468[4]	-
490-550	Anthocyanins [26] (Flavonoids)	536[6]	504[5]-536[6]	-
663	Chlorophylls	664[7]	664[7]	664[3]
-	-	608[8]	606[8]	612[4]

*(X) is used to confirm the presence of a peak in the wavelength range of the evaluated secondary metabolite. 1,2,3,4,5,6,7,8: Number of the maximum absorbance (**Annex 4**)*

Eight peaks can be seen in the spectrum of the methanol extract. Two possible type of SM that could be present in this extract are flavonoids and tannins. The chromophores of the flavonoids are the two benzene rings, and for tannins is the benzoyl of the Gallic acid. The Gallic acid is the basic structure of hydrolysable tannins (**Figure 5**). Gallic acid has an experimental maximum absorbance at $\lambda_{max} = 270\text{nm}$, while flavonoids present two: one (band I) in the range from 300-550nm, and another (band II) in the range from 240-285nm.

In the spectrum, it can be seen that there are maximum absorbance bands in both ranges (**Figure 16**). So these maximum absorbance points in the spectrum may belong to such structures.[65]

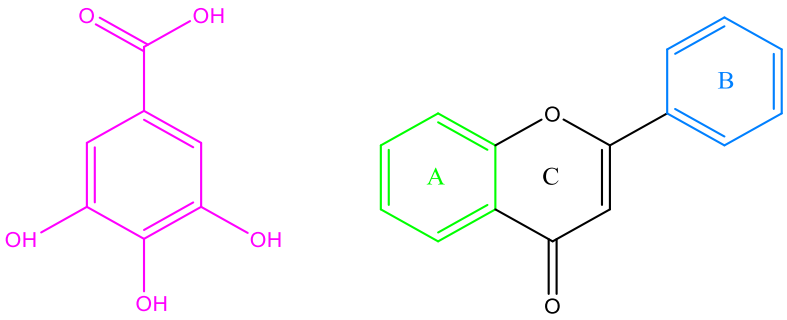


Figure 15. Basic structure of hydrolysable tannins (Gallic acid) and flavonoids.

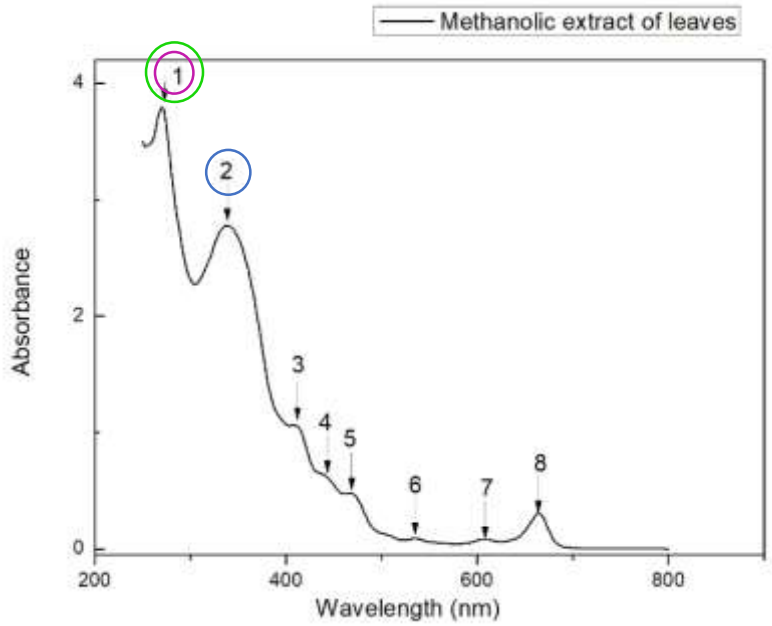


Figure 16. UV-VIS spectra of methanolic extract of leaves.

There are no data from similar studies in Orchidaceae. However, UV-VIS analysis of species extracts from other families has been performed. Zavoi et al[117] performed the UV-VIS analysis of methanolic, ethanolic, and glycerin extracts of *Cynara scolimus*, *Taraxacum officinalis*, *Chelidonium majus*, *Hypericum perforatum*, *Silybum marianum*; *Lycopodium clavatum* revealed maximum absorption values in the ranges for carotenoids, chlorophylls,

and phenols such as flavonoids. The results obtained from the UV-VIS spectra of the species mentioned above are similar to the results obtained from the spectra of the species *Epidendrum coryophorum.* In both studies, the results suggest the presence of carotenoids, chlorophylls, and phenols.

6.5 Determination of antibacterial activity

6.5.1 Agar diffusion test or Kirby-Bauer test

As a result of the antibacterial activity assay on agar, it was observed that methanolic and ethanolic extracts of leaves of *Epidendrum coryophorum* had a moderate antibacterial activity at a concentration of 25 mg/mL with resultant inhibition diameters of 7.8 mm and 7.4 mm, respectively. Both extracts had similar results at a concentration of 10 mg/mL with diameters of 7.8 mm and 7 mm for methanolic and ethanolic, respectively (**Table 8**). The chloroformic extract of leaves of *Epidendrum coryophorum* had a minimum evident antibacterial activity. In contrast with the negative control (DMSO), the halo of the chloroformic extract of leaves have inhibition but not so high as the ethanolic and methanolic extracts. The difference in bacteria concentration in the halos is evident, so the antibacterial activity has to be probed with higher concentrations of the chloroformic extract (**Figure 17**).

Table 8. Antibacterial effect of the methanolic (EHM), ethanolic (EHE), and chloroformic (EHC) extracts of leaves of *Epidendrum coryophorum* against *Escherichia coli.*

Extract	Concentration (mg/mL)	Inhibition diameter (mm)
EHM	25	7.8
	10	7.8
EHE	25	7.4
	10	7.0
EHC	25	7.01
	10	7.0
Kanamycin	0.5	12.1

Kanamycin, a standard antibiotic, was used as a positive control
The antibacterial activity results were expressed using the inhibition diameter (halo)

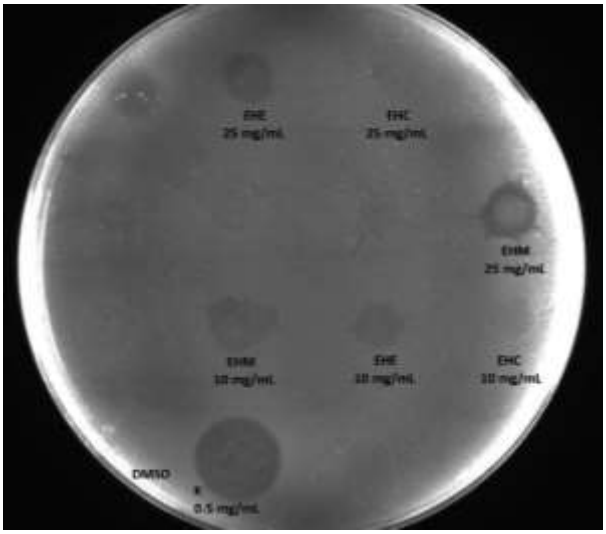


Figure 17. Inhibition of *Escherichia coli* in the presence of two different concentrations of methanolic (EHM), ethanolic (EHE), and chloroformic (EHC) extracts of leaves of *Epidendrum coryophorum*, (10 mg/mL and 25 mg/mL).

In the results of the antibacterial agar diffusion test for methanolic and ethanolic extract of leaves of *Epidendrum coryophorum*, there is a direct relationship between the TPC values and the degree of bacterial inhibition. The lowest TPC value was obtained for the chloroformic extract of leaves with 3.10 mg GAE/g *E. coryophorum*. Also, the minimum antibacterial activity was found for the chloroformic extract of leaves. On the other hand, the methanolic and ethanolic extract of leaves reached the highest values for TPC with 43.57 mg GAE / g of *E. coryophorum* and 19.96 mg GAE / g of *E. coryophorum*. More significant antibacterial activity was found in methanolic extract than in ethanolic extracts of leaves. So, the resulting antibacterial activities tally with the type and quantity of phenolic compounds.

This relationship between the TPC and the antibacterial activity may be due to phenolic compounds are fundamentally responsible for the antibacterial activity against Gram-positive and Gram-negative bacteria. The phenolic compounds with the most significant antibacterial effect are tannins and flavonoids.[119] The most accepted mechanism of action for some flavonoids is the aggregating effect on all bacterial cells and for tannins, the inhibition of bacterial growth.[119] Another SM with antibacterial activity is terpenoids and alkaloids.[88] Terpenoids have antibacterial activity against some multidrug-resistant bacteria.[13] These SM are also present in the methanolic extract of leaves of *Epidendrum coryophorum*.

The antibacterial activity of several Orchidaceae species have been studied, but there are no data from similar studies in species of the *Epidendrum* genus. Orchids exhibit positive activities against Gram-positive and Gram-negative bacteria, also anti-fungal, antioxidant, antidiabetic, and antiviral activities, among others. This is the case of some species of the genus *Vanilla*. They have antibacterial activity against several species and strains of *Listeria.*[1] The orchid species *Flickingueria nodosa* is used in India´s traditional medicine as an expectorant and astringent. The evaluation of the antibacterial activity of this species gave positive results for the inhibition of Gram-positive and Gram-negative bacteria.[88]

6.5.2 Growth inhibition assay

As a result of this assay, the methanolic, ethanolic and chloroformic extracts of leaves of *Epidendrum coryophorum* showed no antibacterial activity at the concentration of 1 mg/mL in DMSO. All the curves of the extracts are under the growth curve of *E. coli*; this is because DMSO has a mild antibacterial activity.[120] The extract curves and the DMSO curve grew at very similar rates, so it is evident that the extracts showed no activity at this concentration (**Figure 18**).

The percentage of growth rate inhibition of *E. coli* was the highest for DMSO at 11.16%. The percentage of growth rate inhibition of *E. coli* for EHM, EHE, and EHC, were 6.29%, 7.18%, and 6.73%, respectively. Since the values of the percentage of the growth rate inhibition of the extracts are lower than the value of the solvent, the antibacterial activity is absent at this concentration. The experiment should be repeated using higher concentrations of the extracts (**Table 9**).

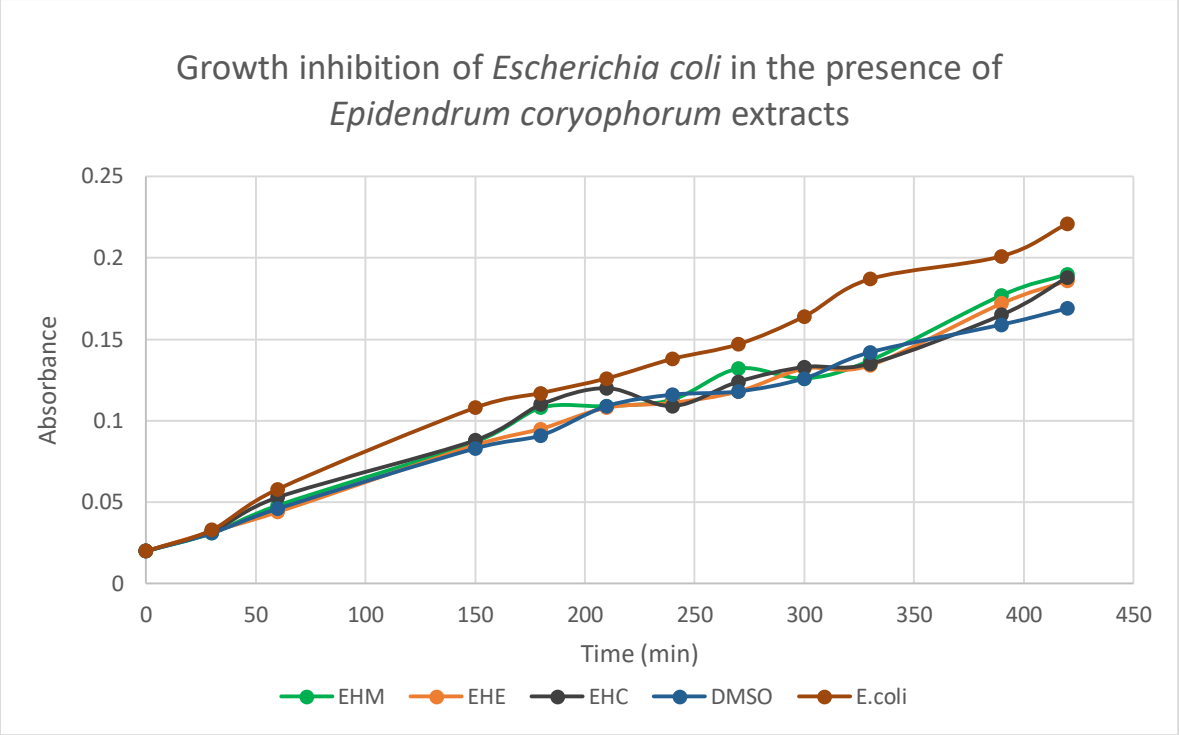


Figure 18. Growth inhibition curve of *Escherichia coli* in the presence of methanolic, ethanolic, and chloroformic extracts of leaves *of Epidendrum coryophorum.*

Table 9. Growth inhibition percentage of leaves extracts of *Epidendrum coryophorum.*

Sample	N_0	N	μ	Percentage of growth rate inhibition %
EHM	24000000	228000000	0,321613114	6,2910721
EHE	24000000	223200000	0,318573486	7,1767335
EHC	24000000	225600000	0,320101384	6,7315473
DMSO	24000000	202800000	0,30488092	11,166358
E.coli	24000000	265200000	0,343204347	0

*μ: proportionality constant, N: number of bacteria at the beginning of a time interval, N_0: number of bacteria at the end of the time interval (**Annex 5**)*

The percentage of growth rate inhibition of *Escherichia coli* for *Epidendrum coryophorum* has not been reported in the literature. The antibacterial activity of several Orchidaceae species have been studied, but there are no data from bacterial growth inhibition assays. Dávila[98] evaluate the antibacterial activity of the *Allium ampeloprasum* by measuring the

growth kinetics of an oily extract. The ethanolic extract was not employed for this test, preliminary due results of antibacterial activity realized by agar diffusion were negative. For the oily extract, the inhibition diameters in the agar diffusion test were 8.4 mm and 7.4 mm for *Sta. aureus* and *E. coli*, respectively. The value of the resultant inhibition diameter in the agar diffusion test with *E. coli* for *Allium ampeloprasum* is similar to the inhibition diameter for the methanolic extract of leaves of *Epidendrum coryophorum*. The percentages of growth rate inhibition of *E. coli* obtained by oily extract of *Allium ampeloprasum* were 89.19%, 91.89%, and 72.97% at a concentration of 17.8 mg/mL, 8.9 mg/mL, and 4.4 mg/mL, respectively. For *S. Aureus*, they were 72.65%, 40.17%, and 16.14% at concentrations of 17.8 mg/mL, 8.9 mg/mL, and 4.4 mg/mL, respectively. Finally, the percentages of inhibition obtained for *B. cereus* were 65.13% and 14.86% at concentrations of 17.8 mg/mL and 4.4 mg/mL, respectively. The percentages of growth rate inhibition of *E. coli* for *Allium ampeloprasum* are greater than obtained by *Epidendrum coryophorum*. These difference could be due to higher concentrations of the extract of *Allium ampeloprasum* were used to perform the test.

6.6 Determination of anti-inflammatory activity

6.6.1 Microencapsulation of extract

As a result of the microencapsulation of the ethanolic extract of leaves of *Epidendrum coryophorum*, 8.97 g of microcapsules were obtained. The microencapsulation yield was 60.77%, and it is the effect of the adhesion losses of microcapsules on the walls of the thermal camera in the dryer process with the mini spray drier. The composition of the microcapsules consists of 1.16% of pure extract, 33.87% of maltodextrin, 33.87% of Gum Arabic, and 31.1% of *Plukenetia volubilis* oil (**Table 10**).

Table 10. Microencapsulation data.

Composition of microcapsules	Pre-microencapsulation mass (g)	Percentage of the total composition (%)	The total mass of microcapsules	Yield percentage
Gum Arabic	5.00	33.87	8.97 g	60.77%
Maltodextrin	5.00	33.87		
Dry extract	171 mg	1.16		
Plukenetia volubilis oil	4.59	31.10		
TOTAL	14.76	100		

6.6.2 Inhibition of heat-induced hemolysis

The assay of membrane stabilization in an isotonic media gave positive for the extract tested in this experiment. The microcapsules of ethanolic extract of leaves gave the percentage of hemolysis inhibition of 18.19% at 1 mg/mL, 38.98% at 1.5 mg/mL, and 40.94% at 2.5 mg/mL. The commercial aspirin, positive control, gave percentage of 65.33% at 1 mg/mL, 72.36% at 1 mg/mL, and 73.75% at 2.5 mg/mL (**Figure 19**).

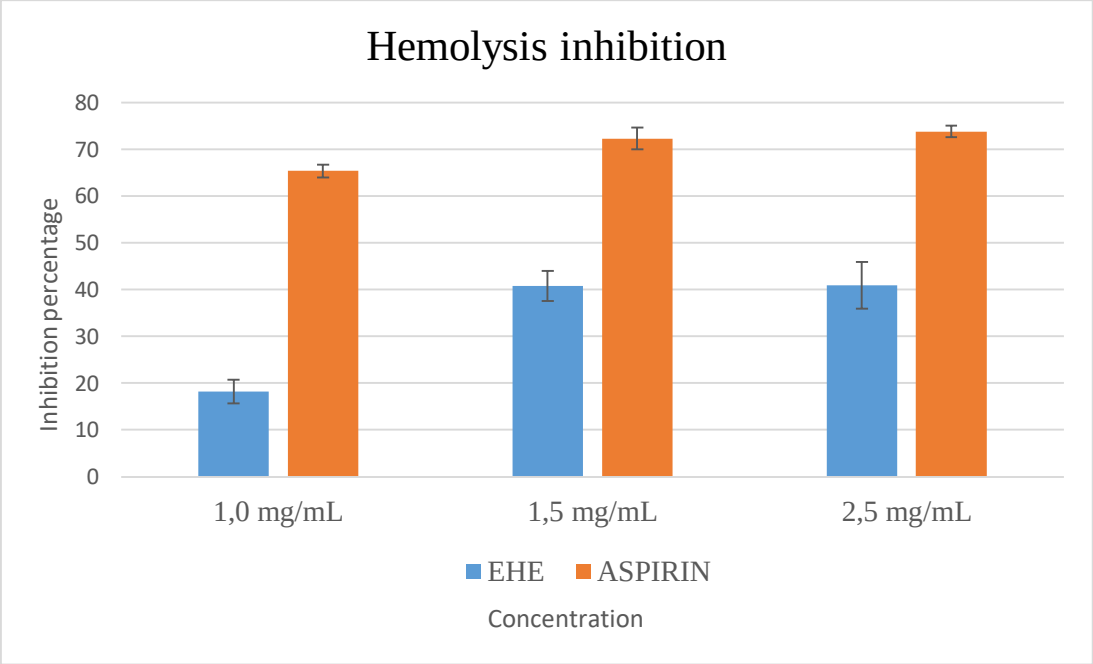


Figure 19. Diagram of percentages of hemolysis inhibition of microcapsules of the ethanolic extract of leaves of *Epidendrum coryophorum*.
Commercial aspirin was used as a positive control (**Annex 6**).

Sterols, steroids, and flavonoids are secondary metabolites that are present in the ethanolic extract of leaves. These metabolites have anti-inflammatory activity, and its effect is due to they have different mechanisms of inhibition of the synthesis of pro-inflammatory molecules.[83] These secondary metabolites are present in the ethanolic extract of *Epidendrum coryophorum*. So, the percentages of hemolysis inhibition obtained by the ethanolic extract could be the result of the presence of these SM.

The anti-inflammatory activity of *Epidendrum coryophorum* has not been reported in the literature. However, studies of anti-inflammatory activity of orchids from other genus have been reported. Gomez[102] evaluated the anti-inflammatory activity of 70% ethanolic extract of the Orchidaceae species *Aerides maculosum, Coelogyne breviscapa, Dendrobium macrostachyum, Pholidota pallida,* and *Vanda testaceae* employing the stabilization of the human erythrocyte membrane test. The maximum inhibition percentages were obtained at the concentration of 10 mg/mL with values of 80.73%, 70%, 87.86%, 57.56%, and 95.16% for *A. maculosum*, *C. breviscapa*, *D. macrostachyum*, *P. pallida*, and *V. testaceae*, respectively. The lowest percentages of inhibition were obtained at a concentration of 2 mg/ mL with percentages of 7.68%, 7.67%, 12.31%, 3.58%, 22.44% for *A. maculosum*, *C. breviscapa*, *D. macrostachyum*, *P. pallida,* and *V. testaceae*, respectively. The percentages of hemolysis inhibition of the extracts of these five species at concentrations of 2 mg/mL are similar to the percentages obtained by extract of *Epidendrum coryophorum* at a concentration of 1 mg/mL.

7. CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

- *Epidendrum coryophorum* contains in their leaves phenols, coumarins, flavonoids, tannins, terpenoids, steroids, sterols, Cardiotonic glycosides, carbohydrates, and reducing sugars.
- The highest content of phenolic compounds in *Epidendrum coryophorum* was obtained in the methanolic extract of leaves (43.57 mg GAE / g *Epidendrum coryophorum*).
- UV-VIS spectroscopy confirmed the presence of flavonoids and simple phenols.
- Extracts of leaves of *Epidendrum coryophorum* show antibacterial activity against *Escherichia coli* at concentrations of 10 mg / mL in DMSO and 25 mg / mL in DMSO through disk diffusion method.
- No extract has evident activity at a concentration of 1 mg /mL in DMSO through the growth inhibition method.
- In the stabilization of the erythrocyte membrane test, the microcapsules reached the highest percentage of inhibition at a concentration of 2.5 mg / mL in PBS with a value of 40,94%.

7.2 Recommendations

- Separate the compounds contained in the extracts by chromatography or TLC.
- Characterize the isolated compounds by FT-IR and NMR.
- Evaluation of the biological activity of identified compounds.

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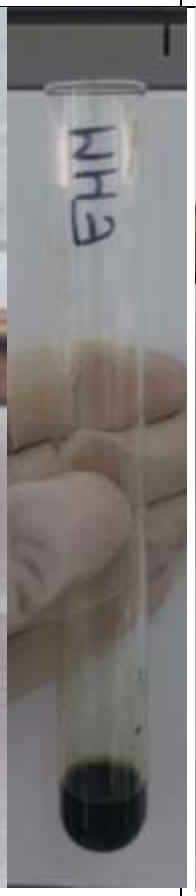
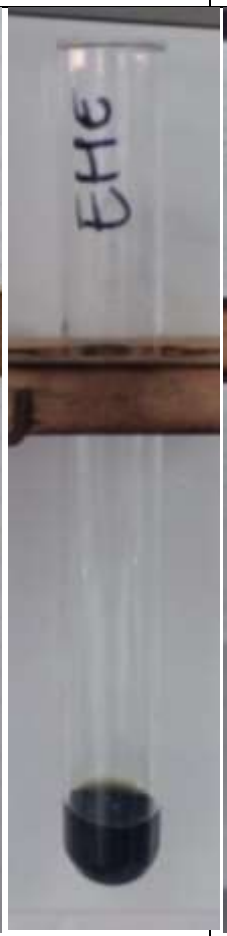
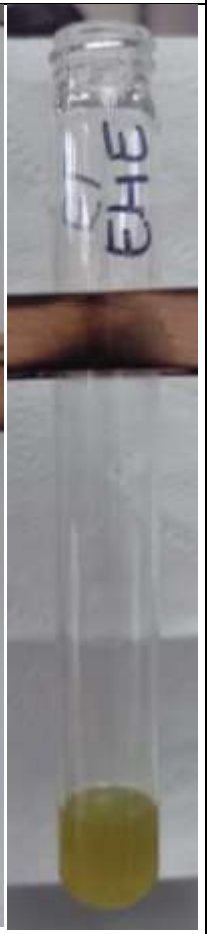
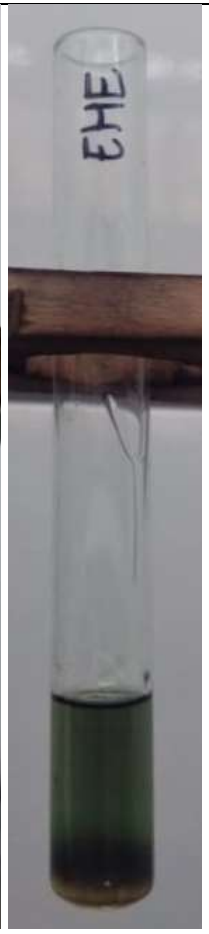
9. ANNEX

Annex 1. Positive results of the phytochemical screening of *Epidendrum coryophorum*

METHANOLIC EXTRACT OF LEAVES							
PURE EXTRACT	**PHENOLS**	**COUMARINS**	**TANNINS**	**TERPENOIDS**	**STEROIDS**	**REDUCING SUGARS**	**CARBOHYDRATES**
							

ETHANOLIC EXTRACT OF LEAVES							
PURE EXTRACT	**PHENOLS**	**COUMARINS**	**FLAVONOIDS**	**TANNINS**	**STEROIDS**	**CARDIOTONIC GLYCOSIDES**	**CARBOHYDRATES**
							

CHLOROFORMIC EXTRACT OF LEAVES								
PURE EXTRACT	**PHENOLS**	**COUMARINS**	**TANNINS**	**FLAVONOIDS**	**STEROIDS**	**CARDIOTONIC GLYCOSIDES**	**REDUCING SUGARS**	**CARBOHYDRATES**
								

Annex 2. Absorbance values for the calibration curve with Gallic acid for TPC determination

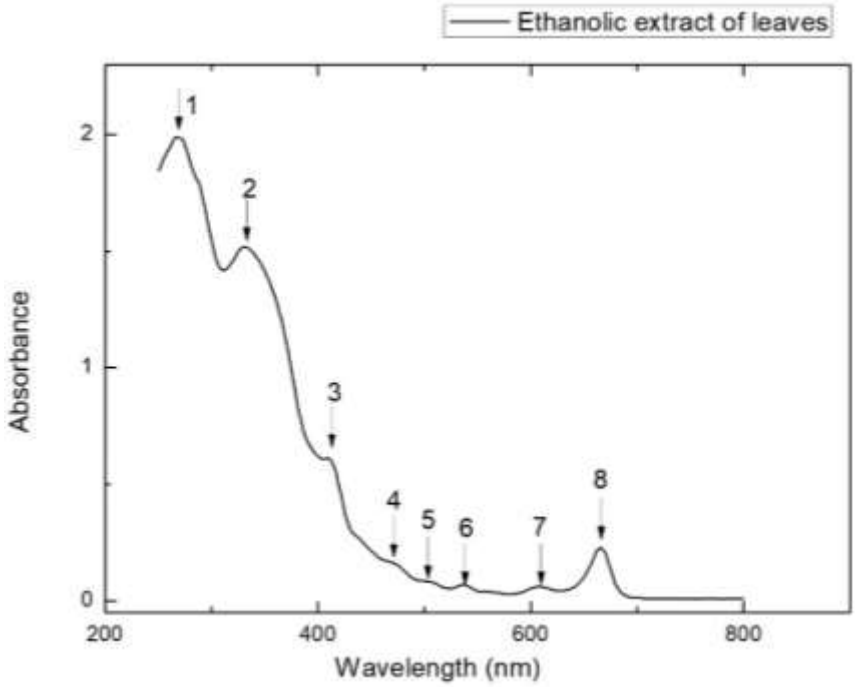
Concentration of Gallic acid (mg/mL)	Absorbance
0,02	0,17
0,04	0,375
0,06	0,557
0,08	0,745
0,10	0,914
0,12	1,142
0,14	1,291
0,20	1,815
0,30	2,644

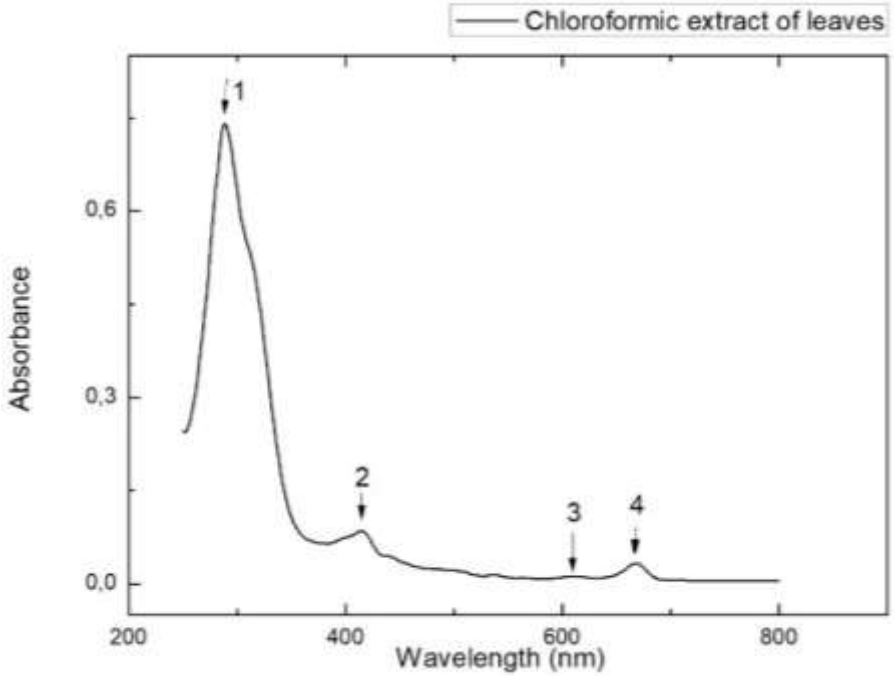
Annex 3. Total phenolic content values of extracts of *Epidendrum coryophorum*

Extract	Absorbance 1	mgGAE/g*E.c*	Absorbance 2	mgGAE/g*E.c*	Absorbance 3	mgGAE/g*E.c*	Average	SD
EHM	1,145	41,994	1,159	42,523	1,256	46,185	43,567	2,282
EHE	0,620	22,171	0,525	18,584	0,539	19,113	19,956	1,936
EHC	0,797	2,885	0,858	3,116	0,907	3,300	3,100	0,208

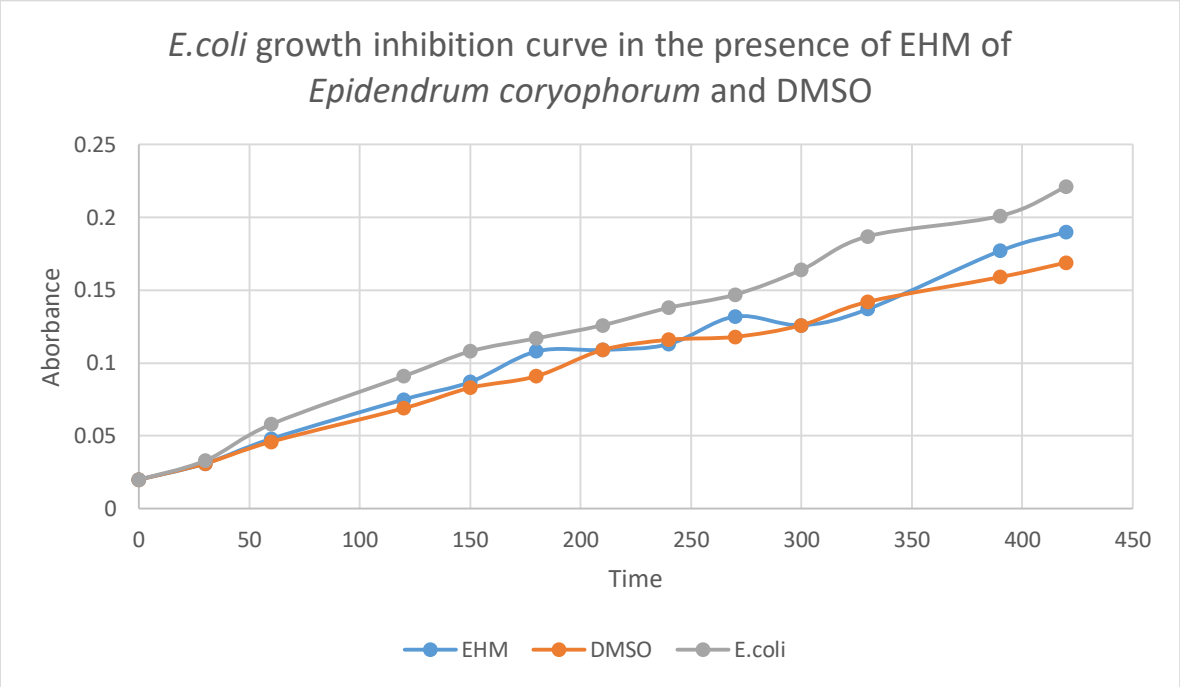
Each extract was analyzed by triplicate. The resultant absorbances values for each extract are absorbance1, absorbance 2, absorbance3
E.c: Epidendrum coryophorum
SD: standard deviation

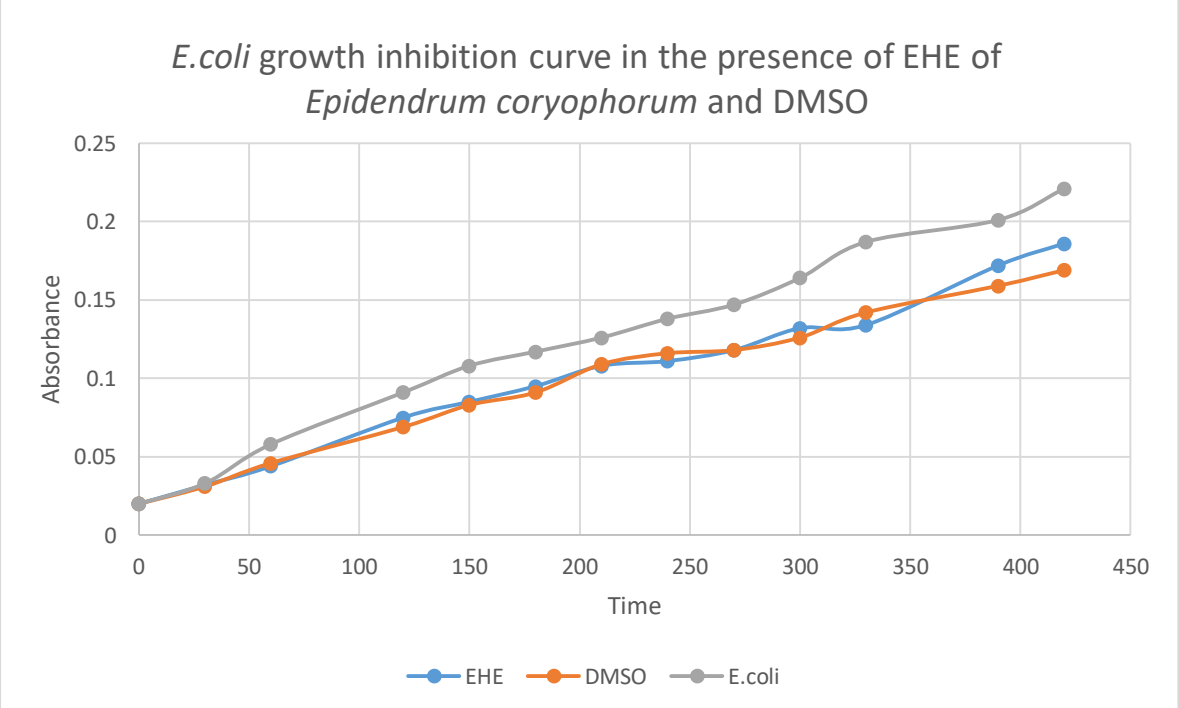
Annex 4. UV-VIS spectra of extracts of *Epidendrum coryophorum*

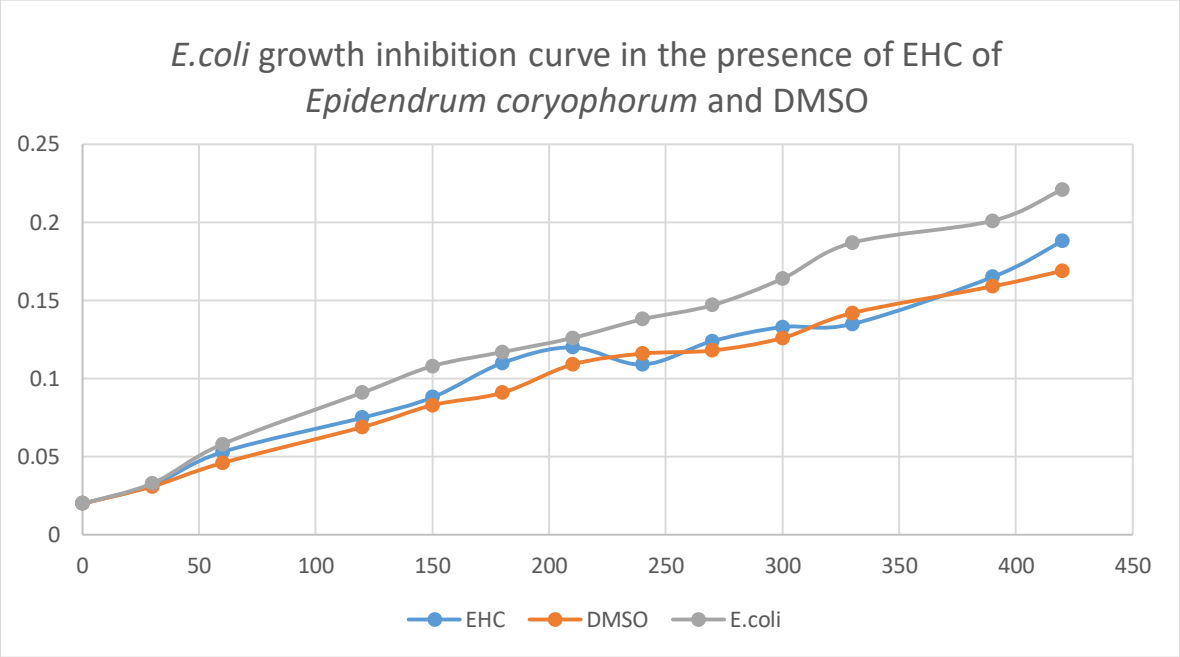




Annex 5. Growth inhibition curve of *Escherichia coli* in the presence of methanolic, ethanolic, and chloroformic extract of leaves of *Epidendrum coryophorum*







Annex 6. Anti-inflammatory activity data of ethanolic extract of *Epidendrum coryophorum*

Microencapsulated	% of hemolysis inhibition	% of hemolysis inhibition	% of hemolysis inhibition	Average	SD
1 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	5,01
Commercial aspirin					
1 mg/mL	65,70	66,50	63,78	65,33	1,39
1,5 mg/mL	73,76	73,40	69,61	72,36	2,13
2,5 mg/mL	75,13	73,42	72,71	73,75	1,24

SD: standard deviation