UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY TECH

Escuela de Ciencias Químicas e Ingeniería

Título: Qualitative phytochemical study, determination of total phenolic content, UV-VIS analysis, and determination of antimicrobial and anti-inflammatory activities of *Alternanthera porrigens*

Trabajo de integración curricular presentado como requisito para la obtención del título de Química

Autor:
González Falconí Daniela Estefanía

Tutor:

Ph. D. Morera Córdova Vivian

Urcuquí, Abril 2020

Urcuquí, 24 de abril de 2020

SECRETARÍAGENERAL
(Vicerrectorado Académico/Cancillería)
ESCUELA DE CIENCIAS QUÍMICAS E INGENIERÍA
CARRERA DE QUÍMICA
ACTA DE DEFENSA No. UITEY-CHE-2020-00028-AD

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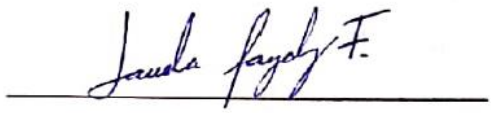

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Dedicatoria

Para mi querida mamita Cristina, porque cada recuerdo suyo me llenaba el corazón para poder seguir adelante y no darme por vencida. Porque al llegar a casa y verla sonreír era lo mejor que podía pasarme. Porque con un abrazo suyo bastaba para hacerme sentir que cada viaje valía la pena. No pasará un solo día sin que deje de recordarla y atesorarla en mi mente y corazón.

Para mis abuelos, quienes han sido un pilar fundamental en mi desarrollo académico, sin ellos nada de esto hubiese sido posible.

Para mi madre y mi padre.

Para mi hermano, por ser mi confidente y demostrarme que se puede salir adelante y ser más fuerte cada día.

Para mi hermana, por quien intento ser mejor cada día.

Daniela González Falconí

Acknowledgments

This work would not have been possible without the guidance of my advisor Ph.D. Vivian Morera, whose support and patience were essential for handling and overcoming adversities.

Furthermore, I sincerely thank Ph.D. Orestes Darío Lopez, for his help and support during the development of total phenolic content and anti-inflammatory activity.

Also, thank Ph.D. Manuel Caetano for his help with the measurements of the UV-VIS spectra and for always giving us a motivating smile in the laboratory.

And last but not least, thank Ph.D. Nelson Vispo, who helped us with the antimicrobial activity and optical density, thank you for the knowledge during the career and for always motivate us to get involved in science.

Thanks to my family for support.

Thanks to all my friends, Bryan, Iri, Andre, Georgie, for being an essential part of my life through these years, for always supporting me and for all the great moments we shared. You will always be in my heart, love you guys.

And finally, thanks to Luis, whose support and love have been essential in my life.

Daniela González Falconí

Abstract

Ecuador is a mega-diverse country with many species of medicinal plants that grow in the Ecuadorian Andes. Cerón, in 2006 reported ethnomedical information on 432 medicinal plant species. Of these, 273 are plants marketed in the herbal stores of local markets, and 255 are wild plants. Among the species, it was reported *Alternanthera porrigens* or commonly known as "moradilla". The therapeutic use of this plant to treat circulatory disorders, as a purgative, to relieve flu, for menstrual disorders appears and most important for inflammatory conditions. This work addresses the preliminary qualitative phytochemical analysis of the ethanolic, chloroformic, and aqueous extracts of the leaves and flowers of *Alternanthera porrigens*. For this, the tissues of the plant were separated, washed, and dried until an electrical mill could spray these until obtaining a fine powder. Then, by maceration, it was obtained three different extracts for each tissue, with ethanol, chloroform, and water. So, it started with preliminary phytochemical screening to the extracts, for the identification of secondary metabolites in the tissues of the plant. Once it was observed the presence of phenols in the extracts with the assays, it proceeded to determine the total phenolic content of the same using the Folin-Ciocalteau method. Also, UV-VIS spectra of the different extracts were obtained to corroborate the presence of the secondary metabolites. Subsequently, the previous results were compared with other species of the genus Alternanthera. Fortunately, these were within the same range, so it was assumed that *Alternanthera porrigens* could have similar biological activities. For that, it was determined the antimicrobial activity of the ethanolic and chloroformic extracts at concentrations of 10 mg/mL and 25 mg/mL, these gave positive results for a Gram-negative bacteria, *Escherichia coli*. So, it was carried out an optical density assay to prove if the extracts had antimicrobial activity at a concentration of 1 mg/mL, but the activity obtained was null at this concentration. And finally, an anti-inflammatory activity was tested for the dried extract of flowers, which results were lower than expected in comparison with other species of the same genus. In the end, it was proved the presence of phenols, terpenes, and other secondary metabolites that contribute to the biological activities in *Alternanthera porrigens*.

Keywords: *Alternanthera porrigens***,** preliminary qualitative phytochemical screening, total phenolic content, antimicrobial activity, anti-inflammatory activity.

Resumen

Ecuador es un país megadiverso con un sinnúmero especies de plantas medicinales que crecen en los Andes ecuatorianos. Cerón en 2006 informó sobre 432 especies de plantas medicinales. De estas, 273 son plantas que se comercializan en las herberías de los mercados locales y 255 son plantas silvestres. Entre las especies, se informó de *Alternanthera porrigens* o comúnmente conocida como "moradilla". El uso terapéutico que se le da a esta planta es para tratar los trastornos circulatorios, como purgante, para aliviar la gripe, para los trastornos menstruales y lo más importante, para las afecciones inflamatorias. Este trabajo aborda el análisis fitoquímico de los extractos etanólicos, clorofórmicos y acuosos de las hojas y flores de *Alternanthera porrigens*. Para esto, los tejidos de la planta se separaron, se lavaron y se secaron hasta que se pudieron pulverizar con un molino eléctrico. Luego, por maceración se obtuvieron tres extractos diferentes para cada tejido, con etanol, cloroformo y agua. Entonces, se comenzó con la marcha fitoquímica de los extractos para la identificación de metabolitos secundarios en los tejidos de la planta. Una vez que se observó la presencia de fenoles en los extractos con los ensayos, se procedió a determinar el contenido fenólico total de los mismos utilizando el método Folin-Ciocalteau. Asímismo, se obtuvo el espectro UV-Vis de los diferentes extractos para corroborar la presencia de los metabolitos secundarios. Posteriormente, los resultados anteriores se compararon con otras especies del género Alternanthera, afortunadamente estos estaban dentro del mismo intervalo, por lo que se asumió que *Alternanthera porrigens* podría tener algunas actividades biológicas similares. Para eso, se determinó la actividad antimicrobiana de los extractos etanólicos y clorofórmicos a concentraciones de 10 mg/mL y 25 mg/mL, estos dieron resultados positivos para una bacteria Gram negativa, *Escheriria coli*. Entonces, se realizó un ensayo de densidad óptica para probar si los extractos tenían actividad antimicrobiana a una concentración de 1 mg/ml. Para esta concentración la actividad antimicrobiana parece ser nula. Y finalmente, se probó con la actividad antiinflamatoria para el extracto seco de flores, cuyos resultados fueron más bajos de lo esperado en comparación con otras especies del mismo género. Al final, se comprobó la presencia de fenoles, terpenos y otros metabolitos secundarios que contribuyen a las actividades biológicas en *Alternanthera porrigens*.

Palabras clave: *Alternanthera porrigens*, marcha fitoquímica cualitativa preliminar, contenido fenólico total, actividad antimicrobiana, actividad antiinflamatoria.

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1 Introduction – Justification

1.1 Medicinal plants in Ecuadorian Andes

Plants have played a fundamental role in the development of Andean cultures since humans arrived in the region 10000 years ago [1]. People have used vegetable resources like food, medicine, fuel, construction materials, and tools. Plants have even occupied an essential place in their beliefs and rites [2]. In the beginning, the resources were obtained by the recollection of wild plants; however, planned and technology-based agriculture developed over time [1]. With this, it began to establish the importance of plants and their functions in the human being. The presence of the Andean mountain range has marked an evident difference in the relief, climate, land soils, and especially in vegetation in the zone. As a result of this, around 10000 plant species are present in the Sierra region, about 64% of the total species in the whole Ecuador [3]. From this number, 3118 species belong to the 206 plant families used for medicinal purposes in the country. From this, the 75% of the species are native species, and 5% are endemic plants, most of them are herbs, shrubs and trees [4]. This, accompanied with the vast culture and traditions variability in this region, provoke a fantastic combination of empirical knowledge, where medicinal plants are the principal actors. In this way, medicinal plants contribute to the health system of local communities, due to their extensive use in most of the rural populations [5]. Nowadays, a large percentage of the worldwide population, especially in developing countries, use plants to meet the primary needs of medical assistance [6].

1.2 *Alternanthera porrigens*

Ecuador is one of the countries with the most exceptional biodiversity around the world [7], which gives it a significant potential in the use of plants, as a source of traditional medicine. In this way, *Alternanthera porrigens* is an annual or perennial subharbe plant native of the Andean region, it not only grew up in Ecuador but throughout all the mountain range [8]. This plant, also known as "Lancetilla", "Sanguinaria", "Alcancel" but most common "Moradilla" [9] belongs to the amaranth family.

The leaves are arranged opposite. The inflorescence is a spike or rounded head that appears in the armpits of leaves or at the ends of branches. The flowers have five tepals, and its color is dark purple when the plant is mature [10]. There are 3 to 5 stamens that merge at one edge at the bases,

and five pseudostaminodes, appendages between the stamens that are not true staminodiums. The fruit is a utricle that contains a seed [11].

Table 1: Detailed taxonomic information about Alternanthera porrigens. [11]

Kingdom	Plantae
Class	Magnoliopsida
Order	Caryophyllales
Family	Amaranthaceae
Genus	Alternanthera
Species	Alternanthera porrigens

Figure 1: Alternanthera porrigens.

According to Bussmann 2010 [9], moradilla is commonly used for cleansing the womb after childbirth. Tene (2007) said that this plant could be used for hepatic pain, influenza, and kidney problems [6]. And Cerón, (2006) [8], affirmed that it is applied for inflammation [12], circulation, as purgative, for influenza and menstrual disorders.

There are some studies about other species of the same genus, and most of the species match in that the variety of phytochemical constituents of the Alternanthera genus is pretty wide. Between the secondary metabolites, most studied in this genus are flavonoids, glycosides, alkaloids, sugars, amino acids, hydrocarbons, and steroids [13].

1.3 Phytochemistry

Phytochemical studies generally are based on previous ethnobotanical and ethnopharmacological knowledge about plants and often constitute hypothesis-driven studies. The general methodology for studying secondary metabolites (SM) from plants comprises several stages, extraction from natural sources, the phytochemical screening of extracts to determine the main chemical classes of SM present in the plant, the purification of individual components and the elucidation of their chemical structures, the biological activity studies through in vitro/in vivo assays and the toxicity-cytotoxicity studies on organisms or cells qualitatively.

1.4 Secondary metabolites

Plants are autotrophic organisms. In addition to the primary metabolism present in all living beings, they have secondary metabolism that allows them to produce and accumulate compounds of a very diverse chemical nature. The compounds derived from secondary metabolism in plants are called secondary metabolites (SM) [14].

The SM of the plants constitutes a large and varied group of organic compounds that are synthesized in small quantities. And, they have no direct function in essential processes such as photosynthesis, respiration, solute transport, protein synthesis, nutrient assimilation, and the differentiation or formation of carbohydrates, proteins, and lipids. They appear in plants as a result of chemical conversions. And, even when many of their functions are unknown, it is believed that SM are related to the defense of the plant against predators and pathogens, they also act as allelopathic agents that influence growth, survival, and reproduction of other plants, attract seed pollinators and serve to face adaptation to sudden changes in temperature, humidity, light intensity, and drought [15–17]. The SM of the plants have a differential distribution between taxonomic groups in the Kingdom of the plants, and therefore they are useful for Systematic Botany [18].

The study of biological functions and the structure of SM are of great importance because of this knowledge, and it has been possible to use them in different industries. Many SM are used as aromas, resins, gums, flavor enhancers, as insecticides and herbicides [19–23]. On the other hand, the majority of SM have found utility in the pharmaceutical industry, given a large number of pharmacological activities that are known about them [24].

1.4.1 Classes of secondary metabolites in plants

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 large groups: terpenes, phenolic compounds, and alkaloids [25].

1.4.1.1 Terpenes

Terpenes constitute the largest group of SM in plants to which more than 40,000 different molecules are allocated [25]. From the chemical point of view, they are non-saponifiable lipids since fatty acids do not intervene in their formation. They are also known as isoprenoids since the basic structural unit that forms them is the isoprene molecule (figure 2) [26].

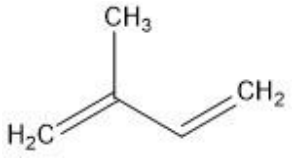
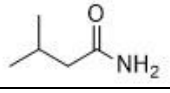
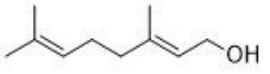
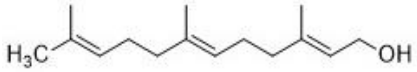
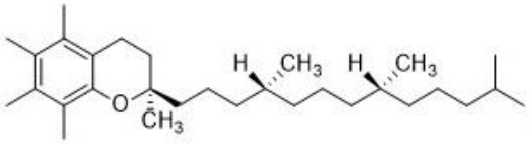
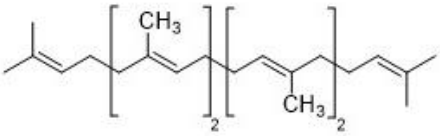
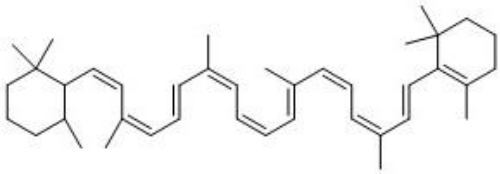
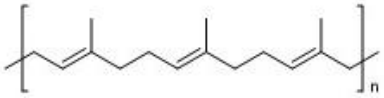


Figure 2: Isoprene.

They are classified according to the number of isoprene units they contain. The simplest class of all is hemiterpenes with a single isoprene unit and five carbons in its structure. The best-known hemiterpene is isoprene, a volatile product that emerges from photosynthetically active tissues. With two units, the terpenes are classified in monoterpenes, with three units in sesquiterpenes, with four in diterpenes, with six in triterpenes, with eight in tetraterpenes, and with more than 10 in polyterpenes (Table 1) [27,28].

Table 2: Classes of terpenes according to the number of isoprene units.

Class	Number of isoprene units	Number of carbon atoms in the structure	Examples	Structure
Hemiterpene	1	5	Isovaleramide	

Monoterpenes	2	10	Geraniol	
Sesquiterpenes	3	15	Farnesol	
Diterpenes	4	20	Vitamin E	
Triterpenes	6	30	Squalene	
Tetraterpenes	8	40	Carotene	
Polyterpenes	>9	>40	Rubber	

Many plants contain terpenes in their flowers and fruits as mixtures of volatile compounds with specific odors; among them, we can mention lemon, mint, eucalyptus, ginger, and great basil [29]. Terpenes have several biological functions and participate in both the primary metabolism and the secondary metabolism of plants. In the primary metabolism they are photosynthetic pigments (carotenes), regulators of plant growth and development (gibberillins, strigolactones, brassinosteroids), are part of cell membranes and participate in protein glycosylation (phytosterols), and some work as electron carriers (ubiquinone and plastoquinone) [30]. The last one, fulfill a very important function in light dependent photosynthesis, as it can be observed in figure 3, plastoquinone (PQ) is responsible to transport electrons from photosystem I during photosynthesis. A novel thylakoid membrane protein (*ssr2016*, PGR5) transfers the recycled electron from ferredoxin to plastoquinone. Then, plastoquinone is reduced to plastoquinol (figure 4) by four electrons generated when a water molecule is divided into O_2 and H^+ in photosystem II [31].

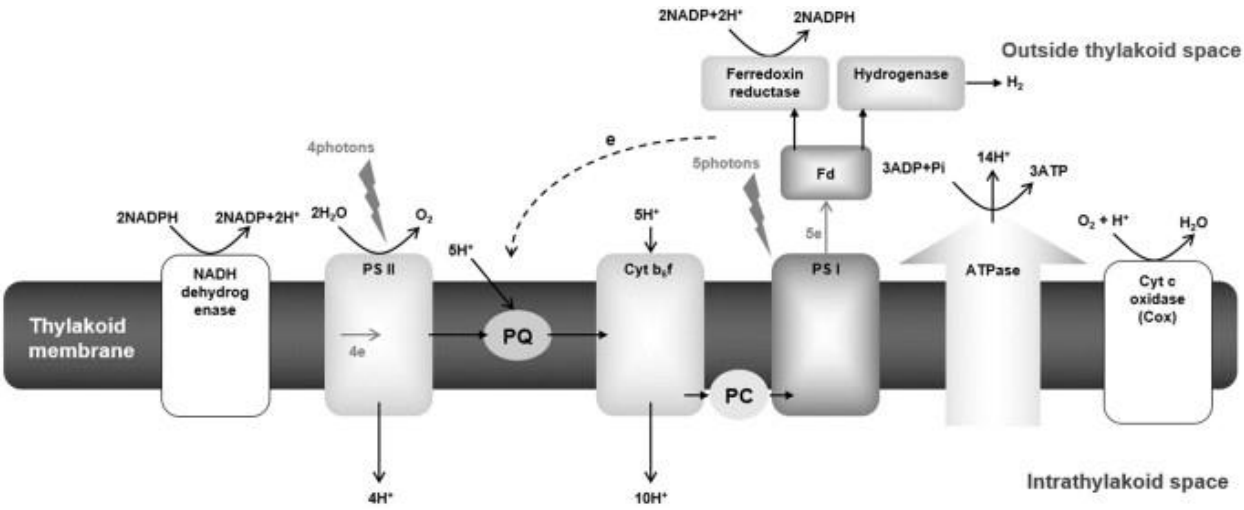


Figure 3: Schematic of light-dependent photosynthesis. Taken from Hong [31].

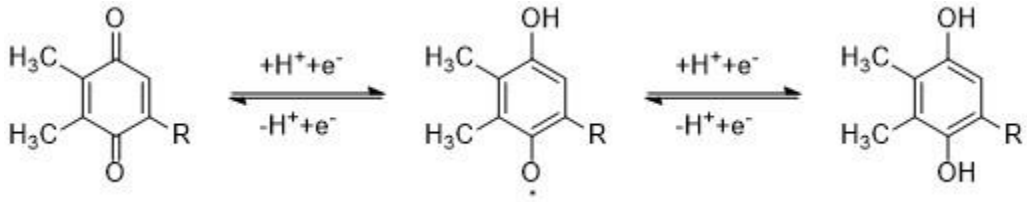


Figure 4: The reduction (from left to right) of plastoquinone (PQ) to plastosemiquinone ($PQH^{\cdot}$) to plastoquinol (PQH_2).

In secondary metabolism they participate as defense molecules, toxic compounds and food deterrents for insects. In some plants, they are the responsible molecules for attracting pollinators, or they function as dispersers [32–34].

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 [29].

1.4.1.2 Phenolic compounds

Phenolic compounds 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 [35–38].

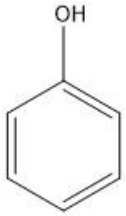
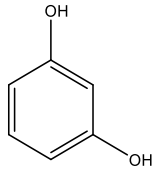
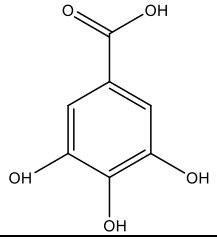
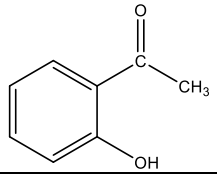
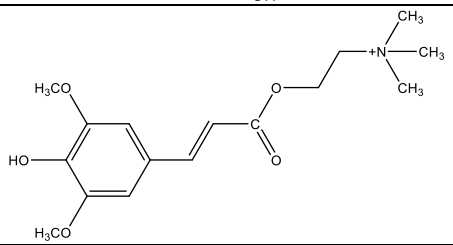
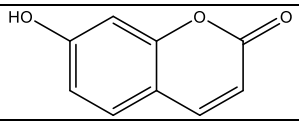
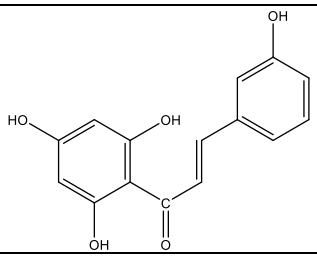
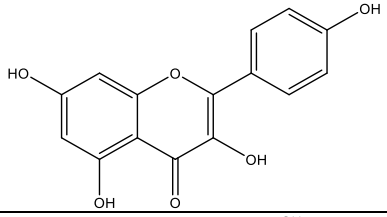
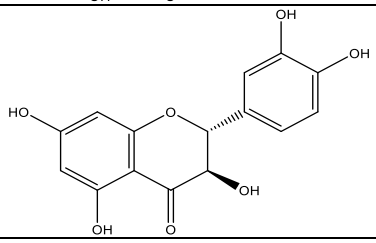
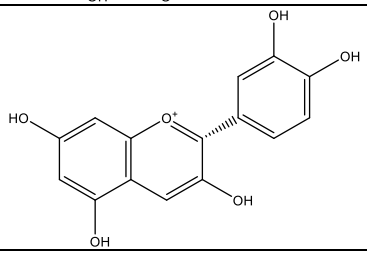
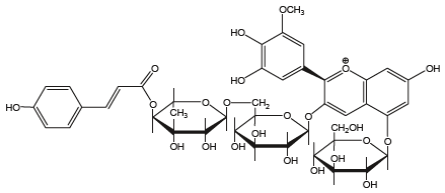
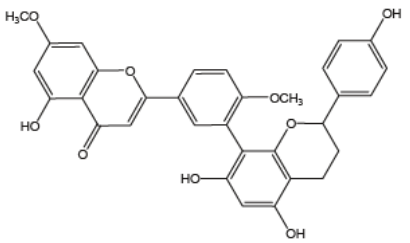
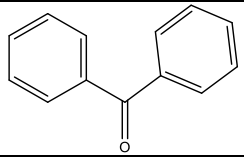
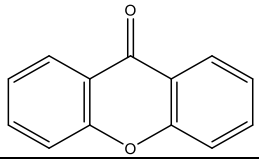


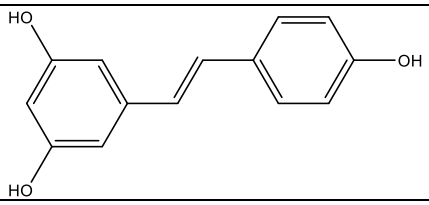
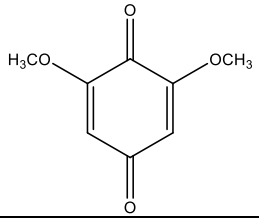
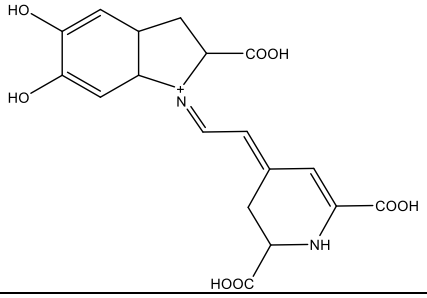
Figure 5: Phenol.

The most important criterion for classifying phenolic compounds 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. Lignans, neolignans, tannins, and phlobaphenes also belong to this group. The latter are polymers and have more complex structures [39,35].

Table 3: Classes of phenolic compounds according to the number of carbons in the structure.

Skeleton structure	Class	Examples	Structure
C6	Simple phenolics	Resorcinol	
C6 - C1	Phenolic acids and related compounds	Gallic acid	
C6 - C2	Acetophenones and phenylacetic acids	2-hydroxyacetophenone	
C6 - C3	Cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols	Sinapoyl choline	
C6 - C3	Coumarins, isocoumarins, and chromones	Umbelliferone	

C6-C3-C6 (C15) Flavonoids	Chalcones, aurones, dihydrochalcones	Butein	
	Flavones	Kaemferol	
	Flavanonols	Taxifolin	
	Anthocyanidins	Cyanidin	
	Anthocyanins	Pentanin	
C30	Biflavonyls	Ginkgetin	
C6-C1-C6	Benzophenones	Benzophenone	
	Xanthones	Xanthone	

C6-C2-C6	Stilbenes	Resveratrol	
C6, C10, C14	Quinones	2,6-dimethoxybenzoquinone	
C18	Betacyanins	Betanidin	

Phenolic compounds are synthesized in plant cells by the shikimic acid pathway or the malonate/acetate pathway (or both, for example, flavonoids) [36]. 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) [37,38]. The poly acetate pathway provides quinones and xanthones. The mixed pathways combine precursors of both the shikimic acid pathway and the poly acetate pathway. This is the case of flavonoids [40,41].

Phenolic compounds fulfill various functions in plants: they oxidize easily and act as antioxidants [42–44], they act as plant growth inhibitors [45], seeds accumulate significant amounts of phenols that act as filter so that oxygen does not reach the embryo and inhibit its germination [46]. Phenols also accumulate on surfaces of leaves capturing up to 90% of UV radiation [47]. Phenols confer aromas and colors to the fruits making them appealing for herbivores, which favors the dispersion of seeds through feces [48]. Plants compete with each other to preserve their territories, and in this process (allelopathy), the phenols participate [49]. Plants also defend themselves against the attack of pathogens by synthesizing phytoalexins that are toxic to microorganisms, and their presence prevents infections [50]. Phenols also protect plants by generating bitter flavors or textures that are unpleasant for herbivores [51].

1.4.1.3 Alkaloids

Alkaloids constitute another large and diverse group of SM that includes molecules isolated primarily from vascular plants [52]. Plants generally produce a complex mixture of alkaloids, in which a major constituent dominates [52]. In a given plant, the biosynthetic origin of the alkaloids present is common, even if their structures are slightly different [52]. 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 [53]. Alkaloids are also found in fungi, bacteria, and animals [54]. They include an atom of nitrogen in their structure, are toxic compounds and respond to common precipitation reactions [55,56].

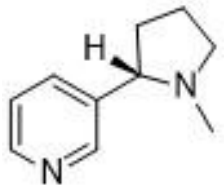
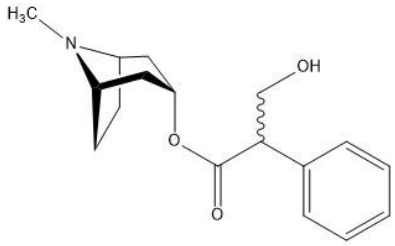
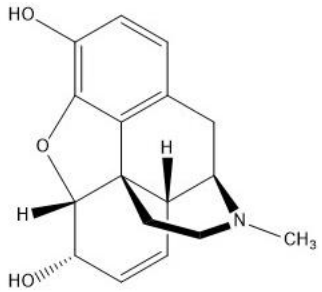
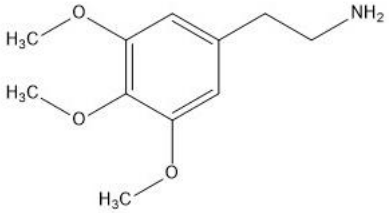
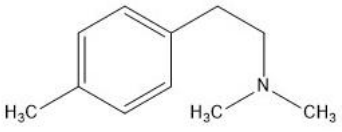
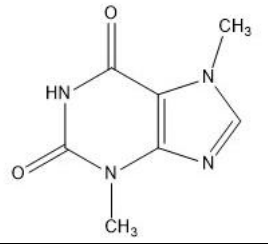
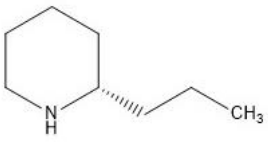


Figure 6: Nicotine a common alkaloid.

Even when there is no uniform classification of alkaloids, several criteria have been used to classify them: biosynthetic origin, presence of a basic heterocyclic nucleus in the structure, pharmacological properties, and distribution in plant families [57]. 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 [58]. True alkaloids strictly comply with the fundamental characteristics of the alkaloids. The majority of the 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 true alkaloids have been derived from anthranilic and nicotinic acids [58,59]. The protoalkaloides 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 [56]. The pseudoalkaloids contain heterocyclic rings with nitrogen but are not derived from amino acids. They are formed by subsequent incorporation of nitrogen into compounds originally free of this element. To this group belong terpenic alkaloids [59].

Table 4: Classes of alkaloids according to their biosynthetic origin.

Class	Name	Structure
True alkaloids	Atropine	
	Morphine	
Protoalkaloids	Mescaline	
	Hordenine	
Pseudoalkaloids	Theobromine	
	Coniine	

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) [60].

Alkaloids have remarkable physiological properties and toxicological that are exerted primarily on the nervous system central, with predominance in some of its levels. For these reasons, they can be used as drugs. Prolonged use of any of these compounds produced in man accustoming, which constitute true drug addictions, with physical and psychic dependence and an increase in the tolerance [58,60]. To date, around 15,000 alkaloids have been isolated from plants. If it is considered to have been examined less than 25% of the upper plant species of the planet, it is clear that there is still a wide field for his research. Because of its pharmacological and medicinal importance, there is a great motivation for continue with the chemical-biological study of the alkaloids. This is one of the most important secondary metabolites of plants with therapeutic interest [59].

1.5 Pharmacological activities of secondary metabolites

Plants have the ability to synthesize a huge and diverse group of SM. 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 an important source of substances that can be used for improving health and curing diseases. Among the beneficial pharmacological activities of the plants stand out antitumor, antioxidant, and antibacterial activities [61,62].

1.5.1 Antitumor activity

Special attention has been devoted to the antitumor activity of SM. According to the World Health Organization, among the causes of death that most affect humanity today cancer is found [63]. Even when there are numerous alternatives for cancer treatment, research is continuing today to find new molecules from natural sources with better treatment effectiveness or able to alleviate the toxic effects of treatments [64]. Examples of isolated metabolites of plants with antitumor activity are lupeol, Asiatic acid, celastrol, aurapten, ursolic acid, saidmanetin and indole-3-carbinol and hypericin. These substances have been shown to affect signaling to control cell growth and

apoptosis, immune response, and stromal microenvironment [64,65]. In this case, there is an anthraquinone called emodin, obtained from aloe vera. Aloe-emodin induces ROS and Ca^{2+} production to inhibit transcription factors, ER stress, dysfunction of mitochondria by the reduction of its membrane potential ($\Delta\Psi m$), cytochrome c release, capspase-9 and -3 activation and it also induces AIF release, finally leading to apoptosis. Aloe-emodin may be a chemotherapeutic drug candidate for the treatment of tongue squamous cancer in the future [66].

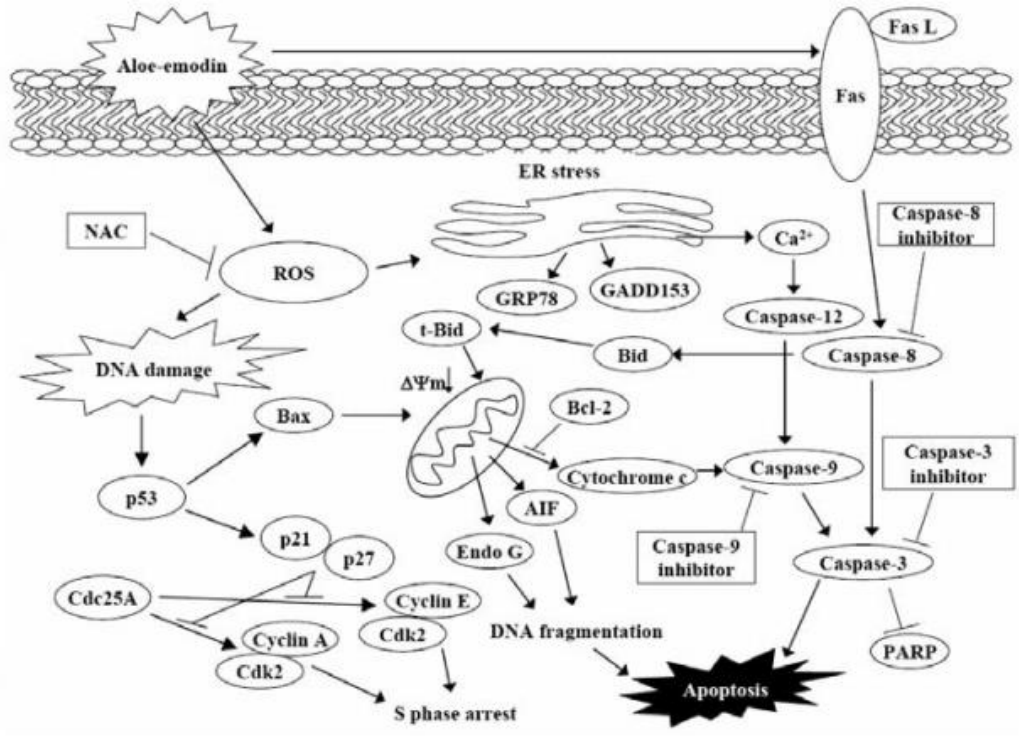


Figure 7: Proposed model of aloe-emodin-mediated cell cycle arrest and apoptosis in human tongue squamous cancer SCC-4 cells. Taken from Chiu [66].

According to computational investigations of the theoretical bond dissociation enthalphy (BDE) and density functional theory (DFT) predicted that the 3-OH group of emodin is important for its anti-oxidant role as radical scavenger. For protein binding it has been shown that the 3-OH of emodin is essential for the interaction with the amino acid residues Asp175 and Lys68 within the binding pocket of CK2 (that is a protein kinase responsible for phosphorylation of substrates in various pathways within the cell) facilitating the inactivation of various downstream pathways [67].

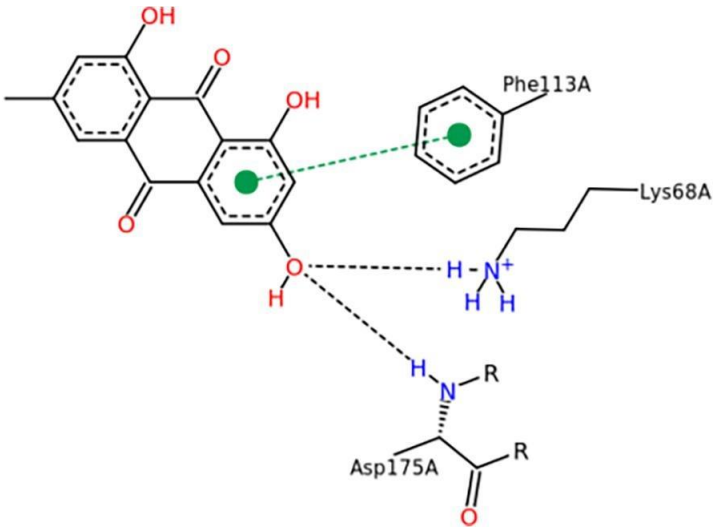


Figure 8: 2D diagram of amino acid residues facilitating binding of emodin within CK2 binding pocket. Taken from Deitersen [67].

1.5.2 Antimicrobial activity

Another health problem that has been in focus on the action of medicinal plants is the antimicrobial resistance. It is estimated that around 25 thousand patients die per year in the European Union, due to infections caused by resistant bacteria [68]. In the United States, it is estimated that resistant bacteria cause around 77 thousand deaths per year. These estimates give a clear idea that the search for new molecules with antimicrobial activity is a priority in basic research and the necessity of the pharmaceutical industry. The antimicrobial activity of many plant extracts has demonstrated to be effective against Gram-positive and Gram-negative [69,70]. Also, 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 vital 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 have the ability to break the cell membranes of microorganisms, 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 [74,75].

1.5.3 Antioxidant activity

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. Besides, flavonols such as quercetin can chelate transition metal ions such as iron or copper, preventing the formation of reactive oxygen species [57,76].

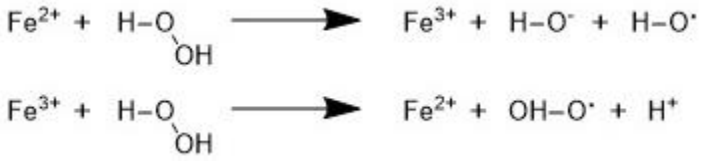


Figure 9: Fenton reaction for antioxidant activity.

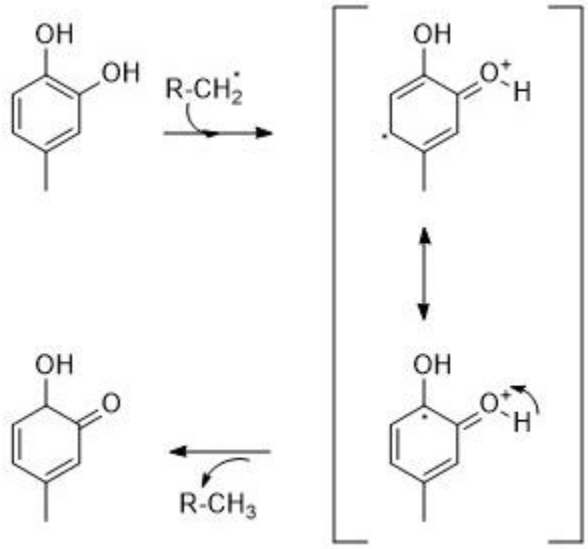


Figure 10: Mechanism of the antioxidant activity of polyphenols.

1.6 Methods

1.6.1 Extraction

The initial step during extraction is the preparation of plant tissues. The extraction can be done on clean and ground leaves, barks, roots, fruits, and flowers, from fresh or dried plant material. It is recommended that the interval between harvest and the initiation of extraction does not exceed 3 hours since the plant tissue is fragile and tends to deteriorate faster than dry tissue, to maintain the freshness of the samples and avoid possible chemical damage [77]. Otherwise, it is preferable to dry the plant by air-drying, microwave-drying, oven-drying, or lyophilization. Each of these methods

has advantages and disadvantages that should be considered [78–81]. Another important point to consider during pre-treatment of the plant is the particle size of plant material. The smaller the particle size, the greater the area of contact between the plant material and the solvent and consequently the more effective the extraction of the chemicals [82].

Extraction is the process that allows separating SM from the plant by using solvents of different polarity. As a result of the extraction remains two phases: a liquid phase containing solubilized metabolites and a solid containing the insoluble cell debris. Conditions as temperature and time, are important factors to achieve high-quality extracts [83]. Most common extraction methods are maceration, infusion, decoction, Soxhlet or continuous extraction, ultrasound-assisted extraction (UAE) [84].

1.6.1.1 Maceration

Maceration is a solid-liquid extraction technique [54]. The method consists of using a solvent or a mixture of solvents having different polarities and certain affinity with compounds that are going to be extracted. The mixture (plant-solvent) is placed in a container with lid and let it rest for two or three days until the compounds could be transferred from vegetal tissues to the solvent. This method is widely used with soft vegetal material [85].

1.6.1.2 Infusion

The infusion is a maceration process too but uses shorter extraction times, and the solvent usually is cold or boiling water. This method is used to obtain a diluted solution of compounds that are easily extracted [83].

1.6.1.3 Decoction

The decoction is a more convenient method for extracting water-soluble compounds from roots and barks that are stable at high temperatures and usually results in oil-soluble compounds compared to maceration and infusion [86]. This method is carried out boiling the vegetal material in water by 15 minutes, then cooling and filtering [83].

1.6.1.4 By soxhlet

Another way to conduct the extraction of SM is by using a Soxhlet apparatus. In this method, a Soxhlet extractor, a condenser, and a round bottom flask are used. The finely ground 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 solvent vapors go into the thimble and then return to the flask after being condensed. The system is left, at least for sixteen hours [87]. The main advantage of Soxhlet extractor is the use of a smaller quantity of solvent compared to maceration. However, the exposure to hazardous and flammable organic solvents, with potential toxic emissions is high [84].

1.6.1.5 The ultrasound-assisted extraction

The ultrasound-assisted extraction (UAE) facilitates the partition of analytes with the occurrence of the fragmentation of cell wall provoked by the collisions between the electromagnetic waves and the particles. There are two forms of applying it: in direct contact with the sample or using an ultrasound bath, where the contact is given through the walls of the bottle. In the first case, the efficacy is 100 times higher than the second one. The procedure is simple, low cost, and can be used in both small and large-scale extraction [88].

1.6.2 Qualitative phytochemical screening

The phytochemical screening is a fast and cheap procedure to determine the main classes of SM or groups of substances that a plant contains. Since each class or group of SM is related to specific biological activities, based on the results obtained in the preliminary qualitative phytochemical screening, it is possible to guide further research to determine the biological activity of the species in question and the active principles involved. The phytochemical screening consists of executing chemical reactions on aliquots of the plant extracts. The reactions can be based on a liquid-liquid partition with solvents in chemical reactions that produce colorimetric changes, fluorescence, or precipitates of a specific color. Among the SM to be analyzed are alkaloids, anthraquinones, flavonoids, phenols, saponins, sterols, tannins, quinones, coumarins, and terpenoids. Numerous reviews are summarizing the principles of chemical reactions and the qualitative changes that can be observed [84,89–91]. A summary of the experimental protocols for the phytochemical screening methods is shown in Table 6.

Table 5: A summary of the qualitative phytochemical screening methods.

Secondary metabolite	Name of test	Reactants	The expected result if positive present
Alkaloid	Dragendorff's test	A solution of potassium bismuth iodide	A red precipitate
	Wagner´s test	Iodine in potassium iodide	A brown/reddish precipitate
	Mayers 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 a 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
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

1.6.3 Total phenolic content

Phenolic compounds are present in all the vegetal tissues. These secondary metabolites constitute one of the micronutrients groups that form part of the human diet. Nowadays, it is essential to study phenols because of its antioxidant properties, its possible beneficial application for human health such as treatment and prevention of cancer, cardiovascular diseases, and inflammatory pathologies [92]. According to Shahidi y Nack, 2004 [93], some phenols are essential for the growth and reproduction of the plant. These also have action as antibiotics, natural pesticides, and UV protective agents. So, it is crucial the determination of the total phenolic content of plants to take advantage of all the benefits that it can provide.

The quantification of phenolic compounds remains difficult. There are a lot of different quantification methods developed according to the phenolic group of the compound [94]. Gas chromatography and high-performance liquid chromatography or its combinations are commonly used to quantify phenolic compounds with the help of mass spectroscopy [93].

On the other hand, spectroscopy is one of the most accessible techniques for the quantification of total phenolic content. In this way, Folin-Ciocalteau is the most used for plant phenols. Folin-Ciocalteau is a method based on an oxidation-reduction chemical reaction in a basic pH [94], and this reaction presents as a result, a blue coloration that can be measured at 765 nm. Folin-Ciocalteau reactive contains a mixture of sodic wolframato and sodium molybdate in phosphoric acid; this has a yellow color, which, when being reduced by the phenolic groups, gives as a result, a dark blue complex [95]. Acid gallic is used for the calibration curve of this assay, due to this compound is a natural phenolic compound. So, the results are also expressed in grams of gallic acid per gram of extract (g of GAE/g of extract). The disadvantage of this method is that not only phenolic compounds react with the Folin-Ciocalteau reagent but also ascorbic acid, aromatic amines, and sugars [96].

1.6.4 UV-VIS analysis

The constituents in plants can be measured in very diluted solutions. These measurements must take into account the solvent in which the extract is diluted. Because it can be the reason for the peaks instead of the components in the plant. The spectral measurements can identify such phytochemical constituents, and with this, these can be monitored if the extract solution is

fractionated [97]. This is very important to consider that the wavelength measurements are not specific for a metabolite but a family of metabolites.

1.6.5 Antimicrobial activity

Nowadays, common therapeutic antibiotics are used to treat microbial infections. The disadvantage of this is that pathogen microorganisms have become resistant to the compounds of these drugs. Also, these common antibiotics are related to adverse effects such as hypersensitivity, immunosuppression, and allergic responses [98]. So, it is essential the discovery of new phytoconstituents that can deal with microbial infections without difficulties. For this, there are some methods to determine if a plant has antimicrobial activity, within the most important are Disc diffusion agar (DDA) and Well diffusion agar (WDA) [99,100].

The aliquots of the different concentrations of the extract solution are needed to carry out the disc diffusion agar. These are placed on paper discs, and the solvents evaporate naturally. Then, the discs with the extracts are distributed on a plate with different microorganisms. So, the late is incubated, and the inhibition zones of growth are determined in mm [99].

On the other hand, for well diffusion agar, different wells are created on the surface of the agar in the Petri dishes with the help of Pasteur pipettes. So, the incubated bacteria are spread on the agar. Then, different solutions of the extract solution are poured into a well each one, and finally, the Petri dishes are incubated [98].

It is crucial to consider the use of positive and negative controls to then compare with the results of the extract solutions.

1.6.6 Growth inhibition of bacteria

When talking about spectroscopy measurements, it is said that the absorbance is directly dependent on the concentration and chemical composition of the studied sample. So, the optical density is useful to describe the dynamics of bacterial growth, where the bacterial density is defined as the dry weight of cells per unit volume of a culture or as the number of doublings of bacterial density per unit time [71]. The curve obtained illustrates the events occurring through the time when bacteria are growing.

Another method to known if extracts can inhibit the growth of bacteria is by the periodical measurements of the relative electric conductivity of bacteria samples that contain extract solutions. It consists in measuring the change in electrical conductivity that is correlated with the change of cell wall permeability. When an extract has growth inhibition activity on bacteria, the cell wall of the bacteria breaks. So small ions such as K^+, $Na^{+,}$ and H^+ are released, and so the electric conductivity increases in the medium. Thus, while more bacteria dead, the electrical conductivity increases too [75].

1.6.7 Anti-inflammatory activity

Inflammation is a process that begins as a response of the immunological system to mechanical damage, burns, microbial infections, and others. Also, it can be said that inflammation is a protective response, whose main objective is to rid the body of the element that causes cellular damage, such as microbes and toxins [101], and the consequence of this damage, with the formation of necrotic cell and tissues. Without inflammation, infections would spread, and wounds would never heal. On the other hand, inflammation that was not correctly cured is the basis of hypersensitivity reactions and chronic diseases, such as rheumatoid arthritis, atherosclerosis, and pulmonary fibrosis [102]. In this way, some flavonoids have the capacity to inhibit the inflammation through different mechanisms. One that has been studied in vivo with a mouse is the way these metabolites are able to regulate the enzymatic activity of COX [101]. However, for the test *in vitro*; it is commonly used the erythrocyte membrane stabilization [103]. It is based in the stabilization of lysosomal membrane, with this, is possible to limit the inflammatory response [104], by avoiding the release of lysosomal constituents such as proteases and bactericidal enzymes, which are the responsibility of cause tissue inflammation [103].

2 Problem statement

Through the years, human beings have found in nature the satisfaction of basic needs, food, footwear, clothing, transport, flavors and odors, and last but not least, is the use that humanity has made of natural sources to cure diseases since ancient times. Plants have formed the basis of traditional medicine systems, which have existed for hundreds of years. These systems continue playing an essential role in health today. Despite the fact that the use of medicinal plants has some disadvantages such as the interaction between medicinal herbs and conventional medicines, accidental contamination of plants with toxic bacteria and fungi, the lack of proper standardization of plants due to genetic diversity and changes in the environment in which they develop (which conditions a great variability in the concentration of the substances contained in them) and the most important and common, the ignorance of the adverse effects derived from the toxic substances present in plants. The World Health Organization (WHO) has estimated that approximately 80% of the world's inhabitants have used traditional medicine in their health care [105]. An analysis of the prescriptions presented in pharmacies in the United States showed that until 2008 around 35% contain plant extracts or active ingredients derived from them [106]. On the other hand, no less than 250 chemical substances, derived from 145 species, can be considered of importance in many countries, of which 74% were discovered as a result of chemical studies or isolation of the active ingredients of plants used in traditional medicine [54]. So, it is easy to imagine how many more phytoconstituents with bioactive and physiological activities could be discovered. In this aspect, *Alternanthera porrigens* is a medicinal plant used in Ecuador because of its multiple benefits for the human being mainly for the treatment of inflammation, to treat circulation, as purgative, and for menstrual disorders. Then, the study of its secondary metabolites lies on to find which ones could have biological activities, and the antimicrobial and anti-inflammatory activities help to affirm the ancestral uses that people give to this plant.

3 Objectives

3.1 General objective

To determine the presence of secondary metabolites in different extracts of flowers and leaves of *Alternanthera porrigens,* and to analyze the presence of biological activities by *in vitro* assays.

3.2 Specific objective

- To analyze the qualitative phytochemical screening of ethanolic, chloroformic, and aqueous extracts of leaves and flowers of *Alternanthera porrigens.*
- To determine the total phenolic content of the ethanolic, chloroformic, and aqueous extracts of leaves and flowers of *Alternanthera porrigens.*
- To characterize the ethanolic, chloroformic, and aqueous extracts of leaves and flowers of *Alternanthera porrigens* by UV-VIS analysis.
- To determine the antimicrobial activity of the ethanolic, chloroformic, and aqueous extracts of leaves and flowers of *Alternanthera porrigens.*
- To examine the anti-inflammatory activity of dried ethanolic extract of flowers of *Alternanthera porrigens.*

4 Methodology

4.1 Reactive and equipment

4.1.1 Reactants

Analytical reactive were used in the present work.

- Methanol (99,92% Alpha Chemika)
- Ethanol (96% NOVACHEM, ECUADOR)
- Chloroform (99,5% NOVACHEM, ECUADOR)
- Ferric chloride (99,99% Sigma-Aldrich)
- Distilled water (NOVA Laboratorios)
- Hydrochloric acid (37% SCIENCE Company)
- Ammonia (29% NOVACHEM DEL ECUADOR)
- Sodium hydroxide (97% Sigma-Aldrich)
- Sulfuric acid (96% LabChem)
- Acid acetic (99,8% 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 (98% Thomas Scientific)
- Gallic acid (98% Fisher Scientific)
- Dimethyl sulfoxide (99,9% 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)

Any purification was applicated to the reagents.

4.1.2 Equipment

- Zuzi spectrophotometer model 4211/50 (ROGO-SAMPAIC, France)
- PerkinElmer UV/VIS/NIR spectrophotometer lambda 1050 (Perkin Elmer, EEUU)
- Balance Cobos precision HR-150A (COBOS precision, Spain)
- Drying stove SLW 115 POL-EKO-APARATURA (POL-EKO APARATURA, Poland)
- Bante MS300 hot plate magnetic stirrer (BANTE instruments, EEUU)
- P/SELECTA Ultrasound HD (GRUPO SELECTA-Spain)
- BUCHI Rotavapor R-210 (BUCHI Labortechnik, Switzerland)
- Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Fisher SCIENTIFIC, EEUU)
- Thermo Scientific MaxQ 4450 Incubated Benchtop Orbital Shakers (The Lab Depot, Dawsonville EEUU)
- Thermo Scientific Heratherm Refrigerated incubator IMP -180 (Thermo Fisher Scientific, Belgium)
- Telstar AV-30/70 vertical laminar flow bench (Fisher Scientific, EEUU)
- Mini Spray Dryer BUCHI-B290 (BUCHI, Mexico)
- Centrifuge (HettichZentrifugen, Germany)
- Spectrophotometer (UV-VIS Fisher Scientificaccu Skan Go, EEUU)

4.2 Tissues preparation

The plant was collected in the province of Imbabura, the leaves and flowers of the plant were separated and subsequently washed with abundant distilled water, this process was repeated five times to remove all the dust or dirt that were present in these tissues. Then, the excess water in the tissues was removed with paper towels. To finally place them on aluminum foil and let them dry at 25 °C for four days. Once the parts of the plant were dried, they were crushed with the help of an electric grinder, working each organ separately. Finally, reasonably fine powder was obtained, which was weighed and then proceeded with the maceration of the material.

For the maceration, the tissue powder was first placed inside an amber bottle, and then the solvent was added, allowed to macerate for 48 hours. Macerations were performed with three different solvents: ethanol, chloroform, and water. The macerations made from the flowers were made with a dry tissue:solvent of 1:10 (p:v). In the case of the leaves, the extractions with ethanol and

chloroform were performed at a 1:10 ratio (p:v), but with water, it was extracted at 1:9 (p:v). Finally, after each maceration, the amber bottles were ultrasound for 1 hour at 25 °C, the plant tissue was filtered with the help of vacuum and filter paper, and the extracts were stored in amber bottles, protected from light and at 4 °C.

4.3 Phytochemical analysis

Different colorimetric, and precipitation tests with ethanolic, chloroformic, and aqueous extracts from plant tissues should be completed and practiced to perform the phytochemical analysis of the moradilla. It should be noted that there are several different tests to determine the presence of a specific metabolite, and these already have established protocols.

Phenols: to identify these secondary metabolites, the ferric chloride test was used. This test consisted of the addition of 3 drops of 1% ferric chloride aqueous solution to 1 mL of extract solution. The presence of color changed according to the position of the phenolic oxyhydriles: it was yellow for the presence of 1 -OH, greyish green for 2 -OH, and dark blue for 3 -OH [107].

Anthocyanins: 1 mL of 2N chlorihidric acid and 1 mL of ammonia were added to 2 mL of extract solution. If the color changed to blue and violet, then anthocyanins were present [108].

Coumarins: 2 mL of extract solution was mixed with 3 mL of 10% sodium hydroxide. If there was a formation of a yellow color, then coumarins were present [108].

Flavonoids: 2 or 3 drops of 1% ammonia solution were added to the extract solutions of the samples in a test tube. If flavonoids were present, then a yellow coloration should appear [109].

Tannins: For tannins, it was used three different tests: modified Braemer, potassium permanganate, and gelatine. The gelatine test consisted of adding ten drops of a 2% aqueous solution of gelatine (made with an acidic sodium chloride solution) to a test tube with 3 mL of solution extract. An abundant precipitate should appear to demonstrate the presence of tannins [107].

Anthraquinones: Borntrager's test determined the presence of this metabolite. This test was about boiling 1 mL of the extract with 1 mL of 10% chlorihidric acid for a few minutes in a water bath. Then, the solution was filtered, and it was added chloroform in the same volume. Finally, some drops of 10% ammonia were added to the last mixture, and the whole was heated. If the appearance of the solution turned pink, it indicated the presence of anthraquinones [110].

Phlobatanins: hydrochloric acid test was used, in which it is added 1% hydrochloric acid to 2 mL of aqueous solution in a test tube, then it was heated in a water bath. If a red precipitate was observed, then it indicated the presence of phlobatanins [90],[111].

Terpenoids: to determine the presence of this metabolite, Salkowski's test was applied. In a test tube, it was added the extract solution with 2 mL of chloroform, and then 3 mL of concentrated acid sulfuric were poured carefully along the sides of the test tube. A reddish-brown layer was formed to demonstrate the presence of terpenoids [110].

Saponins: to establish the presence of saponins, 1 mL of extract solution was diluted with distilled water until 10 mL, and then the mixture was shaken for 2 minutes. The presence of saponins was confirmed with the presence of a thick layer of foam that remains for a few minutes, the longer the foam lasts, the more saponins are present [112].

Steroids and sterols: it was used Liebermann-Burchard's test. So, it was added 1 mL of chloroform to 1 mL of extract solution, and then it was mixed with 2-3 mL of acetic anhydride, to then it was put 1-2 drops of concentrated sulfuric acid. It should present a dark green coloration for the presence of steroids; red, pink or violet for triterpenoids, and light yellow for steroids or saturated triterpenoids [113].

Cardiotonic glycosides: it was used a modified Kellar–Kiliani. Then, three drops of strong lead acetate solution were added to 2 mL of aqueous extract solution, and this was mixed and filtered. The filtrated was shaken with 5 mL of chloroform, and then the chloroform layer was carried to dry. The rest was dissolved with glacial acetic acid (that contained 2-3 drops of ferric chloride solution). Finally, 2 mL of concentrated sulphuric acid were added along the sides of the test tube carefully. Cardiotonic glycosides were present if the upper layer was bluish-green and if the ring between the two layers had a reddish-brown coloration, respectively [114].

Proteins and amino acids: for this, it was used the ninhydrin test, where some drops of Ninhydrin reagent were added to 1 mL of extract solution, then the test tube was heated in a water bath. If the color became violet/purple, it meant that proteins, peptides, or amino acids were present [110].

Carbohydrates: it was used Benedict's test, where the extract solution and Benedict's reagent were put in a test tube in equal volumes, then the tube was heated gently for a few minutes. If a red precipitate appeared, then it meant the presence of carbohydrates [115].

Reducing sugars: were demonstrated by Fehling's test, in this, the extract solution was treated with Fehling's A and B solution in equal volume, then it was heated in a water bath for some minutes. If there was a formation of a red precipitate, so carbohydrates were present [114].

4.4 Quantification of the total phenolic content

Before beginning the assay, it was diluted the aqueous extract of flowers, the aqueous extract of leaves, and the ethanolic extract of leaves in a proportion 1:20 to be measured by spectrophotometry. The determination of the total phenolic content was performed by reacting with the Folin-Ciocalteau reagent. It was used the method of Singleton [116] with some modifications. First, it was necessary to build a standard calibration curve. For this, gallic acid was used in different concentrations: 0 mg/mL, 0,02 mg/mL, 0,04 mg/mL, 0,06 mg/mL, 0,08 mg/mL, 0,1 mg/mL, 0,12 mg/mL, 0,14 mg/mL, 0,2 mg/mL, 0,3 mg/mL. All of them were maintained protected from light. So, for both gallic acid and extract solutions, to 300 µL of the solution was added 150 µL of 10% Folin-Ciocalteau reagent, then it was let to react for 3 minutes to room temperature. Next, it was added 120 µL of 7,5% sodium carbonate and let it rest for 30 minutes in a dark place to measure the absorbance at 765 nm [117]. This procedure was made for water and ethanol, too, to use them as blank control. After that, the absorbances obtained for the extract solutions were extrapolated in the standard calibration curve to obtain the concentration of them. The results were expressed as mg in equivalents of gallic acid per gr of extract.

4.5 Characterization of extracts by UV-VIS spectroscopy

The extract solutions were diluted to 1:20 v:v in their respective solvent. The spectra were registered in a range of 250 to 800 nm in a UV-VIS NIR Lambda 1050 spectrophotometer [118].

4.6 Biological activities

4.6.1 Antimicrobial activity

4.6.1.1 Extracts, positive controls, and negative controls

First, the ethanolic and chloroformic extracts of leaves and flowers were evaporated in the rotary evaporator until a mass was obtained of each extract. Then, the solute was weighted and labeled in an Eppendorf tube to continue with its dilution in dimethylsulfoxide (DMSO) [119]**.** It was made two concentrations for each extract, 10 mg/mL in DMSO and 25 mg/mL in DMSO. On the other hand, it was used as a solution of 0,5mg/mL of Kanamycin as a positive control. As negative

controls, it was used pure DMSO for the ethanolic and chloroformic extracts and water for the aqueous extracts.

4.6.1.2 Bacterial culture

In order to perform the antimicrobial analysis it was used a Gram-negative bacteria, Escherichia coli. 100 µL of *Escherichia coli* was put in lysogeny broth (LB), previously prepared by the addition of 4 g of Lysogeny broth to 200 mL of distilled water and autoclaved for 1 hour, to inoculate in a shaker at 37°C with 150 rpm for 12 hours before the experiment was carried out.

4.6.1.3 Modified well diffusion agar assay

An *in vitro* antibacterial activity was carried out with the extracts of *Alternanthera porrigens*. It was compared with standard antibiotic Kanamycin by a modified well diffusion agar method [100]. So, lysogeny broth agar, previously prepared with 5 g of LB agar dissolved in 125mL of distilled water and autoclaved for 1 hour, was put in the Petri dish and the Petri dish was put aside until the culture medium got solidified. Then, 16 quadrants at a required distance were drawn in the back of the plate. Next, 300 µL of the cell bacteria were spilled on the solidified culture medium and spread carefully with a cell spreader. Then it was let to dry for a few minutes. Finally, using sterilized micropipettes, it was put the extract solutions with different concentrations, the positive controls, and the negative controls, 3 µL of each one and each in a different quadrant. The Petri dish was incubated at 37°C for 24 hours, and the activity of the extracts was determined by measuring the zones of inhibition (mm) and compared with the positive controls to obtain the percentage of inhibition.

4.6.1.4 Growth inhibition of bacteria

200 µL of *Escherichia coli* were put to inoculate with 3 mL of LB broth 14 hours before taking the measurements with the Nanodrop spectrophotometer. First, it was necessary to carry the absorbance of the bacteria in its culture medium to 0,02 by the addition of LB broth. Then, it was taken 3 mL of the bacteria and put 3 µL of the negative controls or the extracts to be studied. So, all the tubes were carried to incubate in the shaker at 37°C with 150 rpm. The measurements were taken every 30 minutes for 12 hours or until the bacteria reach its declension phase. The following equation is used to measure the growth rate of the bacteria in comparison with the growth rate of bacteria affected by the extracts:

$$R = \frac{\log_2 x_2 - \log_2 x_1}{t_2 - t_1}$$

Equation 1: Equation to calculate the growth rate of bacteria.

Where:

x_2= the value of the initial absorbance and x_1= the value of the final absorbance

t_2= the initial time and t_1= the final time

4.6.2 Anti-inflammatory activity

4.6.2.1 Microencapsulation of extracts by spray drying

The extract was concentrated in a rotary evaporator at 30°C, which allowed the distillation of the ethanol from the mixture. Then, the concentrated extract was weighed, labeled, and stored in an Eppendorf tube at 4°C, avoiding the degradation of the metabolite. Then, the extract was first dissolved in *Sacha inchi* oil until most of the extract homogenize in the oil. So, for the matrix of the microencapsulated extract, it was used maltodextrin, Arabic gum, and water in a proportion of 30:30:30 (v:v:v). The mixtures were taken to a shaker to complete its homogenization. For the microencapsulation, the Mini Spray Dryer was used, this was fed with the respective combination, once the equipment conditions were verified to be adequate (-50 mbar, 150 °C inlet and 90 °C outlet). The resulting microencapsulated product was stored in covers tightly closed.

4.6.2.2 Purification of erythrocyte solution

First, it was prepared a 40% erythrocyte solution following Kumar methodology but with some modifications [120]. It was extracted 3 mL of human blood from healthy volunteers who did not have used anti-inflammatory drugs at least the last two weeks, then the samples were placed in heparinized tubes. Then, it was necessary to homogenize the blood with the anticoagulant, carefully, and then the tube was taken to the centrifuge for 10 minutes at 3000 rpm to achieve phase separation. The supernatant (blood plasma) was discarded, while to 3 mL of the precipitate (erythrocytes) was added the same volume of phosphate buffer solution (PBS) pH 7.4. This mixture was taken to the centrifuge for 5 minutes at 3000 rpm. The last process was repeated four times more, and in the previous wash, it was prepared the 40% erythrocyte solution in PBS (for this, it was taken 800 µL of erythrocytes and 120 µL of PBS), pH 7.4.

4.6.2.3 Determination of anti-inflammatory activity by the method of erythrocyte membrane stabilization

For the determination of anti-inflammatory activity, it was used the erythrocyte membrane-stabilizing method [121] with some modifications. A stock solution was prepared using 0.08g of dry extract in 2 mL of PBS, having a final concentration of 40 mg/mL. From this, 4 different concentrations were obtained (solution taken from the stock solution and graduated to 3 mL with PBS): 1 mg/mL, 1,5 mg/mL, 2 mg/mL and 2,5 mg/mL. For positive control, it was employed aspirin, in its stock solution it was used 500 mg to reach a concentration of 40 mg/mL, from this, some solutions were prepared (solution is taken from the stock solution and graduated to 3 mL with PBS): 1 mg/mL, 1,5 mg/mL, 2 mg/mL and 2,5 mg/mL. The assay was carried out in 3 centrifuge tubes of 15 mL (DO_1, DO_2, DO_3). Each essay follows these conditions:

DO_1: it was put 3 mL of the extract, 30 µL of the 40% red blood cell solution was added, and both were mixed slightly in the test tube. It was allowed to rest for 20 minutes and then centrifugated for 10 minutes at 3000 rpm.

DO_2: it was added 3 mL of the extract with 30 µL of the 40% red blood cell solution in a test tube, then the content together was mixed slightly. The test tubes were subjected to a water bath at a temperature of 54 °C for 20 minutes, and so they were centrifugated for 10 minutes at 3000 rpm.

DO3: it was added 3 mL of the buffer phosphate (PBS) pH 7,4 with 30 µL of the 40% red blood cell solution in a test tube, then the content together was mixed slightly. The test tubes were subjected to a water bath at a temperature of 54 °C for 20 minutes, and so they were centrifugated for 10 minutes at 3000 rpm.

This procedure was carried out for the plant extract, the aspirin, and the buffer phosphate (PBS) with all their different concentrations, using a test tube for each one. Finally, with a spectrophotometer, the absorbance of the supernatants of each assay was recorded at a wavelength of 540 nm. PBS was used as a negative control, and the test was performed in triplicate. It was used the following equation to determine the percentage of anti-inflammatory activity:

$$\%\, Anti - inflammatory\ activity = \left(1 - \frac{DO_2 - DO_1}{DO_3 - DO_1}\right) * 100$$

Equation 2: Equation to obtain the percentage of Anti-inflammatory activity in the dried extract.

5 Results, interpretation, and discussion

5.1 Preparation of leaves and flowers of *Alternanthera porrigens*

Medicinal plants have global importance because of the diverse phytoconstituents these can present [122]. The use of this kind of plant is as ancient as civilization [100], because they are components of phytomedicine. For this reason, scientists started looking for the basis of the pharmacological activity of these plants [123]. It was found that secondary metabolites are bioactive compounds that are responsible for the beneficial actions of these organisms [124].

To start the study of the secondary metabolites in *Alternanthera porrigens*. The plant must be cleaned of all impurities that could affect the assays. Then the tissues must be dried to remove any trace of water. Then, it can proceed with the extraction of secondary metabolites of leaves and flowers of *Alternanthera porrigens*. In the present work, the maceration method was used to extract the secondary metabolites (Table 7). This method was considered to achieve proper extraction and concentration of metabolites [84].

Table 6: Weights of dried material, the volume of each solvent used, and time of maceration for each extraction for leaves and flowers of Alternanthera porrigens.

Tissues	**Leaves**			**Flowers**		
Solvent	**Ethanol**	**Chloroform**	**Water**	**Ethanol**	**Chloroform**	**Water**
Weight of dried material (g)	32,12	31,00	30,95	44,03	47,38	42,00
Volume of solvent (mL)	320,10	310,00	279,00	440,30	474,00	420,00
Time of maceration (h)	23,00	22,00	24,00	19,00	18,00	20,00

It was obtained three different extracts for each tissue, ethanolic extract, chloroformic extract, and aqueous extract. Nomenclature for each extract is shown in Table 8.

Table 7: Nomenclature for the extracts with different solvents for leaves and flowers of Alternanthera porrigens.

Tissue	**Extraction solvent**		
	Ethanol	**Chloroform**	**Water**
Leaves	EHE	EHC	EHA
Flowers	EEF	ECF	EAEF

5.2 Qualitative phytochemical screening for *Alternanthera porrigens*

In order to find the compounds responsible for biological activities in a plant, it initiates with a qualitative phytochemical screening to determine which secondary metabolites are present in the plant.

Through the different qualitative assays, it was identified the presence of some secondary metabolites such as phenols, anthocyanins, coumarins, flavonoids, tannins, terpenoids, saponins, steroids and sterols, cardiotonic glycosides, proteins and amino acids, carbohydrates, reducing sugars in *Alternanthera porrigens*. Likewise, it was noticed the absence of anthraquinones and phlobatanins in all the extracts. It can be appreciated in Table 9.

Table 8: Qualitative phytochemical screening of Alternanthera porrigens.

SM	Secondary metabolites	Tests	Leaves extracts			Flowers extracts		
			EHE	EHC	EHA	EEF	ECF	EAEF
Phenols	Phenols	Ferric Chloride	+	-	+	+	-	+
	Anthocyanins	A)	-	-	+	-	-	+
	Coumarins	B)	-	-	-	+	+	+
	Flavonoids	Ammonia	-	-	-	-	+	+
	Tanins	Modified Braemer	+	-	-	+	-	-
		Potasium permanganate	+	-	-	+	-	-
		Gelatin	+	-	+	-	-	+
	Anthraquinones	Bornstrager	-	-	-	-	-	-
	Phlobatanins	Hydrohloric acid	-	-	-	-	-	-
Terpenes	Terpenoids	Salkowski	-	-	+	-	-	+
	Saponins	Foam	-	-	+	-	-	+
	Steroids and sterols	Modified Lieberman – Burchardt	+	+	-	-	-	-
Others	Cardiotonic glycosides	Modified Kellar – Kiliani	+	-	+	-	-	+
	Proteins and amino acids	Ninhidrina	-	-	+	-	-	+
	Carbohydrates	Benedict	+	+	-	+	-	-
	Reducing sugars	Fehling	+	+	-	+	+	-

(+) means the presence of the metabolite in the extract and (-) indicates the absence of the metabolite in the extract.

A) It was added 2 mL of hydrochloric acid and 2 mL of 0,5M ammonia to 4 mL of extract solution.

B) It was mixed 1mL of extract solution with 1 mL of 10% sodium hydroxide

The extract with more metabolites presents was the aqueous extract of flowers. It showed phenols, anthocyanins, coumarins, flavonoids, tannins, terpenoids, saponins, cardiotonic glycosides and proteins, and amino acids. Then, the aqueous extract of leaves was the second with a higher number of secondary metabolites identified. Between them were phenols, anthocyanins, tannins, terpenoids, saponins, cardiotonic glycosides and proteins, and amino acids. Both aqueous extractions were highly good for attracting saponins due to when the assay was carried on, a lot of foam that resists for a long time was observed.

In the ethanolic extract of leaves, it was possible to detect the presence of phenols, tannins, sterols and steroids, cardiotonic glycosides, carbohydrates, reducing sugars. In the ethanolic extract of flowers, there were phenols, coumarins, tannins, carbohydrates, and reducing sugars. On the contrary, chloroformic extraction was not able to attract many secondary metabolites, only coumarins, flavonoids, and reducing sugars in flower extraction, steroids, sterols, carbohydrates, and reducing sugars in leaves extraction.

All these metabolites have critical biological activities for human beings; for that reason, these were tried to be identifying. For example, phenols have antibiotic activity, these work as natural pesticides, and isolators in cell walls [92]. Sterols can decrease the cholesterol in the organism due to the reduction of the absorption of cholesterol by the bowels [92.] The principal function of terpenoids is its potent antibacterial activity [92.] Anthocyanins are studied for obesity control, diabetes control, cardiovascular disease prevention, and improvement of visual and brain functions [125]. Flavonoids are known due to its anti-inflammatory, antiallergic, antiulcerogenic, antiviral, and anticarcinogenic activity, also because these are used to treat diabetes and heart diseases [92]. Tannins present anti-oxidant and antimicrobial activities [126]. Saponins have several physiological activities such as surfactants and hemolytic properties, have hint effect, insecticide, anti-protozoa, anti-inflammatory, leishmanicidal, anti-trichomonas, anti-platelet, bronchiolitis, and hypo-cholesterolemic activities, also cytotoxic activity against neoplasms [92]. By the way, coumarins have potential effects on the vascular system and photosensitizing properties [92]. And cardiotonic glycosides have an impact on heart diseases [92].

So, with taking into account this, future investigations could be applied to *Alternanthera porrigens* to obtain a powerful compound capable of treat diseases and replace drugs with collateral effect in the human organism.

On the other hand, the presence of proteins, amino acids, carbohydrates, and reducing sugars implicates the nutritional value of the plant for the human being [24].

According to literature, the best way to extract the higher amount of secondary metabolites is the use of solvents with different polarities, and it is selected depending on the polarity of the metabolites expected to extract [127]. Due to the ability of each extract to attract different molecules, a solvent of polarity similar to that of the solute dissolves this more easily. Also, some aspects of the solvent must be considered such low toxicity, easy to evaporate at low temperatures (low boiling point), rapid physiological absorption of the extract, preservative action and it must not have any activity in the extract (to provoke the formation of complex or to dissociate the compounds of the extract) [89].

In this way, ethanol being a polar solvent, is capable of attracting terpenoids, tannins, sterols, polyphenols, especially antioxidants [94], flavonols, alkaloids, polyacetylenes [108], flavonoids [89]. Also, it can extract higher amounts of phenolics [127]. On the other hand, chloroform being a less polar solvent, can attract only terpenoids, flavonoids,[108], and occasionally tannins [89]. And finally, water is capable of catching terpenoids, saponins, anthocyanins, and tannins [108]. It has been demonstrated that aqueous solvent can extract antimicrobial metabolites [89]. So, in comparison with the results obtained, most of the secondary metabolites were extracted in the solvent they supposed to be extracted.

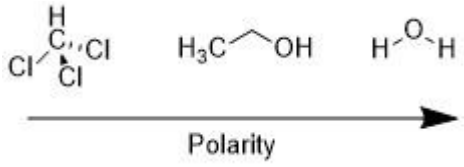


Figure 11: Polarity of solvents used for the extractions.

This is the first qualitative phytochemical screening realized for the species *Alternanthera porrigens*. But, in general, it has been described that plants of the genus Alternanthera have a wide variety of secondary metabolites, such as saponins, tannins, flavonoids [128], glycosides, lipids, sugars, amino acids, steroids, and some species also present alkaloids and hydrocarbon [13]. In the case of the studied species, phlobatanins are not present nor anthraquinones, these secondary metabolites do not appear in any other study of the Alternanthera genus neither.

Alternanthera sessilis is an aquatic plant that is used for the treatment of eyesight and headache to reduce fever and to treat gastrointestinal problems, also for stomach disorders, diarrhea, and dysentery. Also, it has been proved that this species has an anti-inflammatory effect, cytotoxic effect towards pancreatic cancer cell lines, and scavenging activity towards free radicals [129]. According to Hossain (2014) [130], it was demonstrated the presence of saponins, flavonoids, alkaloids, and tannins in the methanolic extract of leaves of *Alternanthera sessilis*. Additionally, Sivakumar, (2016) [100], described the presence of amino acids, carbohydrates, phenols, steroids, terpenoids, and glycosides in the ethanolic extract of the leaves of *Alternanthera sessilis*. *Alternanthera porrigen*s presents all the metabolites mentioned, but in its ethanolic extract of leaves does not present proteins and amino acids as *Alternanthera sessilis* [100].

Da Silva, (2010) [99], revealed the presence of flavonoids, phenolics, and steroids compounds in the hydroalcoholic extract of the leaves of *Alternanthera brasiliana* that is a flowering plant known due to its antimicrobial, analgesic and anti-inflammatory properties. On the contrary, *Alternanthera porrigen*s does not present flavonoids in any extract of its leaves.

Biella (2008) [51], and Salvador (2006) [55], mentioned that *Alternanthera tenella*, a flowering plant that has the presence of flavonoids, tannins, saponins, and reducing sugars in its aqueous extract. This plant is used for infections, fever, inflammation, as a diuretic and antifungal and antimicrobial activity have been proved. In this way, *Alternanthera porrigens* presents the same metabolites in its extracts except reducing sugar that was found in its ethanolic and chloroformic extracts in this work.

Dutta (2015), affirmed that glycosides, flavonoids, tannins, saponins, amino acids, and reducing sugars were present in the aqueous and ethanolic extracts of leaves of *Alternanthera philoxeroides*. It is a flowering plant considered a weed, commonly used for dysentery and asthma, in the same way, alkaloids, terpenoids, and steroids were absent in this plant [131]. On the other hand, in the aqueous and ethanolic extracts of leaves of *Alternanthera porrigen*s, there is not the presence of flavonoids. Still, it has terpenoids and steroids present in the present work.

In this way, the use of the same solvents or ones with similar polarities helps to the determination of the presence of the metabolites mentioned in the different species of Alternanthera genus, being these the same as the ones found in *Alternanthera porrigens*. Considering this and the diverse uses

and pharmacological activities that have been attributed to these plants, it could be assumed that *Alternanthera porrigens* could also have some of these activities.

5.3 Determination of the total phenolic content of extracts of *Alternanthera porrigens*

The Folin-Ciocalteau method is the simplest method available for the measurement of phenolic content in solutions [132]. This method can detect antioxidant compounds such as flavonols, flavones, flavanones, flavonols, proanthocyanidins, isoflavones, anthocyanins, and phenolic acids [96].

Folin-Ciocalteau method is widely used for the measure of the total phenolic content in natural products [133]. And, its fundamental principle is an oxidation/reduction reaction with the phenolic group being oxidized, and the metal ion is reduced [95].

Folin-Ciocalteau reagent is composed of heteropoly-phosphotungstates/molybdates [134]. It is supposed that the Folin-Ciocalteau reaction includes a sequence of reversible one or two-electron reduction reactions. In this reagent, molybdates are more easily reduced than tungstates (Equation 3).

Reaction at pH∼10

$$Na_2WO_4/_{Na_2MO_4}\,(yellow) \rightarrow [(Phenol - PMoW_{11}O_{40})^{-4}](blue)$$

$$Mo^{+6}(yellow) + e^{-}(from\ phenolics\ compounds) \rightarrow Mo^{+5}(blue)$$

$$Mo^{+5}e^{-}(from\ phenolics\ compounds) \rightarrow Mo^{+4}(blue)$$

Equation 3: Reduction of molybdates in Folin-Ciocalteau reaction [96].

It is suggested that in the assays, most of the electron-transfer reactions are between the reductants and the molybdates. During this reaction, Folin-Ciocalteau reagents are at pH∼10. This pH is reached by the addition of sodium carbonate. These basic conditions allow the dissociation of a phenolic proton that leads to the formation of a phenolate ion, which is responsible for reducing the Folin-Ciocalteau reagent. Showing as a result of the reaction a blue color that possibly could be molybdotungstophosphate heteropolyanion [$(PMoW_{11}O_{40})^{4-}$] [134].

In this case, the phytochemical screening gave positive results for several phenols, such as anthocyanins, coumarins, flavonoids, and tannins. So, it was considered appropriate for the measurement of total phenolic content in the extracts of *Alternanthera porrigens*.

The total phenolic content determination was carried out using a standard calibration curve with gallic acid, in a concentration of 0.02 mg/mL to 0.3 mg/mL (Figure 12.) It can be appreciated that the R^2 =0,9987, explaining the excellent preparation of the solutions at different concentrations and also the superb work when pipetting and taking measurements.

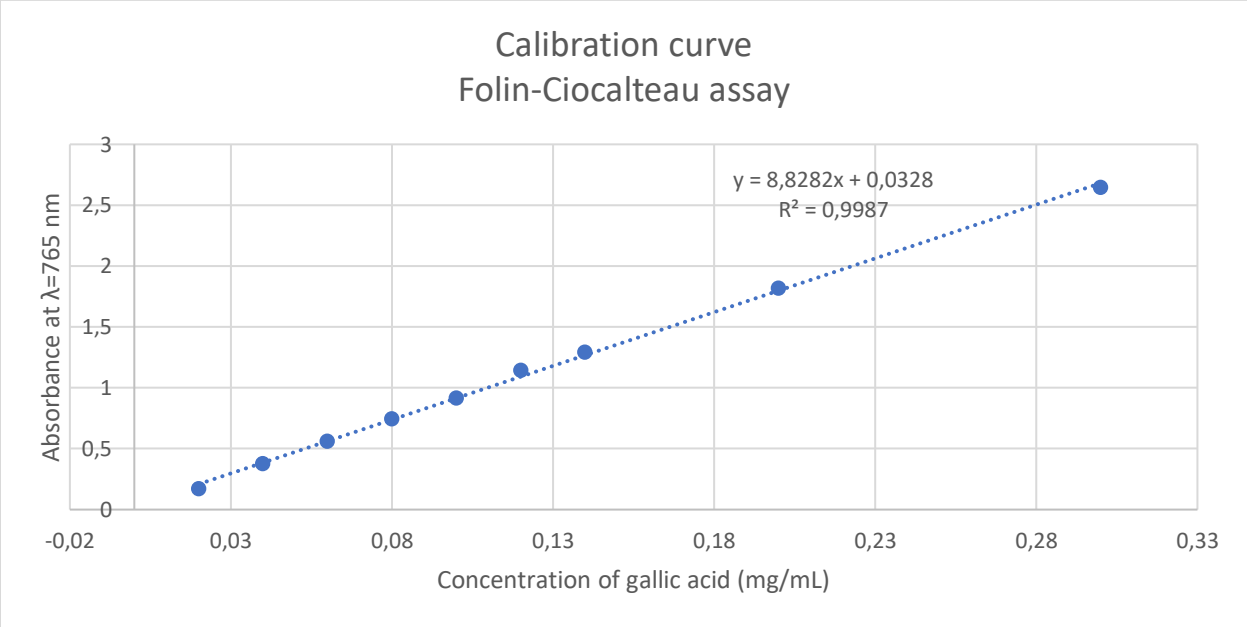


Figure 12: Curve calibration with gallic acid for the determination of the total phenolic content of the extracts of Alternanthera porrigens.

The determination of the total phenolic content of the different extracts of *Alternanthera porrigens* is shown in Table 10. The concentration values were obtained by the equation of the line; which was y = 8,8282x + 0,0328, where y is the absorbance, and x is the concentration. So, if x is cleared, by substitution with the absorbances measured, it can be obtained the concentration of each extract solution in mg of gallic acid per gram of extract (mg of GAE/g extract). Then, it was obtained the highest total phenolic value for the aqueous extract of leaves, with 32,0809 ± 1,4673 mg of GAE/g extract. Next, the highest total phenolic values were obtained for the ethanolic extract of leaves (18,6096 ± 1,2553 mg of GAE/g extract) and the aqueous extract of flowers (14,7331+ 0.83321,2553mg of GAE/g extract).

The results obtained demonstrate that the extracts have phenols in their composition; this information can be corroborated with the one achieved in the qualitative phytochemical screening. The total phenolic content of chloroformic extracts is pretty low. Also, it can be seen in the phytochemical screening that both extract solutions have few or none presence of phenols in their composition according to the colorimetric assays.

Table 9: Total phenolic content of the different extracts of Alternanthera porrigens.

Extract	**mg of GAE/g of extract of *Alternanthera porrigens***	**Dielectric constant of the solvent (at 25℃)**
EHA	32,0809 ± 1,4673	79,0
EHE	18,6096 ± 1,2553	24,5
EAEF	14,7331 ± 0,8332	79,0
EEF	7,4076 ± 0,1171	24,5
EHC	2,1089 ± 0,5112	4,8
ECF	0,1115 ± 0,0733	4,8

Also, it must be considered that the dielectric constant is a quantitative indicator of the polarity of a dissolvent. This constant can measure the relative capacity of a substance to interfere with the attraction between charges of opposite sign. So, while higher is the dielectric constant, more polar is the solvent and, thus, can attract polar compounds. As is presented in Table 10, water and ethanol have a higher dielectric constant, and the result of total phenolic content for the extracts with these solvents is higher too. On the contrary, chloroform has a pretty low dielectric constant, and the result for the total phenolic content for this solvent is quite small also.

According to Jara, (2013) [135], an ethanolic extract of the whole plant of *Alternanthera porrigens* gives as a result 14.0 ± 0.7 mg of GAE/g extract, which result is comparable with the one obtained in this study.

On the other hand, Salvador (2006) [136], analyzed the total phenolic content of the ethanolic extraction of *Alternanthera tenella,* and it was pretty high in comparison with the results obtained in this study, giving as a result 216,36 ± 3.62 mg of GAE/g extract. This fantastic difference between the values obtained could be due to, for *Alternanthera tenella,* the extraction was made first with hexane and then with ethanol. Letting isolated phenols and making measures according to the net weight of the polar part of the extract and not according to the weight of the whole extract as the other plants.

Conferring to Othman (2016) [129], the total phenolic content obtained for the ethanolic extract of *Alternanthera sessilis* red was 35,92 ± 0,70 mg of GAE/g extract and for the aqueous extract was 4,46 ± 0,16 mg of GAE/g extract. On the other hand, the values obtained for the *Alternanthera sessilis* green were smaller, 3,70 ± 0,21 mg of GAE/g extract, and 0,86 ± 0,02 mg of GAE/g extract, respectively. The difference between these results and the ones for *Alternanthera porrigens* is that the total phenolic values in the aqueous extract were higher than for ethanolic extracts. This could be due to the extracts of *Alternanthera sessilis* were lyophilized before the total phenolic content assays. Lyophilization could benefit the quantification of total phenolic content because it has the capacity to maintain pent the compound of the extracts. On the contrary, the extract solutions used in our work were not subjected to any drying, and so, the mixture could be altered with time.

Pereira (2013) [137], obtained 32,7± 0,36 mg of GAE/g extract for the ethanolic extract of leaves of *Alternanthera brasiliana*, a very similar result as for *Alternanthera porrigens* leaves. Both plants have very similar characteristics, such as their flowers and the uses people do of these [99].

In this way, between five different species of the same genus, only one has pretty high values of total phenolic content, the rest has values very similar to the studied plant. *Alternanthera brasiliana* has been considered because of its anti-inflammatory, analgesic, and antimicrobial properties. Due to these properties, Alternanthera brasiliana is known as "penicillin" or "Terramycin" in Brazil [99]. And as it was described, its total phenolic content is not as high as it would be expected as our plant, but anyway, it has been demonstrated that it is a powerful plant.

The phenolic compounds of medicinal plants have several biological effects such as anti-inflammatory properties, antioxidant properties, and play a fundamental role in the prevention of some diseases [138]. The proven presence of phenols in *Alternanthera porrigen*s supports a preliminary analysis of the traditional use that this plant has had in the Andean region for the relief of inflammation.

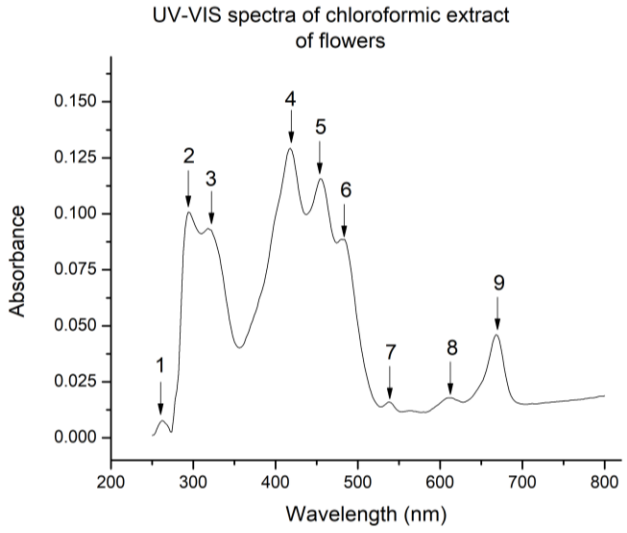
5.4 UV-Vis analysis

UV-Vis analysis allows a qualitative study and identification of metabolites in plant extracts. It is not a very selective technique since each family of metabolite absorbs at a different wavelength. Still, it is challenging to find a wavelength for each specific metabolite; for this, there are libraries

with spectral information [97]. At the time of graphing, the blank measurement must be taken into account, so that this does not influence the metabolite measurements [127].

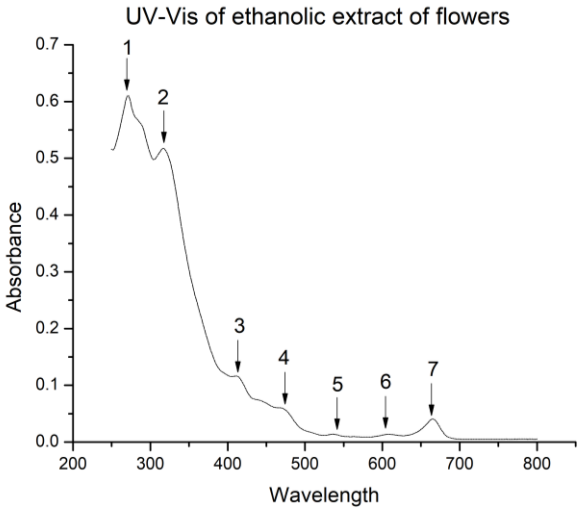
Aromatic molecules are strong chromophores in the UV-VIS range [108]. Pigments such as carotenoids also have characteristic spectra that allow their identification [126]. Likewise, phytoconstituents with π-bonds and σ-bonds can be analyzed by the UV-VIS spectra too.

Once the metabolites have been separated and counted, it is known what kind of them are present in the extracts, which means, what metabolites families exist in the sample. Still, it is unknown the exact molecular formula of each one. In this way, it can be observed the UV-Vis spectra of the different extract solutions of flowers used in this work. For the chloroformic extract of flowers (figure 13), there are present peaks in 262 nm, 294 nm, 320 nm, 418 nm, 454 nm, 484 nm, 538 nm, 612 nm, and in 668 nm. For the ethanolic extract of flowers (figure 14), there are peaks in 272 nm, 318 nm, 410 nm, 536 nm, 564 nm, 609 nm, and 666 nm. And for the aqueous extract of flowers (figure 15), there is only one peak present at 266 nm.



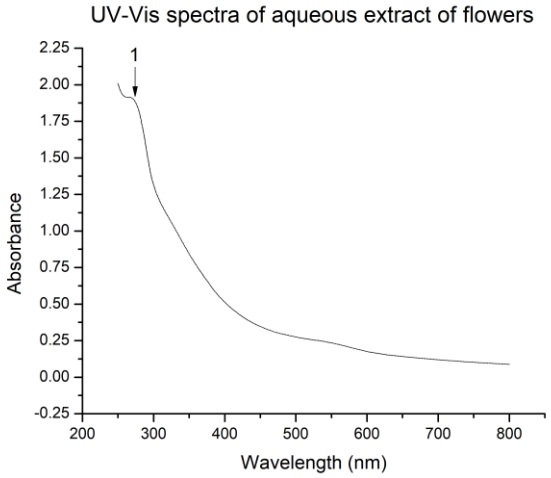
Peak	λ (nm)
1	262
2	294
3	320
4	418
5	454
6	484
7	538
8	612
9	668

Figure 13: UV-Vis spectra of chloroformic extract of flowers of Alternanthera porrigens



Peak	λ (nm)
1	272
2	318
3	410
4	536
5	564
6	609
7	666

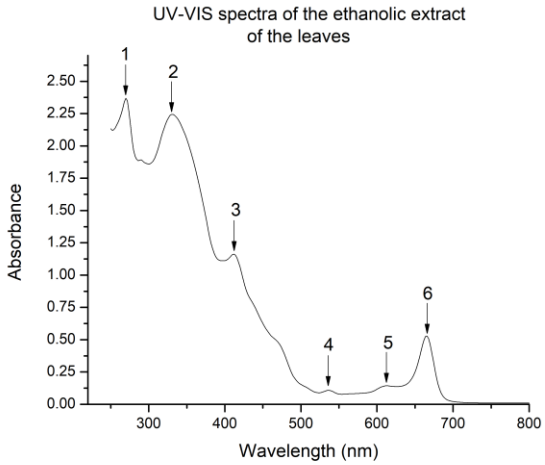
Figure 14: UV-Vis spectra of ethanolic extract of flowers of Alternanthera porrigens.



Peak	λ (nm)
1	266

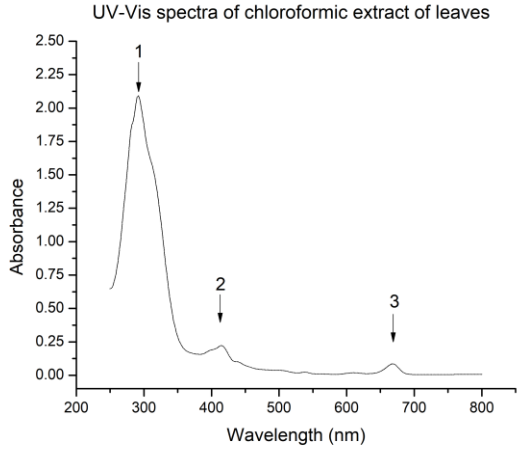
Figure 15: UV-Vis spectra of aqueous extract of flowers of Alternanthera porrigens.

On the other hand, in figure 14 it is observed the UV-Vis spectra for the different extracts of leaves. The ethanolic extract of leaves (Figure 16) presents peaks in 270 nm, 330 nm, 412 nm, 536 nm, 614 nm, and 666 nm. The chloroformic extract of leaves (Figure 17) shows peaks in 292 nm, 414 nm, and 668 nm. And the aqueous extract of leaves (Figure 18) presents two peaks, one in 266 nm and other in 324 nm.



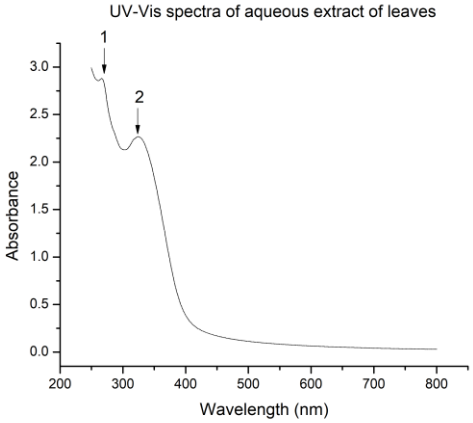
Peak	λ (nm)
1	270
2	330
3	412
4	536
5	614
6	666

Figure 18: UV-Vis spectra of ethanolic extract of leaves of Alternanthera porrigens.



Peak	λ (nm)
1	292
2	414
3	668

Figure 17: UV-Vis spectra of chloroformic extract of leaves of Alternanthera porrigens.



Peak	λ (nm)
1	266
2	324

Figure 16: UV-Vis spectra of aqueous extract of leaves of Alternanthera porrigens.

In Table 7, it is presented the wavelength range for each metabolite family, according to Harborne (1974) [97].

Table 10: Possible secondary metabolites present in the different extracts of Alternanthera porrigens.

Wavelength range (nm)	Secondary metabolite	Family	Leaves			Flowers		
			EHA	EHE	EHC	EEF	ECF	EAEF
200-290	Chalcones and aurones	Flavonoids	X	X		X	X	X
230-290	Simple phenols	Phenols	X	X		X	X	X
240-280	Flavonoids	Phenols	X	X		X	X	X
250-270	Flavonols and betacyanins	Flavonoids and phenols	X				X	X
300-560	Flavonoids	Phenols	X	X		X	X	
315-330	Coumarins	Phenols	X			X	X	
330-350	Flavones	Flavonoids						
370-410	Chalcones	Flavonoids						
400-430	Napthtoquinones	Phenols		X	X	X	X	
430-470	Chlorophylls						X	
475-550	Anthocyanins	Flavonoids		X		X	X	

In this way, it can be affirmed that the chloroformic extract of flowers presents peaks for chalcones and aurenes, simple phenols, flavonoids, flavonols and betacyanins, coumarins, naphthoquinones, chlorophylls, and anthocyanins. In contrast, the ethanolic extract of flowers presents peaks for chalcones and aurenes, simple phenols, flavonoids, coumarins, naphthoquinones, chlorophylls, and anthocyanins. The aqueous extract of the same tissue only shows a peak that can be for chalcones and aurenes, simple phenols, flavonoids or flavonols, and betacyanins, due to all they are in the range of the maximum wavelength peak.

On the other hand, the peaks present for the ethanolic extract of leaves signs the presence of chalcones and aurenes, simple phenols, flavonoids, phenols, flavonoids, naphthoquinones, and anthocyanins. The chloroformic extract of leaves shows the presence of naphthoquinones, and the aqueous extract of leaves presents the existence of chalcones and aurenes, simple phenols, flavonoids, flavonols, and betacyanins phenols and coumarins.

Taking into account that this is the first study of the UV-VIS spectra of the ethanolic, chloroformic and aqueous extracts of *Alternanthera porrigens*, this information, and the one obtained previously and presented in Table 9, it is possible to corroborate the presence of some secondary metabolites in *Alternanthera porrigens*, such as phenols, flavonoids, coumarins, and anthocyanins. Especially

the presence of phenols in all the extracts which demonstrate the results obtained with the total phenolic content. For this, it should be taken into account that the extract solutions were diluted 1:20. Hence, the secondary metabolites are in more concentration that they are perceived in the UV-VIS spectra. Also, it must be mentioned that according to the peaks obtained, it could be present naphthoquinones, chalcones, aurones, chlorophylls, flavonols, and betacyanins in the plant. It must be considered for future analysis.

5.5 Antimicrobial activity

According to literature, it is possible to make a comparison of antimicrobial activity between commercial drugs and plant extracts [139]. But it must be considered the concentration of the extracts and, if possible, its constituents due to the possibility of being a potential antibiotic [140]. If the concentration of the plant is above 100 mg/mL, then its antimicrobial activity is weak, and the plant can not be considered as a potential antibiotic. On the other hand, if the plant concentration is minor than 100 mg/mL, then the plant can be considered as a potential antibiotic against bacteria. So, more studies can be applied to the extracts to determine which compounds are responsible for this effect [99].

In this way, considering the previous results obtained for the extracts of *Alternanthera porrigens*, it was applied a preliminary antimicrobial activity using a modified agar well diffusion method. For this, the extracts were tested against *Escherichia coli* and taking Kanamycin as positive control and DMSO as the negative control. Kanamycin was in a higher concentration than the extracts, it was at 0,5 mg/mL of extract per DMSO, and the extracts were at 10 mg/mL and 25 mg/mL of extract per DMSO. It is said that the kanamycin was more concentrated because it is composed of isolated compounds that work properly against bacteria. On the contrary, the extracts were not fractionated. So, the concentration of these consists of several compounds that can have antimicrobial activity and others that do not necessarily possess this activity. In Figure 19, it is observed the antibiogram with the preliminary results of the antimicrobial activity, at the naked eye, it is found that all the extracts had antimicrobial activity. Still, it is noted that the halo for chloroformic extract of leaves is the biggest of all extracts. Then the ethanolic extract of leaves is the second more significant.

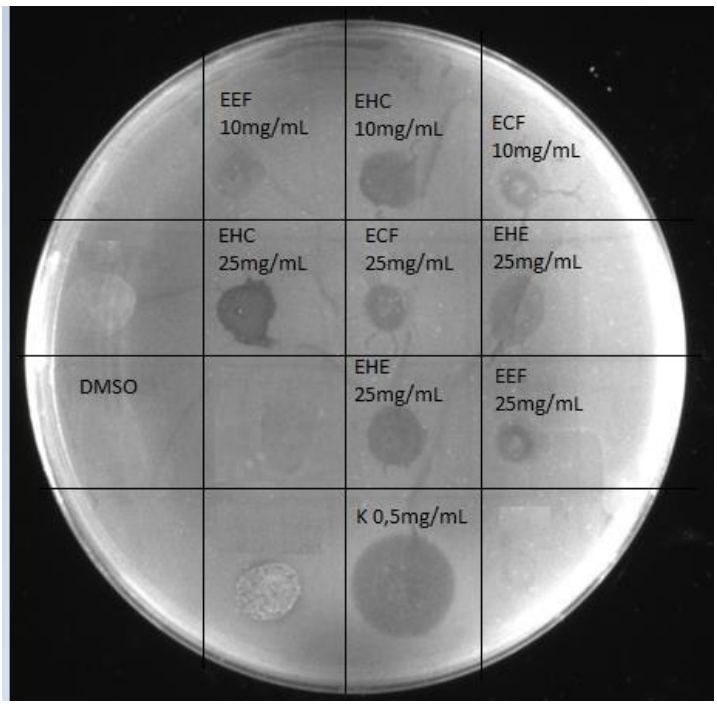


Figure 19: Antibiogram of Escherichia coli *in the presence of the inhibition of different extracts of* Alternanthera porrigens

As it is shown in Table 12, the halo of inhibition of kanamycin was of 13,6 mm. Next, the highest values were obtained for EHE at 10 mg/mL of DMSO with 8,5 mm of the halo of inhibition, then EEF at 10 mg/mL of DMSO with a halo of inhibition of 8,2 mm and EHC at 25 mg/mL of DMSO with 7,5 mm of the halo of inhibition. These represent a moderate antimicrobial activity in comparison with kanamycin. The rest of the extract solutions gave halos of inhibition so small that it represents a deficient antimicrobial activity.

These results obtained for the antimicrobial activity are compatible with the results obtained in the total phenolic content, being EHE and EEF two of the extracts with the highest amounts of phenolic content. The relationship between the amount of total phenolic content and the percentage of inhibition is due to the presence of secondary metabolites as terpenoids and tannins, which are bioactive compounds responsible for antibacterial activity. While higher the amount of total phenolic content in the extract, it is probable that the number of terpenoids and tannins could be higher too, and so the inhibition is more significant than for other extracts that did not show a high amount of total phenolic content.

The mechanism in which terpenoids and tannins work is not established, but the hypothesis is that these secondary metabolites can enter through the cell wall and destroy the cytoplasmatic membrane. This provokes the leakage of internal cell material; the minimum change in the bacteria can affect the cell metabolism and carry the bacteria to its death. This effect increases with the increase of the concentration of the antimicrobial compound [75].

Table 11: Antibacterial effect of kanamycin and ethanolic and chloroformic extract solutions of Alternanthera porrigens

Extract	Concentration (mg/mL of DMSO)	Halo of inhibition (mm)
Ethanolic extract of flowers	10	8,2
	25	4,1
Chloroformic extract of flowers	10	4,8
	25	4,8
Ethanolic extract of leaves	10	8,5
	25	6,8
Chloroformic extract of leaves	10	6,8
	25	7,5
Kanamycin	0,5	13,6

Antimicrobial activity of other species of the genus Alternanthera have been studied and have given positive results against bacteria, between the principal species are, *Alternanthera maritima* [141], *Alternanthera caracasana* [142], *Alternanthera sessilis,* and *Alternanthera philoxeroides* [100]. All of them were tasted with different Gram-positive bacteria and Gram-negative bacteria, between them *Escherichia coli.* On the other hand, *Alternanthera brasiliana* did not present antimicrobial activity [99]. This means an excellent precedent for the testing of *Alternanthera porrigens* with different types of strains due to it would be interesting to study its interaction with Gram-positive bacteria. Because the morphological constitution of Gram-positive bacteria and Gram-negative bacteria is different, principally for their cell wall, while Gram-negative bacteria cell walls are ticker and are composed by a phospholipid membrane that makes the bacteria impermeable in front of antimicrobial components, the Gram-positive bacteria only have a peptidoglycan layer that is not a very good impermeable wall [100]. So, taking into account this, it can be said that the extracts will have potent antimicrobial activity for Gram-positive bacteria.

5.6 Growth inhibition assay

The growth inhibition assay was realized with an *Escherichia coli* strain, and it was analyzed the inhibitory action of dried ethanolic extracts of flowers and leaves and the dried chloroformic extracts of flowers and leaves of *Alternanthera porrigens*. DMSO was used as negative control due to this solvent was used to dilute the dried extracts, and it is recognized due to its lack of antimicrobial activity. So, in the graph, there is a comparison between the strain (which is pure, it means without any extract or solvent that influence in its growth), the DMSO and the studied extracts diluted to a concentration of 1mg/mL of DMSO.

To demonstrate that the extracts can inhibit the bacteria growth, the absorbances obtained for them must be under absorbances for bacteria strain and DMSO. It is not possible to appreciate that the extracts possess a remarkable antimicrobial activity (Figure 20) due to all the curves are almost at the same level, and as it can be seen DMSO curves and extracts curves are touching each other all the time, which means that both have the same pretty low inhibition capacity on *Escherichia coli*. Although this, it is possible to appreciate that the curves for the dried chloroformic extracts of flowers and leaves are a little lower than the ones for DMSO and for dried ethanolic extracts. It could be due to the presence of some secondary metabolites that could be extracted in chloroform and not in ethanol and that can present an antimicrobial activity.

The way growth inhibition can be analyzed by the growth rate, while less is the value for the growth rate (R) then, the extract has more inhibition in the bacteria (Table 13). In this case, the growth rate for Escherichia coli was 0,00526, the R for DMSO was 0,00481, the R for ECF was 0,00481, the R for EHC was 0,00499, the R for EEF was 0,00482, and the R for EHE was 0,00518. In this case, it is affirmed with numbers that the extracts at the concentration used did not have inhibition activity on the growth of the bacteria. As it was mentioned before, it seems that both chloroformic extracts have more antimicrobial activity than the ethanolic ones. It can be proved that the R for the chloroformic extract of flowers is lower than the R for the ethanolic extract of flowers, and the R for the chloroformic extract of leaves is lower than the R for the ethanolic extract of leaves. So, it is corroborated the hypothesis of presence of secondary metabolites responsible of antimicrobial activity in the extraction made in chloroform solvent.

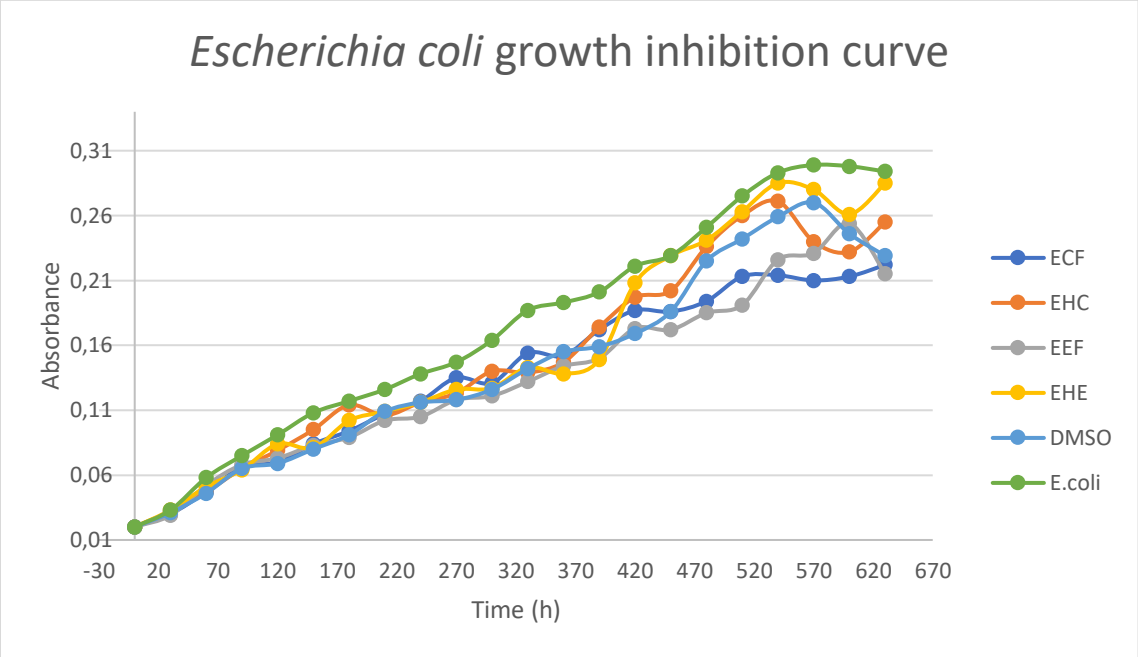


Figure 20: Growth inhibition curve of Escherichia coli *inhibition curve in the presence of different extracts of leaves and flowers of* Alternanthera porrigens.

Table 12: Table of initial and final values of absorbance and growth rate (R) for the bacteria, DMSO, and different extracts used.

Sample	X2 (arb. u.)	X1 (arb. u.)	Growth rate (R)
E. coli	0,033	0,294	0,00526
DMSO	0,031	0,229	0,00481
ECF	0,03	0,222	0,00481
EHC	0,032	0,255	0,00499
EEF	0,029	0,215	0,00482
EHE	0,033	0,285	0,00518

5.7 Anti-inflammatory activity

It was necessary to microencapsulate the extract before the respective assays due to the oily consistency of the extract to determine the anti-inflammatory activity of the dried extract of flowers of *Alternanthera porrigens*. It is diluted in oil because it serves as a vehicle between the polymers and the extract, so it facilitates their interaction

So, it was microencapsulated with gum Arabic and maltodextrin. The yield percentage of the microencapsulated mass of dried extract was 62,39% (Table 10) due to the loss of the powder in the walls of the spray dryer.

Table 13: Quantitative data of the microencapsulation of dried extract of flowers of Alternanthera porrigens.

Microencapsulated	Pre-microencapsulation mass (g)	Proportion	The total mass of microencapsulated	Yield percentage
Gum Arabic	4,660	31,570	9,21g	62,39%
Maltodextrin	5,000	33,870		
Dried extract	0,095	0,646		
Sacha inchi oil	5,000	33,870		
Total	14,760	100,000		

Of microencapsulated material, only 0,64% is the dried extract of flowers; on the contrary, aspirin was used pure, without any additive.

The erythrocyte membrane stabilization method studies the in vitro anti-inflammatory activity of a compound [143]. According to Parvin (2015) [103], it is not known as the mechanism in what compounds stabilize the membrane yet. Still, it is believed that the membrane inhibits the hemolysis induced by the hypotonicity of the erythrocytes membrane because this is contracted. That is because the erythrocyte membrane is similar to the lysosomal membrane, so the pharmacological or extract effect on the stabilization of erythrocyte membranes can also stabilize the lysosomal membrane. The lysosomal membrane stabilization is essential to restrict the inflammatory response by avoiding the release of lysosomal compounds of activated neutrophils, such as proteases and bactericides that are the ones that cause tissue damage and more inflammation [144].

In Figure 21, it is shown the column diagram of the results obtained from the comparison of the percentages of anti-inflammatory activity of the microencapsulated extract and the anti-inflammatory commercial control (aspirin). The sample of microencapsulated extract presented 25,26% as an average of the maximum inhibition percentage at a concentration of 2,5 mg/mL of PBS. On the other hand, the standard of the maximum inhibition percentage for aspirin was 73,75% at the same concentration. It can be observed a considerable difference between both values, due to aspirin is an anti-inflammatory drug, on the other hand, the dried extract was not fractionated, and all its weight is a mixture of a lot of different compounds that can have or not anti-inflammatory activity. Also, it can be observed that the anti-inflammatory activity of the aspirin and the microencapsulated extract increases according to the increase of the concentration.

The secondary metabolites responsible for anti-inflammatory activity are flavonoids and tannins [13], alone, or in combination [103]. Some flavonoids, such as quercetin, are effective in reducing severe inflammations. Other flavonoids have potent inhibitory activity against a lot of enzymes such as phosphodiesterase, phospholipase A2, protein kinase C, Tyrosine kinase proteins [145]. So, as it was observed in the preliminary phytochemical screening, tannins and flavonoids were present in the extracts of flowers of *Alternanthera porrigens*, it can be supposed that these metabolites are the responsible of anti-inflammatory activity in the plant.

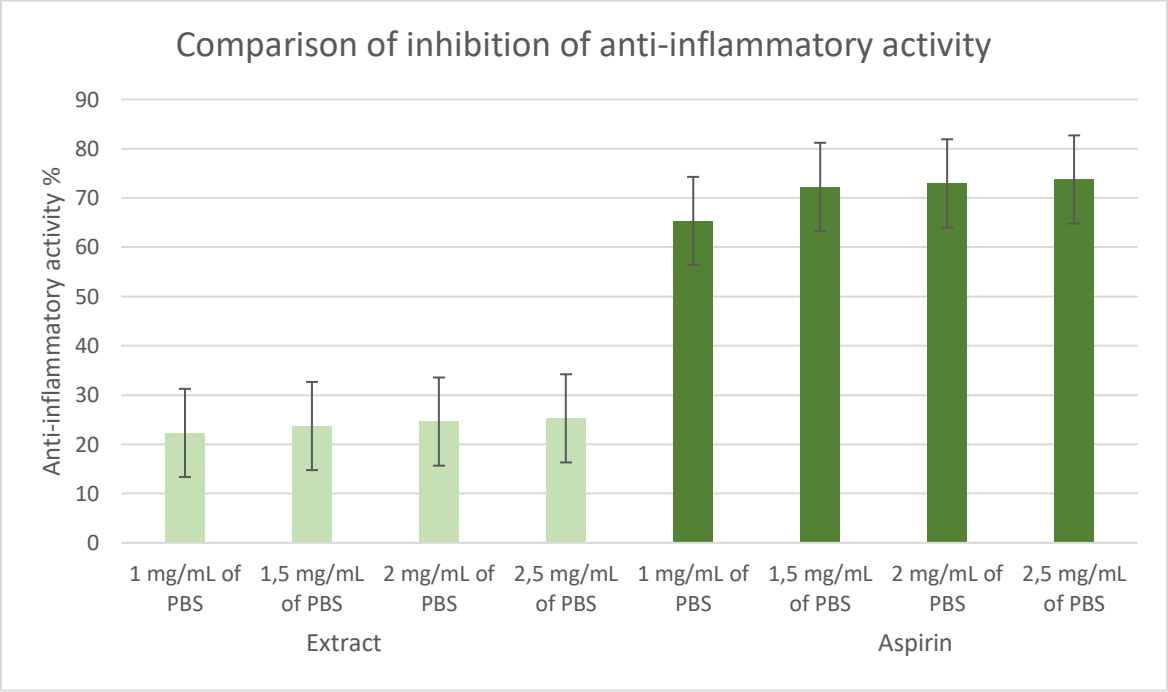


Figure 21: Comparison of the percentage of anti-inflammatory activity between the microencapsulated extract and the aspirin.

The ethanolic extract of leaf of *Alternanthera sessilis* at a concentration of 500 µg/mL gave a result of 73,8% for the maximum inhibition, and ethanolic extract of leaf of *Alternanthera philoxeroides* showed a percentage of 62,2% at the same concentration [104]. It has been reported that *Alternanthera brasiliana* [146], *Alternanthera dentae* [13], *Alternanthera tenella* [13], and *Alternanthera sessilis* [129,147] has anti-inflammatory uses. [13] So, with this information, it can be assumed that most species in the Alternanthera genus can possess this activity. That means that this genus can have a specific phytoconstituent that provokes this effect.

The presence of a preliminary anti-inflammatory activity in the extract solution of *Alternanthera porrigens* and the investigation about other species of the same genus reaffirm the ethnopharmacological use of this plant in Ecuador to treat inflammations [135]. This information can lead to futures researches to find the compound responsible for this effect.

6 Conclusions and recommendations

6.1 Conclusions

- *Alternanthera porrigens* contains phenols, anthocyanins, coumarins, flavonoids, tannins, terpenoids, saponins, steroids and sterols, proteins and amino acids, cardiotonic glycosides, carbohydrates and reducing sugars.
- The highest amount of total phenolic content was obtained in the aqueous extract of leaves of *Alternanthera porrigens* (32,0809 $\pm$ 1,4673 mg of GAE/g of extract).
- UV-VIS spectra confirmed the presence of phenols, flavonoids, coumarins, and anthocyanins in the different extracts of *Alternanthera porrigens*.
- Ethanolic extracts of leaves and flowers of *Alternanthera porrigens* presented a moderated antimicrobial activity against *Escherichia coli*.
- Dried extract of flowers of *Alternanthera porrigens* presents a maximum inhibitory activity of 25,36% in comparison with aspirin.

6.2 Recommendations

- It is essential to consider the solvents to extract the secondary metabolites. It is suggested the use of non-polar solvents first to extract chlorophylls and secondary metabolites that are not in the family of phenols. Then, it must be used a polar solvent to extract all the phenols of the plant.
- It is recommended to dry the extract solutions immediately after the maceration has finished.
- When the total phenolic content takes place, it is crucial to consider the same time of rest for all the samples and the equal amounts of reagents for all the extracts. It is necessary to be precise because these factors can influence the results.
- For the antimicrobial activity, it is crucial to take into account the solvent in which the extract was dissolved so that results can be compared. Also, it must always be used as a pharmaceutical drug in which efficiency is known. It is recommended to repeat the assays in Petri dishes with different extract concentrations. Also, It would satisfy the analysis of antimicrobial activity with other bacteria strains.
- It is recommended the separation of the extracts by liquid chromatography and the characterization of each fraction by thin-layer chromatography, IR, NMR. Then, it would be appealing the evaluation of the antimicrobial, antioxidant, and anti-inflammatory activity for each fraction, due to the phytoconstituents more critical can be found in one specific fraction.

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Annexes

Total phenolic content

Annex 1: Data for the calibration curve with gallic acid for the quantification of the total phenolic content.

Concentration (mg/ml)	Absorbance
0,02	0,17
0,04	0,375
0,06	0,557
0,08	0,745
0,1	0,914
0,12	1,142
0,14	1,291
0,2	1,815
0,3	2,644

UV-Vis spectra

Annex 2: Maximum values of absorption of UV-VIS spectra of the different extract solutions of leaves and flowers of Alternanthera porrigens.

Leaves						**Flowers**					
EHA		**EHE**		**EHC**		**EEF**		**ECF**		**EAEF**	
$\boldsymbol{\lambda(nm)}$	**Abs.**	$\boldsymbol{\lambda(nm)}$	**Abs.**	$\boldsymbol{\lambda(nm)}$	**Abs.**	$\boldsymbol{\lambda(nm)}$	**Abs.**	$\boldsymbol{\lambda(nm)}$	**Abs.**	$\boldsymbol{\lambda(nm)}$	**Abs.**
266	2,8802	270	2,3692	292	2.0906	272	0,6100	262	0,0070	266	1,9135
324	2,2669	330	2,2428	414	0.2214	318	0,5172	294	0,1008		
		412	1,1625	668	0.0855	410	0,1163	320	0,0930		
		536	0,1085			536	0,0137	418	0,1293		
		614	0,1436			564	0,0095	454	0,1156		
		666	0,5285			609	0,0134	484	0,0884		
						666	0,0403	538	0,0160		
								612	0,0179		
								668	0,0461		

Anti-inflammatory activity

Annex 3: Percentage of hemolysis inhibition for the microencapsulated dried extract.

Extract of flowers of *Alternanthera porrigens*	**% of hemolysis inhibition**	**% of hemolysis inhibition**	**% of hemolysis inhibition**	**Average**	**SD**
1 mg/mL of PBS	24,91	21,75	20,27	22,31	2,37
1,5 mg/mL of PBS	28,26	20,69	22,20	23,72	4,00
2 mg/mL of PBS	29,70	21,56	22,58	24,62	4,43
2,5 mg/mL of PBS	28,71	21,08	26,00	25,26	3,87

Annex 4: Percentage of hemolysis inhibition for the aspirin.

Commercial aspirin	**% of hemolysis inhibition**	**% of hemolysis inhibition**	**% of hemolysis inhibition**	**Average**	**SD**
1 mg/mL of PBS	65,70	66,50	63,78	65,33	1,39
1,5 mg/mL of PBS	73,76	73,40	69,61	72,36	2,13
2 mg/mL of PBS	73,15	72,54	73,21	72,97	0,37
2,5 mg/mL of PBS	75,13	73,42	72,71	73,75	1,24