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Escuela de Ciencias Químicas e Ingeniería

TÍTULO: Preliminary phytochemical analysis of the leaves of the *Coriaria Thymifolia* (Shanshi) plant

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

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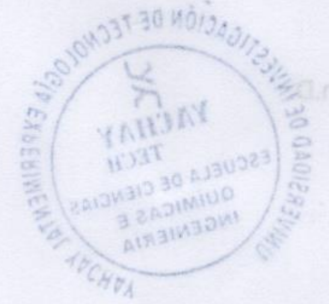

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Dedicatoria

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Resumen

Coriaria Thymifolia (Shanshi) es una planta encontrada en América latina desde México hasta Chile. Esta planta es conocida por tener efectos tóxicos y alucinógenos, dicho efecto es otorgado a un alcaloide llamado *coriarin* presente en las hojas y a un glucósido llamado *coriamyrtine* presente en los frutos. El consumo excesivo de los frutos puede generar efectos no deseados incluso causar la muerte, siendo más peligroso para los niños. *Coriaria Thymifolia* tiene aplicaciones principalmente centradas en sus frutos ya que pueden usarse como tintes, venenos para animales, los pétalos en la fabricación de vinos. También posee aplicaciones medicinales, ya que puede usarse para tratar la diarrea, por su efecto astringente. En la mayoría de la literatura reportada existen estudios centrados específicamente en los frutos de esta planta más no en las hojas. El objetivo de esta investigación es presentar un estudio del extracto de las hojas de *Coriaria Thymifolia*, la cual estuvo centrada en la evaluación fitoquímica de los metabolitos secundarios, mediante técnicas cualitativas y cuantitativas. Se probaron diferentes test cualitativos en el extracto etanólico de *Coriaria Thymifolia*, encontrando taninos, flavonoides, glucósidos y esteroides. El estudio fitoquímico estuvo acompañado por un análisis espectroscópico UV-VIS tomado en un rango de longitud de onda de 250-900 nm. Se utilizó el sistema UPLC-MS junto con el software *Progenesis QI* para el estudio metabolómico y pudimos identificar algunos compuestos utilizando bases de datos masivas. El exitoso protocolo casero de blanqueo de β-caroteno fue desarrollado para evaluar las propiedades antioxidantes de los extractos obtenidos. Hemos descubierto que uno de los extractos posee propiedades antioxidantes más fuertes que nuestro control positivo (vitamina C). Finalmente, se realizó un estudio antibacteriano preliminar y una de las fracciones mostró cierta actividad contra la bacteria DH5-alfa *Escherichia coli*.

Palabras Clave:

Coriaria thymifolia, Shanshi, antioxidante, β -caroteno, antibacteriano, metabolómica.

Abstract

Coriaria thymifolia (Shanshi) is a plant found in Latin America from Mexico to Chile. This plant is known to have toxic and hallucinogenic effects; this effect is attributed to an alkaloid called *coriarin* present in the leaves and a glycoside called *coriamyrtine* present in the fruits. Excessive consumption of the fruits can generate unwanted effects even cause death, being more dangerous for children. *Coriaria thymifolia* has applications mainly focused on its fruits since they can be used as dyes, animal poisons, petals in winemaking. It also has medicinal applications, since it can be used to treat diarrhea, due to its astringent effect. In most of the reported literature, there are studies focused specifically on the fruits of this plant but not on the leaves. The objective of this work is to obtain, separate by chromatography and analyze the ethanolic extract of the leaves of *Coriaria thymifolia,* focusing on the phytochemical evaluation of the secondary metabolites, using qualitative and quantitative techniques. Different qualitative tests were tested on the ethanol extract of *Coriaria thymifolia*, finding tannins, flavonoids, glycosides, and steroids. The phytochemical study was accompanied by a UV-VIS spectroscopic analysis taken in a wavelength range of 250-900 nm. UPLC-MS system together with software *Progenesis* Q1 for metabolomic study were used and we could identify some compound using mass databases. Successful homemade β-carotene bleaching protocol was developed for evaluation antioxidant properties of obtained extracts. We have found that one of the extract possess stronger antioxidant properties than our positive control (Vitamin C). Finally, some preliminary antibacterial study was carried out and one of the fraction have shown some activity against DH5-alpha *Escherichia coli* bacteria.

Key Words:

Coriaria thymifolia, Shanshi, antioxidant, β -carotene, antibacterial, metabolomics.

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CHAPTER 1

1. INTRODUCTION-JUSTIFICATION

1.1. BACKGROUND OF *CORYARIA THYMIFOLIA*

The *Coriaria thymifolia* plant grows on the slopes of the Imbabura Volcano, and become somehow famous in local community after some people consumed the *shanshi*, confusing it with the fruits of the mortiño. This plant to be hallucinogenic can cause severe toxic effects when ingested, produces emotional disturbances and causing fatal poisonings [1, 2].

No medical uses have been reported or studied for any species of *Coriaria*, however, in some countries people use them taking advantage from the toxic properties or as a dyer. In Southern Chile, the fruits of *Coriaria ruscifolia* (another plant from the same family) are used as rodent poisons [3]. While in Mexico they are used as a poison for dogs and cats [4]. The characteristic dark purple color of the fruits in these species provides an intense stain when crushed by hand. That is why the sap is used as a dye, for example, *Coriaria thymifolia* seeds can be used as a colorant to dye the threads for handicrafts [5]. Besides, in Ecuador and other South American countries black ink is used for writing, although the color is transitory and loses its intensity [6].

According to Chicaiza *et al.* (2014) the fruits of *shanshi* are also made up of saponins, so they are used as soaps. *Shanshi* leaves have tannins so they can be used to tan skins. Having astringent properties in some regions they are used to treat diarrhea [7].

On the other hand, there are also applications focused on the fleshy petals of certain species since these can be used for the manufacture of wine [4]. For example, in New Zealand a pleasant drink is produced from the petals that are believed to be non-poisonous unlike fruits [6]. Finally, *Coriaria* extracts have been used in traditional Chinese medicine, with the purpose of treating mental diseases, however, in some cases the side effect were seizures [8].

The fruit of *Coriaria thymifolia* sometimes is used as a recreational drug, whose effects after 30 minutes of being consumed cause: a sensation of flight. However, usually followed by less pleasant experiences as vomiting, diarrhea, abdominal pain, dizziness, seizures, increased respiratory activity and heart rate, and in more severe cases, can cause

poisoning and death from asphyxiation or cardiac arrest [2, 4, 5]. Therefore, this plant is dangerous for people especially for children, since its effect is found to be stronger in young children. There are reports of children intoxicated by eating the fruits of the *Coriaria* species, their fruits can be easily consumed by mistake since their taste is pleasant and their appearance is very attractive [4]. For example, in Ecuador, there are reports of the death of two children in the community of Quialó, located in the province of Cotopaxi [5].

1.2. BOTANICAL CHARACTERIZATION OF *CORIARIA THYMIFOLIA*

1.2.1. Taxonomic classification

Kingdom: Plantae

Clade: Angiosperms

Clade: Eudicots

Clade: Rosidae

Order: Cucurbitals

Family: Coriariaceae

Genus: Coriaria

Specie: C.thymifolia

Scientific name : *Coriaria thymifolia*.

Common names : ECUADOR: "shanshi", "sanchi or zhanzhi" "tinta", "piñan". COLOMBIA: "teñidera". MEXICO: "helecho de tierra", "tlalocopetate". PERU: "mio-mio", "saca-saca","mio-venenosa" [9].

1.2.2. Characteristics of the *Coriariaceae* family

The *Coriariceae* family contains a single genus called *Coriaria*. It is known to be a very isolated group of dicotyledonous plants since its floral structure is very different from any other family, that is, it does not share morphological characteristics. That is why

several authors recognize the *Coriariceae* as anomalous, being difficult to define their position in a natural classification [4 6 9].

The species belonging to the genus *Coriaria* are woody plants that differ in size from shrubs to trees. They have angular branches that give rise to several lateral and elongated branches similar to a fern. The leaves, in general, are characterized by being small and pointed, with a variety of shapes from elliptical to roped, with slap nerves [10]. They have inflorescences in terminal clusters, with small reddish-green or yellow flowers, they are hermaphroditic, monoecious. The fruits are dark purple and contain thickened and fleshy petals forming a pseudodrupa. The roots are fibrous and have nitrogen-fixing nodules [4, 10, 11].

According to Wang (2016) in the *Coriaricea* family, it has been found that Sesquiterpene lactones are the main bioactive components. And the plants belonging to the genus *Coriaria* are rich in tannins and flavonoids are present in the leaves [6, 12].

An important point to consider is the discontinuity of *Coriaria* from a geographical point of view. More than 15 species distributed in different regions are known, that is, *Coriaria* is the only genus that occurs in very separate parts of the world. They are found in the Mediterranean region: in Asia from the Himalayas, in southern China, Japan reaching the Philippines. In the Pacific region: from New Guinea to New Zealand. In America: meeting from Mexico passing Central America to Chile [6, 9, 4]. In Ecuador, the *Coriaria* genus is represented by a single species known as *Coriaria thymifolia* [1].

Within the recognized species of *Coriaria*, there are both morphological and distribution variations. The species of the southern hemisphere and America share certain characteristics among them but are different from the species of Eurasia. In this group, for example, four species of America are similar to *Coriaria thymifolia* from New Zealand. The difference between the species is due to the different behavior in the flowering of the southern hemisphere with those of the northern hemisphere. In addition, there are differences in the structure of the inflorescence [6].

1.2.3. Description of the species *Coriaria Thymifolia* (*shanshi*)

Coriaria thymifolia, known as *shanshi* in Ecuador region, is an Andean shrub recognized by its hallucinogen and intoxicating properties. *Shanshi* is one of the 15

species of *Coriaria* [13]. In the interandina region the shanshi develops in arid regions on the slopes, from 2500 to 3500 meters above sea level. In Ecuador it is mainly distributed in the Sierra region between 1000 and 4500 m of altitude. It is also found in streams and rivers, for example, Minga and Vergudo have observed this plant on the banks of the rivers of Cuenca [14]. This plant can be nominated as a tree or shrub since its maximum height is 4 meters. The structure of the plant is characterized by its long branches, reddish-brown quadrangular stems with purple berries covered with fleshy petals [13]. This plant blooms in the month of April, whose flowers are purple grouped in lateral clusters of 6 to 15 cm in length. The stems are provided with elongated branches of 1 m or more in length, from each branch several short lateral twigs are detached, with abundant foliage of small leaves forming a robust bush. The leaves are dark green and are distinguished by being thin and small with an elliptical shape. And the characteristic seeds of this plant are black [5].

1.3. PHYTOCHEMISTRY

1.3.1. Chemical Background of *Coriaria thymifolia* and other species

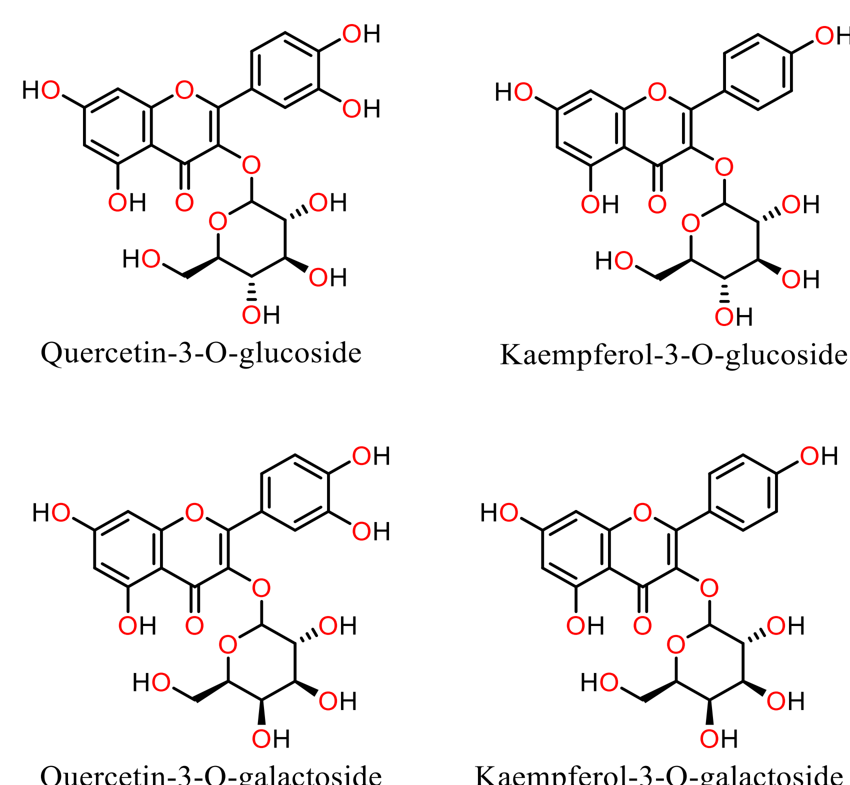
The toxicological properties of *Coriaria thymifolia* are mainly attributed to a specific but undetermined glycoside [13]. However, in a study to analyze the chemical content of the different parts of the *Coriaria thymifolia* plant, an alkaloid called coriarin was found in the leaves, and a sesquiterpenic glycoside called coriamyrtine in the fruits (Figure 1) [2, 7]. Coriamyrtine, having a mechanism of action similar to the alkaloid strychnine, alters the central nervous system causing seizures. In comparative investigations of *Coriaria* species, studies of *shanshi* extracts focused specifically on seeds and fruits were performed, where the fruits were found to be more toxic than the seeds of this plant. These results reported in this species indicated that coriarin and coriamyrtine are also responsible for the neurotoxicity of the berries of *Coriaria myrtifolia* [2]. In addition, the structural study of *Coriaria thymifolia* specimens realized by Bohm *et al.* (1981) has showed a high presence of flavonoids, is to say this plant could have compounds that belong to the group of phenolic compounds [12]. To be more specific, species of kaempferol and quercetin monoglycosides were found, same as a smaller amount of diglycosidic material. In the case of the monoglycoside part, it was determined the existence of 3-O-glucosides, 3-O-galactosides, 3-O-arabinosides, 3-O-xylosides, and 3-O-rhamnoside for

both, kaempferol and quercetin (Figure 2). On the other hand, for the diglycosidic part, kaempferol and quercetin-3-O-rutinoside were found (Figure 3). In the same way, it was determined in the diglycoside fraction, a small amount of two compounds that yielded kaempferol or quercetin and equal amounts of glucose and xylose but the nature of this diglycoside could not be determined [12].

CORIAMYRTINE CORIARIN

Figure 1. Structure of possible compounds responsible of toxic effects of Coriaria Thymifolia.

Monoglycoside part:



Quercetin-3-O-arabinoside

Kaempferol-3-O-arabinoside

Quercetin-3-O-xyloside

Kaempferol-3-O-xyloside

Quercetin-3-O-rhamnoside

Kaempferol-3-O-rhamnoside

Figure 2.Possible structures of the monoglycoside fraction present in Coriaria Thymifolia.

Diglycoside part:

Quercetin-3-O-rutinoside

Kaempferol

Figure 3. Possible structures of the diglycoside fraction present in Coriaria thymifolia.

1.3.2. Phytogeography

1.3.2.1. Geographical distribution of the genus Coriaria and the species *Coriaria thymifolia*

The species of the genus *Coriaria*, unique in the family *Coriariaceae*, are found in the mountains from Mexico to Chile on the American continent; also from the Mediterranean area to the east to Japan; in southern Europe, northern Africa, the Philippines, New Guinea and New Zealand [13, 2]. The *Coriaria thymifolia* species is the only one present in Ecuador, widely distributed in the Sierra region. Also, it is present on the slopes of Mexico, Guatemala and Panama and on the western slopes of the Andes in Colombia, Venezuela and Peru [9].

Figure 4. Geographical distribution of the Genus Coriaria.

Figure 5. Geographical distribution of species Coriaria thymifolia.

1.3.3. SECONDARY METABOLITES

The secondary metabolites are derived from the precursors synthesized in the primary metabolism; they are considered as waste products because their functions are not essential for the survival of the plant. Despite this, secondary metabolites are currently compounds with a great research interest, due to their properties in medicine, pharmacology, food, agronomy, textile industry, cosmetics, etc [15]. For example, in the medical field, molecules with biological functions such as antibiotics, fungicides, antioxidants among others have been identified. Being a key point to generate active ingredients and thus manufacture phytopharmaceuticals, as is the case with paclitaxel [16]. Due to its applications, secondary metabolites are also known as natural products, which were already used in ancient medicine to cure some diseases [17].

The production of secondary metabolites occurs in very low concentrations in most plants, and they are distributed in different parts of them [18]. Secondary metabolism compounds fulfill several functions, they are attractants or insect repellents, they are also pigments since they give color to flowers and fruits attracting insects for pollination. Other compounds act as protectors against predators, giving the plant bitter tastes or poisonous characteristics, and also protect the plant from pathogens since they act as natural fungicides [17].

1.3.3.1. Phenolic Compounds

Phenolic compounds are aromatic metabolites that have one or more hydroxyl substituents attached to their ring. They are synthesized from two routes: shikimic acid and malonic acid. These compounds have anti-inflammatory, antiseptic, antioxidant and pesticide properties [18, 15]. Phenolic compounds are classified in flavonoid compounds and non-flavonoid compounds [19].

Flavonoid compounds

These compounds are known for their pigment characteristics, being responsible for the colors of flowers, fruits and sometimes of the leaves of plants. They are a group that constitutes the low molecular weight phenolic compounds [20, 21]. Its structure consists of a skeleton of 15 carbon atoms (C6-C3-C6) distributed in two aromatic rings linked by a three-carbon bridge [17]. Depending on the degree of oxidation of the three carbons,

flavonoids are classified as: anthocyanins (cyanidine, malvidin), flavones, flavonols (quercetin, kaempferol, myricetin), flavanones, flavanols or catechins and chalcones [19].

Flavonoids are synthesized in plants from the amino acids phenylalanine and tyrosine and are found in plant vacuoles. In plants its function is to protect the leaves from ultraviolet radiation, in addition to attracting pollinators, to help germinate the seeds [21, 15].

Flavonoid compounds have several biochemical and antioxidant activities that help to reduce or counteract the risk of various diseases such as cancer, cardiovascular disease, Alzheimer's disease, atherosclerosis, etc. They have several applications in the pharmaceutical industry due to their antioxidant, anti-inflammatory, antibacterial effects, antimutagenic and also modulate the key functions of cellular enzymes [21].

Figure 6. General structure of flavonoids.

<u>Non-flavonoid compounds</u>

Within this group are phenolic acids with C6-C1 carbonated structure, hydroxycinnamic acids with C6-C3 structure and C6-C2-C6 stilbenes. The group of non-flavonoid compounds also includes esters of gallic acid, and hydrolysable tannins [19].

1.3.3.2. Carotenoids

Carotenoids are very important isoprenoid pigment of plants, they are responsible for the orange, and yellow color of the leaves, flowers, and fruits of plants. For example, the orange coloring in carrots (*Daucus carota L.*) is due to the presence of carotenoids, as are the red colors of peppers and tomatoes [22]. They have antioxidant, coloring and provitamin properties. Carotenoid consumption is beneficial for protection against macular degeneration, damage caused by UV rays and reducing the risk of some degenerative diseases such as cancer and cardiovascular diseases [23, 24].

The structure of the carotenoids is a tetraterpene, formed by the union of 8 isoprene units, giving rise to a skeleton of 40 carbon atoms with conjugated double bonds. They may have acyclic structures such as lycopene or cyclic structures at one or both ends such as β-carotene [25]. The most stable form is the trans conformation, however, carotenoids can change to their cis form due to isomerization processes. Isomerization takes place due to the degradation of carotenoids caused by exposure to light, oxygen, temperature, and presence of acids [26, 24]. They are prone to oxidation due to the presence of conjugated double bonds in their structure. Isomerization reactions take place due to heat treatments, for example, β-carotene should not have a temperature greater than 40 ℃, or due to acid treatment [25, 26].

Figure 7. beta-Carotene structure.

1.3.3.3. Alkaloids

Alkaloids constitute one of the largest groups of secondary metabolites present in plants. These act as defense compounds in plants, their toxicity helps defend against pathogens and predators. The signals caused by aggressors or unfavorable environmental conditions trigger a specific response for the production of alkaloids, being a key to plant protection. These metabolites are soluble in water, contain at least one nitrogen atom, have biological activity and most are heterocycles [15, 27].

Alkaloids have already been used throughout history for medicine, therapeutic, recreational and religious purpose. Its use has been known since ancient times due to the ability of some of these molecules to modulate the central nervous system. At present, alkaloids are ingested to favor some immune functions, nutrition, and physical performance. For example, gingerol, phenolic alkaloid, has antioxidant and antitumor activities. Besides, they participate in the autonomic nervous system being sympathomimetic, that is, they cause adrenaline effects [15].

The toxic effects of alkaloids are related to dose, exposure time and individual characteristics, such as sensitivity. Therefore, toxicity can be both harmful and beneficial, depending on the pharmacological context. At high doses, they are very toxic, but at low doses, they are therapeutic as muscle relaxants, tranquilizers, and analgesics [17].

1.3.3.4. Glycosides

Glycosides are an important class of secondary metabolites, their name is due to the glycosidic bond that is formed by the condensation of a sugar molecule with another that contains a hydroxyl group [17]. Within this group are: phenolic glycosides (are urinary antiseptics), flavonoid glycosides (are antioxidants), anthraquinone glycosides (have laxative effects), saponin glycosides (belong to steroid glycosides with antitumor properties) and cardic glycosides (used in diseases cardiac) [28, 15].

1.4. BACKGROUND EXTRACTION AND PURIFICATION

1.4.1. Thin layer chromatography (TLC)

This is a qualitative chromatographic technique that allows determining the degree of purity of a compound, allowing to know what compounds are present in a mixture to be analyzed. This chromatography consists of a stationary phase and a mobile phase. Silica gel can be used for the stationary phase, while the mobile or eluent phase contains one or more solvents of different polarity, which will depend on the compound to be analyzed. The mixture is deposited on the surface of the layer and this is placed in the chromatographic chamber, there the most apolar compounds will be dragged to the top of the plate leaving the most polar ones at the bottom. Once the compounds are separated, an analysis is performed by UV light or by developers, these can be vanillin, anisaldehyde and $KMnO_4$ [15, 29].

1.4.2. Column chromatography

Column chromatography is widely used for the separation and purification of solid and liquid compounds. The stationary phase is a solid adsorbent, generally Silica gel is used, while the mobile phase is a mixture of eluents that are allowed to flow through the column either by gravity or by external pressure. The mixture to be analyzed is dropped from the

top of the column and with the help of the mobile phase the compounds descend through the column. The less polar fractions that are weakly retained or almost nothing in the adsorbent, are the first to leave the column. On the other hand, the more polar compounds are retained in the adsorbent, which is why it is necessary to increase the polarity of the eluents according to the need for analysis. When the fractions are separated, it is evaluated which compound is present in each of the fractions by means of TLC [30].

For the laboratory scale, the column should generally be of a diameter of 5 mm to 30 mm and a height ranging from 5 cm to 30 cm.

2. PROBLEM STATEMENT

The *Coriaria thymifolia* plant is a species of which there is little information on both the chemical composition and the structural and bioactive properties. Most of the studies have been focused on investigations of the fruits of *Shashi*, which is why this work seeks to evaluate the content of secondary metabolites present in the leaf extract. Besides, to characterize and determine if the compounds have antioxidant activity and antibacterial activity that may have future applications.

3. OBJECTIVES

3.1. General Objectives

Characterize extracts of *Coriaria thymifolia* leaves using qualitative and quantitative techniques, and evaluate their biological activities.

3.2. Specific Objectives

- Determine the presence of secondary metabolites in the ethanolic extract of the *Coriaria thymifolia* leaves through qualitative methods.
- To evaluate the phytoconstituents of *Coriaria thymifolia* by UV-VIS spectroscopic analysis.
- Characterize the extracts of *Coriaria thymifolia* using chromatographic techniques TLC, HPLC, and UPLC-MS.
- Establish the methodology for compound detections and identification using UPLC-MS and Progenesis IQ software.
- Evaluate the antioxidant property of leaf extracts using the carotene bleaching method.
- Test antibacterial activity using the optical density technique in extracts of *Coriaria thymifolia.*

CHAPTER 2

4. RESULTS AND DISCUSSION

4.1. Solid-liquid extractions of *Coriaria thymifolia* leafs

The solid-liquid extraction was performed two times using slightly different conditions (Figure 8). The extraction 1 corresponds to the extraction obtained by Ultrasound, from which were obtained twelve fractions named as E1C1F1, E1C1F2, E1C1F3, E1C1F4, E1C1F5, E1C1F6, E1C1F7, E1C1F8, E1C1F9, E1C1F10, E1C1F11, and E1C1F12; Some of these fractions were analyzed by qualitative techniques. While extraction 2 obtained through the methodology by ultrasonic bath, it was purified and from this was obtained three fractions denominated as E2C1F1, E2C1F2, and E2C1F3, these were simplified and driven by qualitative and quantitative techniques, and antioxidant and antibacterial activity. Also, from the three fractions mentioned above, one was purified again specifically the fraction E2C1F2. Therefore, a second column (column 2) was carried out, from which results in six fractions named as E2C2F1, E2C2F2, E2C2F3, E2C2F4, E2C2F5, and E2C2F6, same that were study by its antioxidant power.

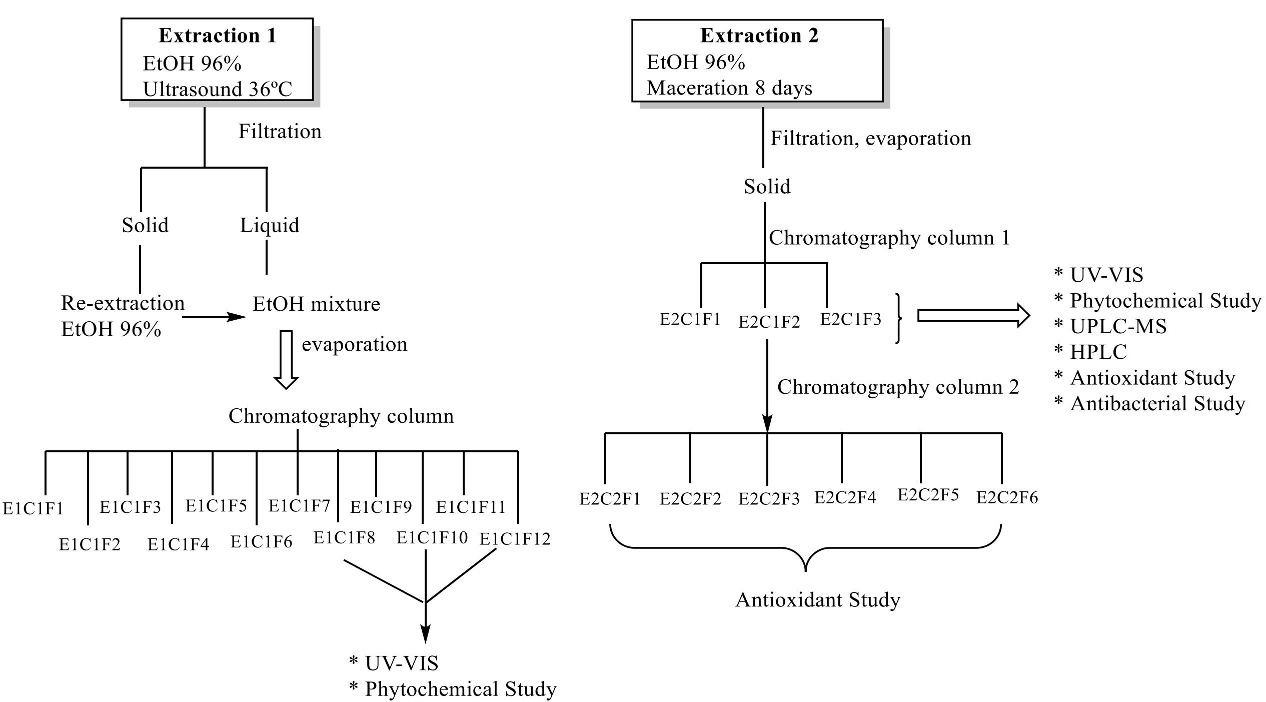


Figure 8.Overview of extractions of Coriaria thymifolia and analysis applied in them.

4.2. Chemical characterization of extracts and fractions of *Coriaria thymifolia* by thin layer chromatography (TLC) and column chromatography (CC)

The first step to characterize the crude extracts of *Coriaria thymifolia*, obtained both by the maceration method and by the ultrasound assisted method, was thin layer chromatography. For this analysis, a mixture of ethyl acetate: petroleum ether (1: 1) v / v was used, which gave an adequate separation to be able to continue with the next step which was the purification of each of the extracts. In the thin layer of Figure 9 the separation of three compounds from the sample (in this case the crude extract) marked on the baseline is observed, however, the initial sample is still retained, which indicates that a more polar solvent system is needed to completely separate the sample. The visualization of the spots was achieved by UV light 254 nm and UV light 366 nm.

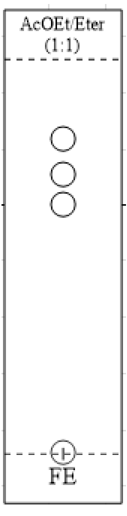


Figure 9. TLC of raw extracts of Coriaria thymifolia; FE= Ethanolic crude extract.

<u>Chromatographic column of the ultrasonic extract (Extraction 1)</u>

From the purification of the ultrasound-irradiated extract, 63 samples of approximately 8 ml each were collected in test tubes. The samples obtained were analyzed by TLC, which allowed determining the number of fractions resulting from this extract, having 12 fractions (Figure 10). Anisaldehyde and Vanillin solution were used in the chromatographic plates as staining where separate spots of various colors are seen, the green color indicates the presence of chlorophylls in the extract, while the pattern and

color of the rest of the bands varies in a range of blue, gray and violet, which can be some kind of secondary metabolite.

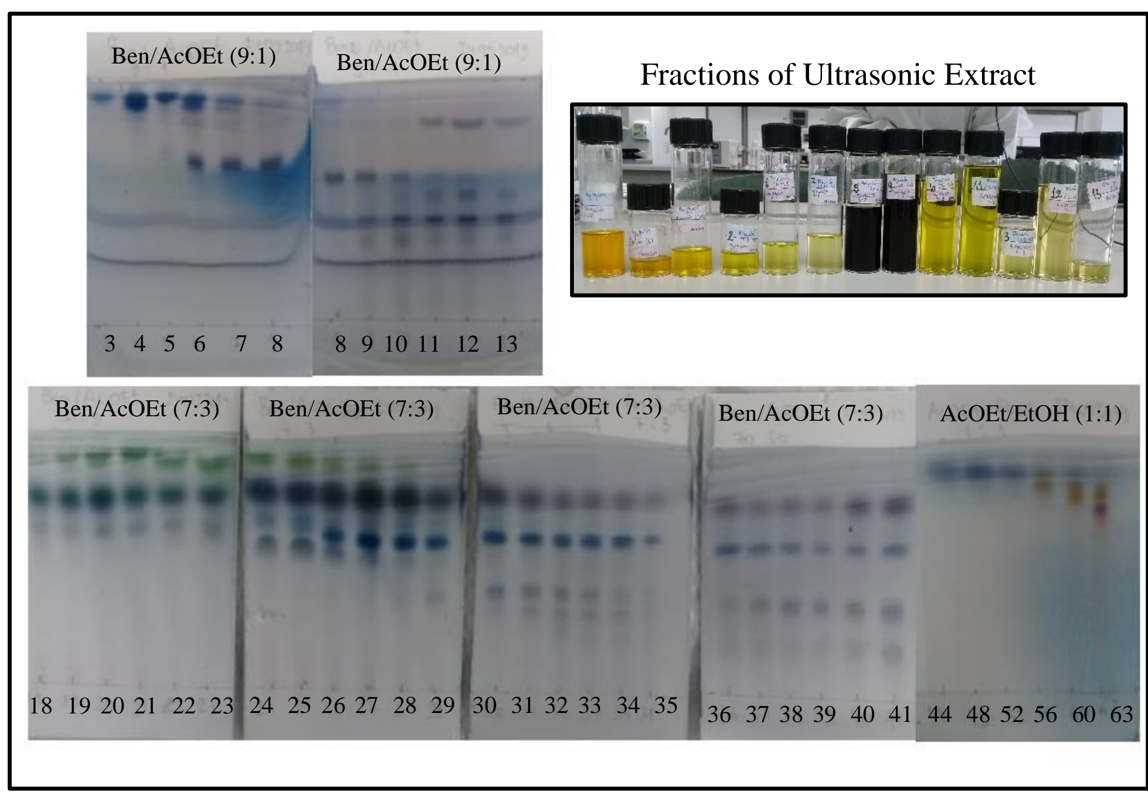


Figure 10. TLC of samples obtained from the purification of the Ultrasonic extract.

Chromatographic column of the Macerated extract (Extract 2; Column 1)

From this extract, a total of 60 test tubes were collected with approximately 8 mL sample in each. TLC of the samples obtained was made to join the samples in fractions, thus, three fractions E2C1F1, E2C1F2, and E2C1F3 were obtained. Figure 11 shows the eluent systems used in each sample, TLCs were visualized by UV light 254 nm and UV light 366 nm. According to TLC results, it was determined that the fractions 1-16 were joined to give Fraction E2C1F1; 17-46 to give E2C1F2 and Fraction E2C1F3 of 47-60.

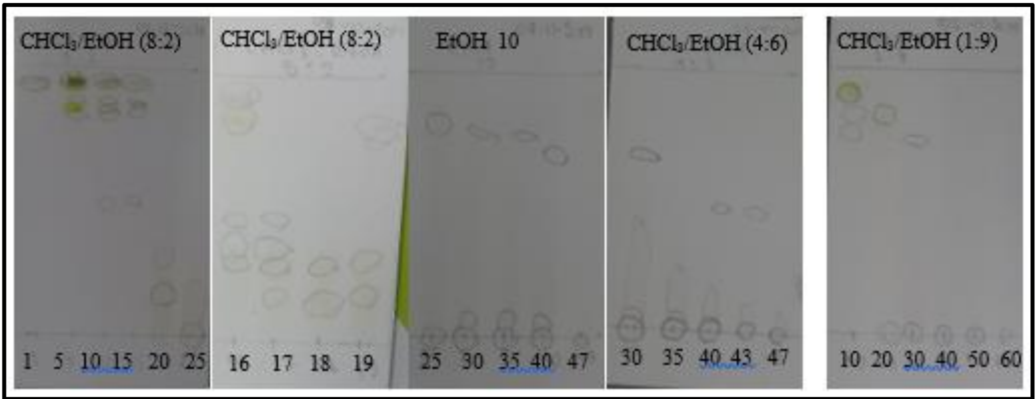


Figure 11. TLC of samples obtained from the purification of the Macerated extract (E2).

A new thin layer was performed with fractions E2C1F1, E2C1F2, and E2C1F3 (Figure 12). The plates show that the initial spots of the fractions are retained at the baseline because the fractions must be made up of polar compounds, which is difficult to move. To better separate the compounds it is necessary to test other eluent systems, however, the repurification of the E2C1F2 fraction was performed taking these results into account. TLCs were visualized by UV light 254 nm and UV light 366 nm.

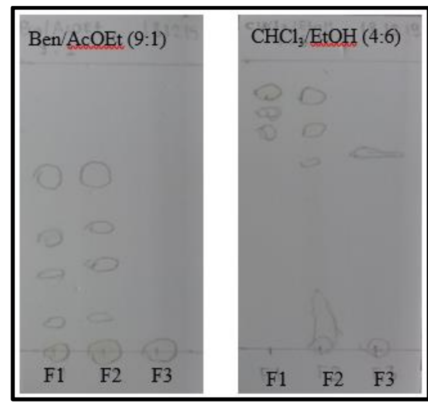


Figure 12. TLC of fractions obtained from the purification of the Macerated extract.

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Re-purification of Macerated extract (Column 2)

Due to a positive antioxidant test of fraction E2C1F2, it was purified by CC. Before carrying out the fractionation, the separation of this sample was evaluated using different solvents, of which a better separation with chloroform: ethanol was obtained in the proportion of (6: 4) (Figure 13). The rest of the fractions were saved for quantitative analysis, as is the case of UPLC-MS.

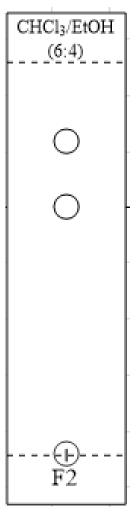

Figure 13. TLC of Fraction E2C1F2.

From this CC 37 test tubes were collected, then, from an analysis by thin layer chromatography of each of the samples (Figure 14), six fractions E2C2F1, E2C2F2, E2C2F3, E2C2F4, E2C2F5, and E2C2F6 were found. TLC analysis was performed with reference to the standard sample of the E2C1F2 (sample pattern) fraction obtained in column 1 and the plates were visuaized using Anysaldehyde stainig and UV light.

Figure 14. TLC of samples obtained from the purification of the Fraction E2C1F2; P=pattern that is the fraction E2C1F2.

Finally, of the six fractions a TLC was made using as chloroform mobile phase: ethanol (7: 3) v / v, but the resulting separation of the compounds was not as good since it is seen that the fractions have still retained compounds (Figure 15). Plates being revealed with anisaldehyde in fraction E2C2F4, spots of different colors are visible, however, by means of this technique, the identity of the present compounds cannot be determined, so the application of additional techniques is required for a better result.

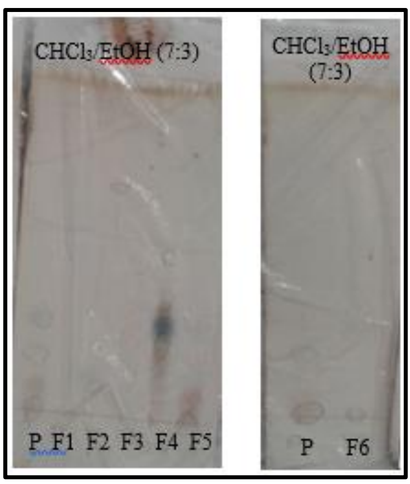

Figure 15. TLC of fractions obtained from the purification of the Fraction E2C1F2

4.3. Qualitative phytochemical screening of the leaves of *Coriaria thymifolia*

The preliminary phytochemical study of the both ethanolic extracts of the leaves of Coriaria thymifolia (Ultrasound and Macerated extracts) shows the qualitative presence of some groups of secondary metabolites such as tannins and phenolic compounds, flavonoids, glycosides and steroids with a total absence of triterpenoids as show Table 1. This study was made following the methodology proposed by Sheel (2014) [31].

By the chemical characteristics of these compounds, and by the type of extraction in which they were obtained it could be inferred in which fractions some of these metabolites predominate. According with the results, E2C1F1 could contain tannins, flavonoids, steroids and a high presence of glycosides; E2C1F2 may contain glycosides, steroids and increased presence of tannins. E2C1F3 was the fraction that had the most positive results regarding the tannin tests, as it possibly contains flavonoids and glycosides. The E1C1F8 fraction could have high glycoside content and also have flavonoid and steroid compounds; E1C1F10 was the fraction in which the lowest amount of secondary

metabolites was found having only tannins. Finally, the E1C1F12 fraction may contain tannins and steroids.

Metabolites	Chemical Test	Ethanolic Extract						Result
		Macerated Extract			Ultrasound assisted Extract			
		F1	F2	F3	F8	F10	F12	
Tannins & Phenolic compounds	5% FeCl3 test	-	+	+	-	-	+	
	Acetic Acid test	-	-	-	-	-	-	
	Diluted iodine test	+	+	+	-	-	-	
	Diluted HNO3 test	-	-	+	-	+	+	
Flavonoids	5% FeCl3 test	-	-	+	-	-	-	
	Alkaline reagent test	+	-	-	+	-	-	
Glycosides	Keller Kiliani test	++	+	+	++	-	-	
Steroids	Salkowski test	+	+	-	+	-	+	
Terpenoids	Libermann-Burchard test	-	-	-	-	-	-	

Table 1. Preliminary chemical tests on ethanolic fractions of the Coriaria thymifolia plant.

4.4. UV-VIS Spectrum

The spectroscopic technique has become a powerful and analytical tool for both qualitative and quantitative studies of biological and pharmaceutical materials, which is why it turns out to be very useful in the determination of phytoconstituents in plant extracts under study. Besides, UV-VIS is a simple, inexpensive and rapid test to detect phytocomponents, which is why it was used in the phytochemical study of the *Coriaria thymifolia* plant.

The qualitative profile of the UV-VIS spectrum of fractions E2C1F1, E2C1F2, E2C1F3, E1C1F8, E1C1F10 and E1C1F12 (the first three obtained from the ultrasound assisted extraction and the last three from the macerated extract) was selected at a wavelength of 250 to 900 nm due to the sharpness of the peaks and the appropriate baseline. The profiles of the fractions of macerated extract are showed on the Figure 17-Figure 19 and the most important peaks are resumed in Table 2. From following data we can conclude that fraction F1 may contains Flavonoids because the band of absorption of the peak 3 belongs to Band I, being in the range of 350-358 nm, which belongs to the conjugation of ring B and the C-4 carbonyl group of flavonoids, this conjugation it is favored by the double bond of ring C (Figure 16). Whereas, peak 4 corresponds to Band II of the 250-280 nm range, originated from the conjugation of ring A with the carbonyl group at C-4. According to Osmany, et al (2015) the position of the band I could favor the identification of the type of flavonoid [32, 20]. Therefore, fraction F1 could be a flavonoid of the group of flavonols (free 3-OH). Considering Fraction F2, it presents the same case as fraction F1, the peaks absorb at wavelengths that are within the range of flavonoids of the flavonol type (free 3-OH). Finally, the F3 fraction could be an isoflavone as it absorbs at a range of 275-295 nm.

Figure 16. General structure of flavonols.

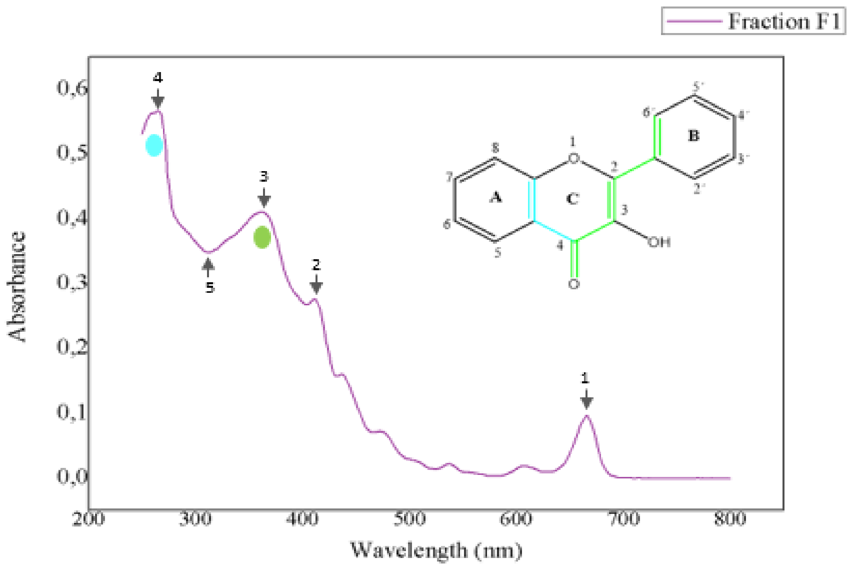

Figure 17. Ultra Violet Visible Spectroscopy Analysis of Fraction E2C1F1 of the Macerated extract.

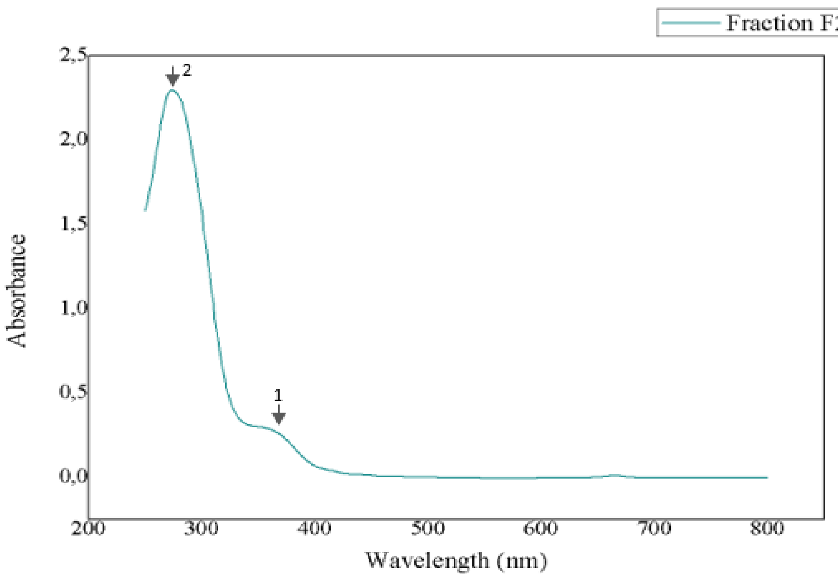

Figure 18. Ultra Violet Visible Spectroscopy Analysis of Fraction E2C1F2 of the Macerated extract.

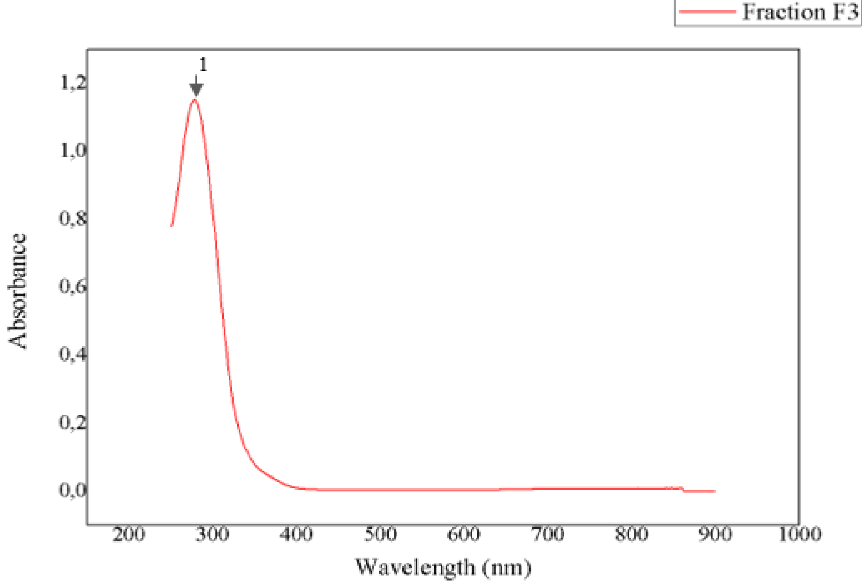

Figure 19. Ultra Violet Visible Spectroscopy Analysis of Fraction E2C1F3 of the Macerated extract.

Sample	Peak No.	Wavelength	Absorbance
Fraction F1	1	668.0	0.0916
	2	414.0	0.2706
	3	368.0	0.4030
	4	268.0	0.5601
	5	314.0	0.3487
Fraction F2	1	370.0	0.251
	2	278.0	2.2768
Fraction F3	1	282.0	1.139

Table 2. UV-Vis Spectrum peak values of fractions E2C1F1, E2C1F2 and E2C1F3 obtained of the Macerated extract.

For the resulting fractions of the ultrasound assisted extract, the UV-Vis profiles are showed on the figures Figure 20-Figure 22 and the most important peaks are resumed in Table 3. In the UV-Vis spectrum of Fraction E1C1F8 peak 6 and 7 have absorption bands in the range of 250-280 nm and 310-350 nm for the band I and band II respectively, which could be a flavonoid of the type flavone. On the other hand, Fraction E1C1F10 has peak 1 and 2 that are within the range belonging to flavones or isoflavones, that is, this fraction could be one of these two types of flavonoids. Finally, Fraction E1C1F12 has absorption values in the wavelength range belonging to flavonols (free 3-OH).

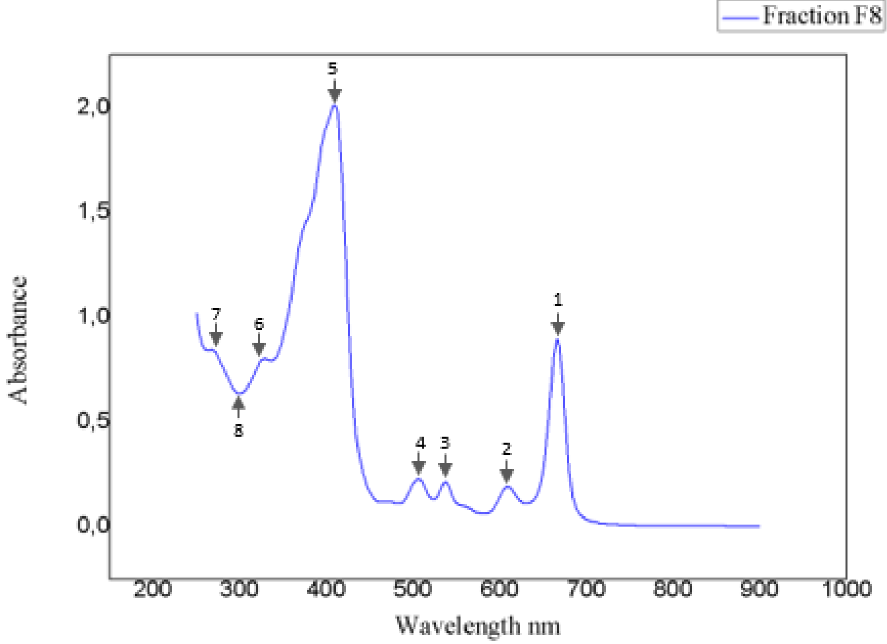

Figure 20. Ultra Violet Visible Spectroscopy Analysis of Fraction E1C1F8 of the Ultrasonic assisted extract.

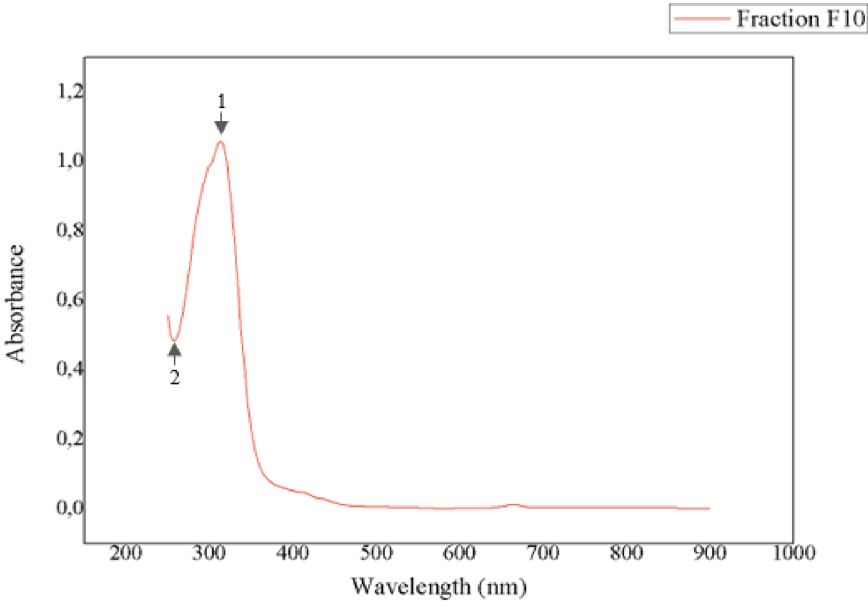

Figure 21. Ultra Violet Visible Spectroscopy Analysis of Fraction E1C1F10 of the Ultrasonic assisted extract.

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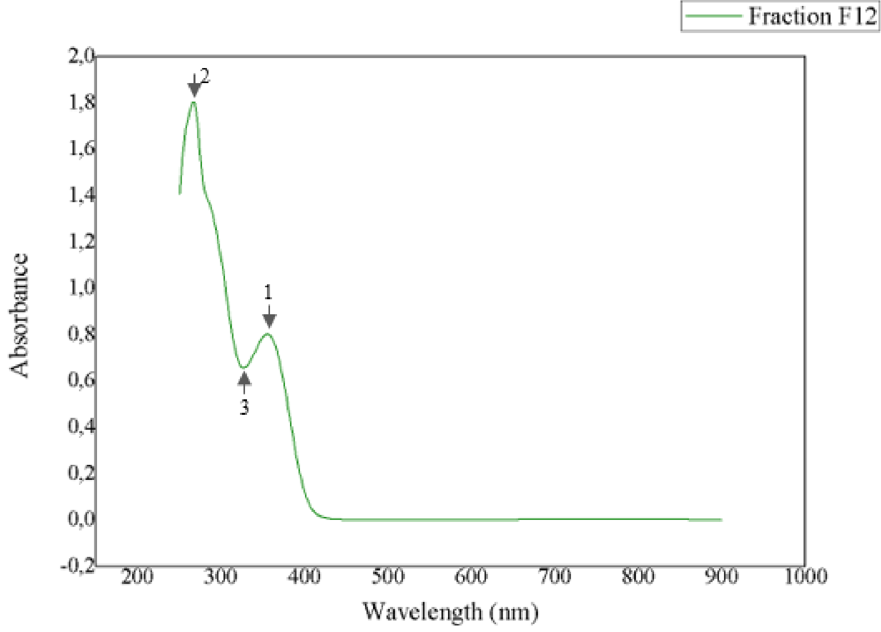


Figure 22. Ultra Violet Visible Spectroscopy Analysis of Fraction E1C1F12 of the Ultrasonic assisted extract.

Sample	Peak No.	Wavelength	Absorbance
Fraction F8	1	668.0	0.8671
	2	616.0	0.1645
	3	540.0	0.2019
	4	512.0	0.1998
	5	410.0	2.0112
	6	332.0	0.7986
	7	274.0	0.8177
	8	304.0	0.6443
Fraction F10	1	316.0	1.0492
	2	258.0	0.4836
Fraction F12	1	362.0	0.7798
	2	268.0	1.8027
	3	330.0	0.6623

Table 3. UV-Vis Spectrum peak values of fractions E1C1F8, E1C1F10 and E1C1F12 obtained of the Ultrasonic extract.

Considering the results of the UV-Vis spectra, these exhibited the presence of flavonoids in the extract of *Coriaria thymifolia*, although the identity of the flavonoid type cannot be determined, the results are consistent with the studies carried out by Bohm *et al.* (1981).

4.5. HPLC, UPLC-MS analysis

Three fractions E2C1F1, E2C1F2 and E2C1F3 were analyzed by HPLC-UV and UPLC-MS separately. Since UPLC-MS chromatogram of the complex mixture as are our three fractions, we decided to try to find the best conditions in HPLC-UV system to have general idea of quantity of main products presents in the fractions. Additionally, we have found that information from both systems cannot be compared using the same eluent condition. For instance, when was used gradient of H_2O-ACN from 0 to 100% of acetonitrile we have obtained very different spectra for both system Figure 23. For that reason, in the next figures, we show the optimized condition for HPLC system (H_2O-ACN from 25 to 35%), 0-100% of ACN gradient for UPLC-MS and we will focus on combined MS spectra of the sample.

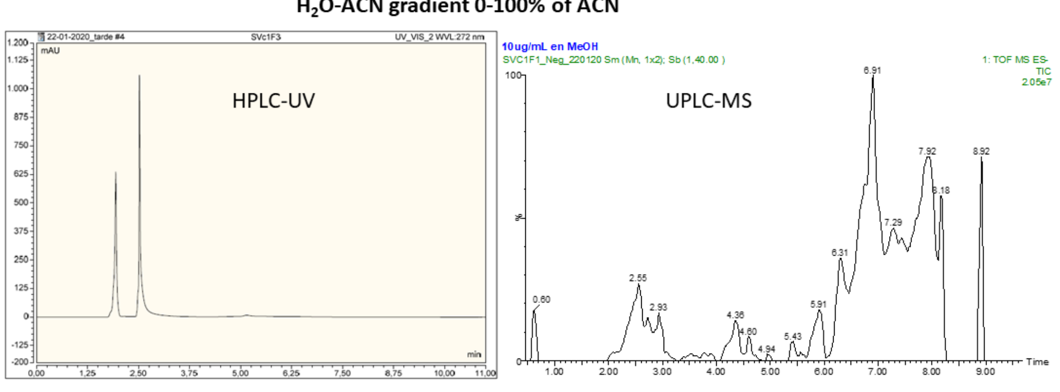


Figure 23. Chromatograms comparison of two system HPLC-UV and UPLC-MS using the same eluent condition and same sample (E2C1F1).

From mass spectrum of sample E2C1F1 (Figure 24) we can clearly see the saponin characteristic fragmentation peaks 507 and 523 m/z[33]. The possible presence of interesting and toxic alkaloid - methyllycaconitine can be associate with the peak of 683 m/z. Other compound with 339 m/z peak can be associate with 6-Caffeoyl-D-glucose, however, the ESI- spectrum seems to be contaminated with alkylbenzene sulfonates that also present peaks 339 and 325 m/z. Presence of palmitic acid and stearic acid is evidence by intense peak at 255 and 283 m/z in ESI- spectrum.

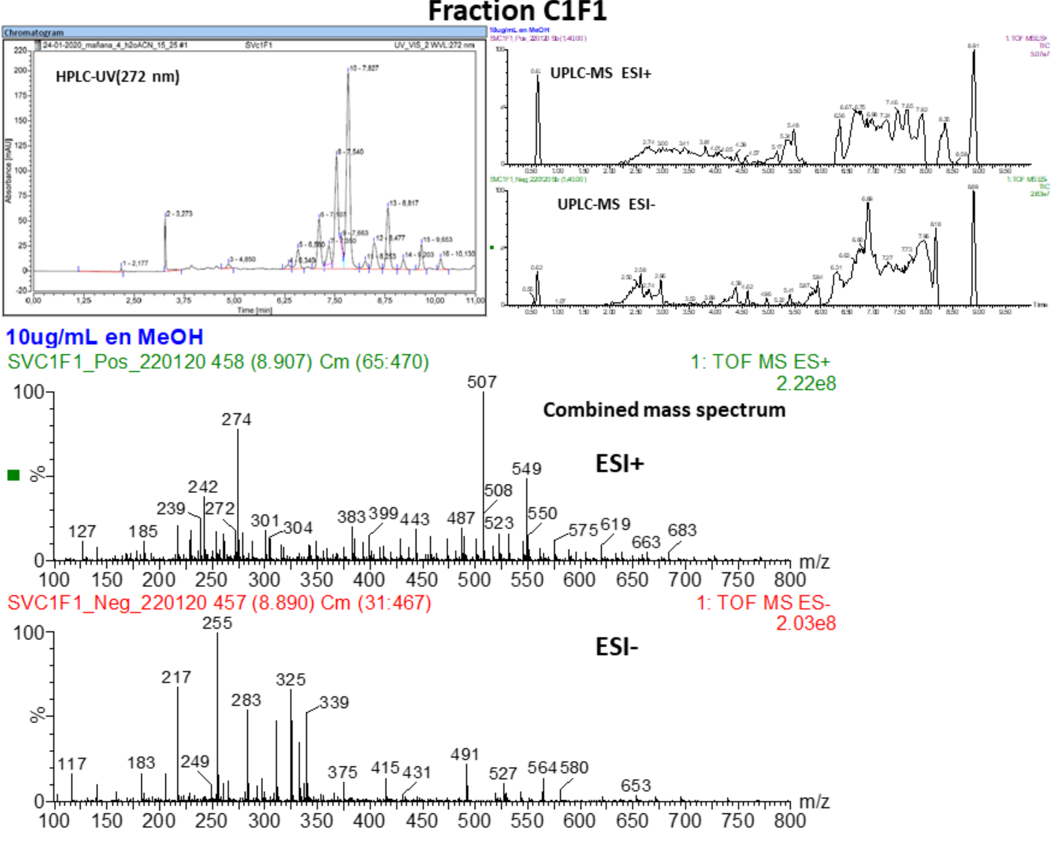


Figure 24. HPLC, UPLC-MS (upper - positive mode and lower -negative mode) chromatograms and Mass spectrum (ESI+ and ESI-) of E2C1F1 fraction.

The fraction E2C1F2 (Figure 25) as fraction 1 and 3 (Figure 26) contain saponin as spectrums present same fragmentation peaks explained before. Manual search of most intensive peak from mass spectrum lead to possible identification of compounds presented in Figure 27. Interestingly some other toxic alkaloid is presented in this fraction as aconitine or macrocycle isomigrastatine.

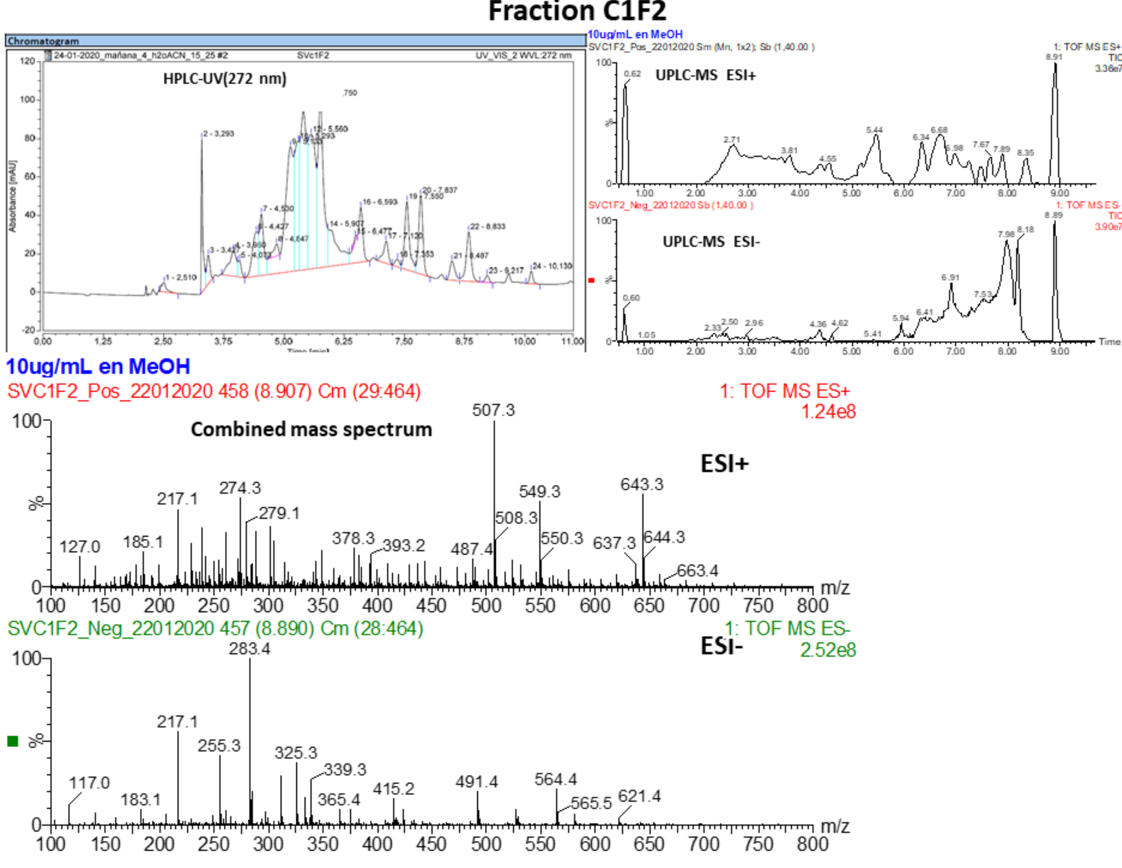


Figure 25. HPLC, UPLC-MS (upper - positive mode and lower -negative mode) chromatograms and Mass spectrum (ESI+ and ESI-) of E2C1F2 fraction.

The last analyzed fraction E2C1F3 (Figure 26) also brings to attention possible presence of interesting compound as it is bioactive deep sea associate alkaloid Meleagrin or antibacterial Jadomycin B.

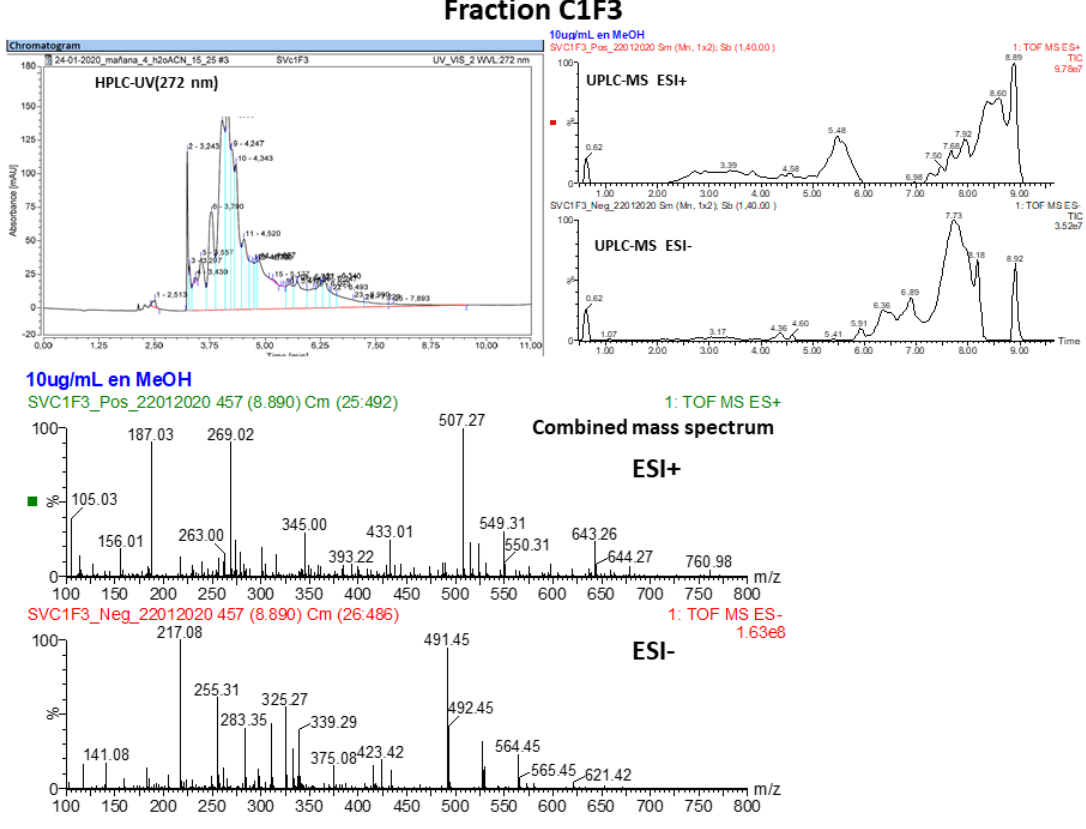


Figure 26. HPLC, UPLC-MS (upper - positive mode and lower -negative mode) chromatograms and Mass spectrum (ESI+ and ESI-) of E2C1F3 fraction.

Of course, to confirm the presence of those structure further purification and other spectroscopic tools should be applied as NMR or IR in the future works.

Methyllycaconitine
683 m/z [M-H]+

6-Caffeoyl-D-glucose
339 m/z [M-H]-

Isomigrastatin
489 [M-H]+

Antibiotic JI-20A
481 [M-H]+

Remikiren
315 Fragment [M-H]+

Aconitine
645.31

Nelfinavir mesylate
663 [M-H]+

Mifepristone
429 [M-H]+

Meleagrin
433

Estradiol mustard
393 [M-H]+

Jadomycin B
549 [M-H]+

Figure 27. Some possible mass identification searched manually. Structure was extracted by isomeric SMILEs from PubChem database.

4.6. Mass analysis using Progenesis Q1

Progenesis is a software for LC-MS data that permits adjust, associate different MS adducts to same compound, normalize and identify masses of different adduct take together different sample measures from the same experiment. In our case, we used data (positive mode) of three fraction E2C1F1, E2C1F2, E2C2F3 and after processing with the software we get data associate with 3600 different compounds (more than 18000

masses), then we filter first most abundant 1000 and using SpiderChem database with KEGG and MassDatabase as a source we found 360 hits. Finally, only those compounds were selected where identification was not exceed more than three possibilities of compounds with the same mass. Results of first 100 hundred compounds is present in Annex 4. On Figure 28 we are showing only first 10 automatically identified and depicted compounds.

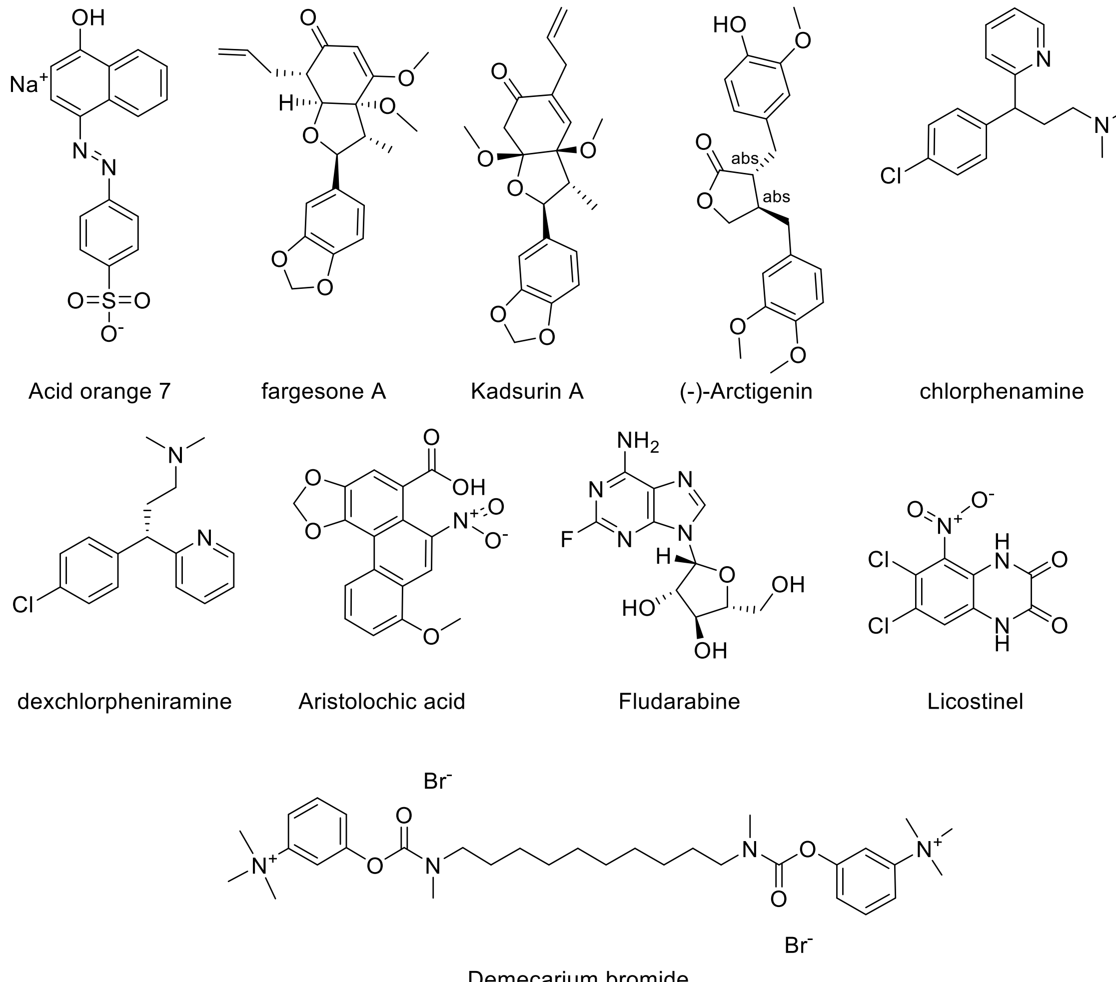


Figure 28. Depiction of ten possible compounds that contains leaves of Coriaria thymifolia. Massases were analyzed by Progenesis Q1 software and compared with reported in database (Spiderchem).

Taking those finding into account we can say that identification of plant using software is much more reliable. The mass spectrum of mixture extracted from plants are very complex and searching manually is very limited, algorithms implements in software can check simple adducts like $[M+H]^+$ and other $[M+Na]^+$ etc. associate one with another and giving relatively viable results. Of course, to be certain about accuracy more study need to be done, better compounds isolation and using different spectrometric tools,

nevertheless this approach gives us a nice overview of what kind of compound (and how much) can be isolated from the plant such as *Coriaria thymifolia.*

4.7. Antioxidant Activity

For antioxidant activity evaluation of the extracts, first we needed to adapt the well-established β-carotene bleaching due the lack of reagents and long terms of shipping. First the β-carotene was extracted from carrot and instead of linoleic acid we used the mixture of isomers of this acid knows as a conjugated linoleic acid (CLA) and used as a diet supplement and available on online store *Mercado Libre*. Finally, instead of Tween 20 surfactant we used Tween 20, which we were able to buy in the local store. Extraction process of β-carotene was not easy since is susceptible to decomposition triggered by light, temperature, oxygen and certain types of solvents. That is why the extraction of *beta* carotene should be done quickly and following a protocol that will not alter and break down this compound. Even after being extracted, care must be taken as to perform analyzes in the shortest possible time, exclude oxygen, avoid high temperatures and contact with acids, and use impurity-free solvents; For example, β-carotene remains stable in petroleum ether and at low temperatures.

After the extraction, column chromatography was performed because of the carrot extraction, the three types of carotenes α, β and γ carotene are obtained. In such a way that the fractions must be separated to obtain only β-carotene. From the column 20 samples were collected in different test tubes, these were analyzed by TLC (Figure 29), finding that the fractions corresponding to the carotene of interest were the first 7, having a value of Rf = 0.86 which corresponds to β-carotene according to literature [34, 35]. In total 6 mg of β-carotene was obtained from 150 milligrams of carotenoid.

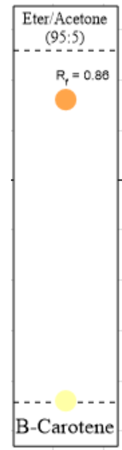


Figure 29. TLC of β -carotene.

In general, carotenoids are prone to isomerization and oxidation due to their highly unsaturated structure (Figure 7). With this in mind, during the beta carotene analysis, preventive measures should be taken into account to ensure the reliability of the analytical results. That is why after the extraction and purification of beta carotene, a 1:10 sample of beta-carotene dissolved in ethanol was prepared and analyzed by UV-VIS spectroscopy, to ensure we obtained it. Figure 30 shows that β-carotene was effectively extracted since the spectra were obtained with the peaks corresponding to this carotenoid, an absorption band at 470 nm, this is consistent with β-carotene spectra reported in the literature [26], additionally the mass spectra have shown the correct mass of the desired compound .

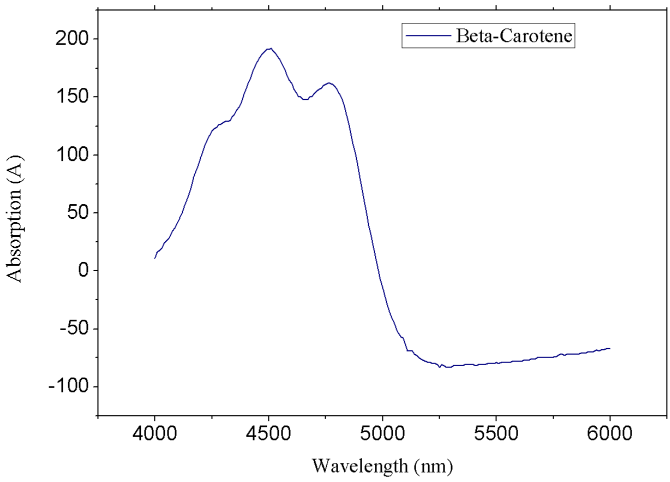


Figure 30. UV-VIS spectroscopy of β -carotene.

β-carotene Bleaching assay of fractions of Macerated Extract

In this test the free radical of linoleic acid generates the discoloration of β-carotene, causing the loss of the chromophore and, therefore, the time dependent loss of orange color which can be measured spectrophotometrically. Antioxidant compounds as ascorbic acid (our positive control) decrease the degree of discoloration measured at a wavelength of 470 nm due the competitive reaction with radicals derivate from linoleic acid and therefore protect the β-carotene.

Firstly, we optimized quantity of CLA so we could observe the time dependent degradation of β-carotene and check that our positive control (ascorbic acid) prevent discoloration of β-carotene in the same assay condition. After successful trials, we decided to move to perform the assay with our extracts.

Figure 31 shows the antioxidant effect of Fractions E2C1F1, E2C1F2 and E2C1F3 all at 1mg / mL, where it is observed that the E2C1F2 fraction generates the highest antioxidant effect very close to the ascorbic acid used as a reference, while E2C1F3 is the one with the least effect. In addition, it is seen that the control sample by itself also prevents bleaching of the carotene in a minimal amount. The test tubes containing Fraction E2C1F2 and ascorbic acid (reference sample) at the end of the measurements were the ones that best maintained the characteristic orange color of β-carotene, results that agree with its greatest effect. Therefore, the stronger the antioxidant power of the compound present in the sample, the brighter the carotene color will be and consequently the absorbance value will be higher.

In the **¡Error! No se encuentra el origen de la referencia.** the data are expressed as the rate of beta-carotene bleaching (R_B), following the equation of Rojas, et al (2008) [36]:

R_B= As 470 nm (t=0) - As 470 nm (t=x)

Where; As (t=0) = absorbance measured at initial time; As (t=x) absorbance measured at time x = 30, 60, 90, 120 and 150 minutes.

In this case, the highest antioxidant effect is attributed to the fraction that has the lowest RB value, this being the E2C1F2 fraction as mentioned above.

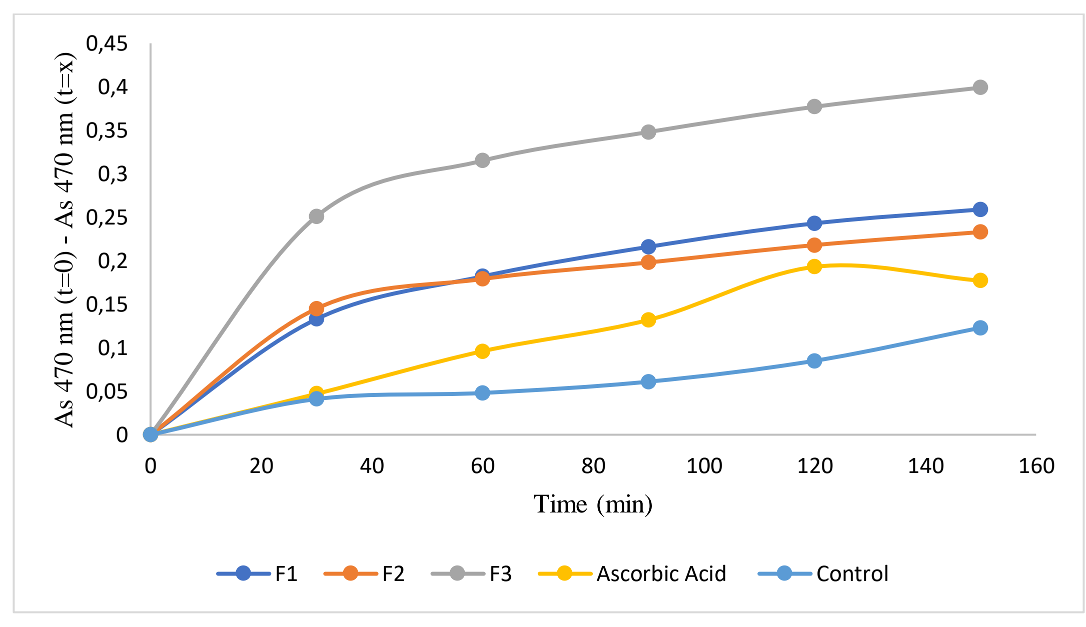


Figure 31. Antioxidant activity of fractions of Macerated extract of Coriaria thymifolia in β-carotene bleaching assay. Fractions and Ascorbic acid at 1mg/mL. Data complete of absorbance of fractions E2C1F1, E2C1F2 and E2C1F3 are detailed in Annex 2.

Beta carotene Bleaching assay of Fraction E2C1F2

Having positive results of Fraction E2C1F2 obtained from column 1 of the macerated extract, an antioxidant test was applied again to the fractions resulting from the purification of Fraction E2C1F2, which is to Fractions E2C2F1, E2C2F2, E2C2F3, E2C2F4, E2C2F5, and E2C2F6. This was done to know in which fraction is the compound that causes the antioxidant effect, and then try to identify the metabolite responsible for this effect. Figure 32 shows the data for beta carotene bleaching as a function of absorbance concerning the measurement time, which was for 2 hours. Following the principle of the antioxidant test, it was found that the E2C2F6 fraction is the one that mainly prevents the bleaching of beta carotene, being very close to the antioxidant activity of the pattern E2C1F2 Fraction. Besides, these two fractions cause an antioxidant effect almost equal to ascorbic acid.

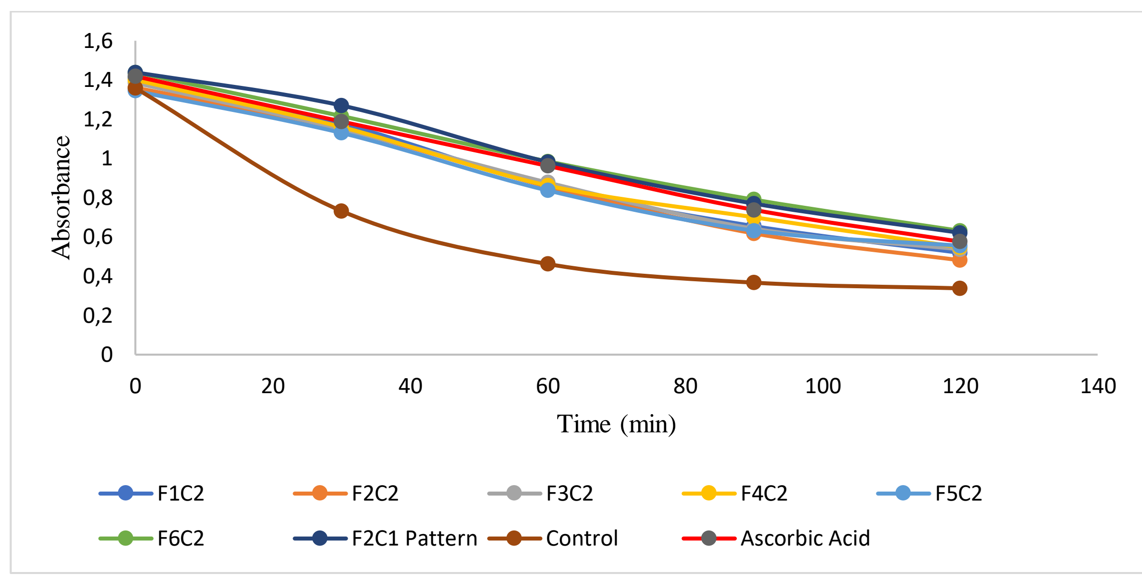


Figure 32. Antioxidant activity of fractions of E2C1F2 of Coriaria thymifolia in beta-carotene bleaching assay. Fractions and Ascorbic acid at 1mg/mL Data complete of absorbance of fractions E2C2F1, E2C2F2, E2C2F3, E2C2F4, E2C2F5, and E2C2F6 are detailed in Annex 3.

The data of Fractions E2C2F1, E2C2F2, E2C2F3, E2C2F4, E2C2F5, and E2C2F6 are also expressed as the rate of beta-carotene bleaching (R_B). Verifying that the E2C2F6 fraction has a good antioxidant capacity since it has the lowest R_B value. While the Fractions E2C2F1 and E2C2F2 have the highest value of R_B being weak antioxidants.

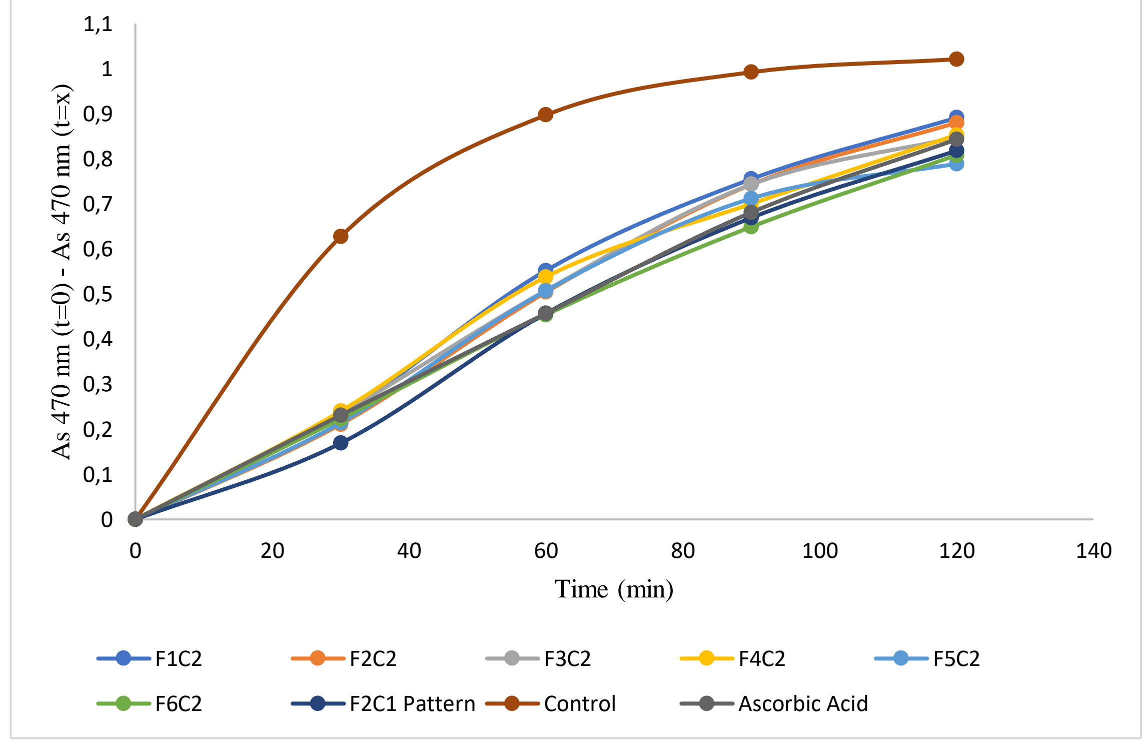


Figure 33. Antioxidant test expressed as the rate of beta-carotene bleaching. Fractions and Ascorbic acid at 1mg/mL.

The percent inhibition was expressed following the formula used by Bhardwaj *et al.* (2017) [37]:

$$\% \textbf{ Inhibition} = \frac{\textbf{Control absorbance} - \textbf{sample absorbance}}{\textbf{control absorbance}} * \textbf{100}$$

The Fraction F6C2 had an inhibition percentage of 86% being higher than the Standard Fraction F2C1 83%. While ascorbic acid had a 70% percent inhibition. Therefore, the F6C2 Fraction proved to be even better antioxidant than ascorbic acid.

4.8. Antibacterial Activity

The three fractions from chromatographic column from extraction 2 were tested against *E. Coli*. The bacteria was cultivated together with 5uL (1mg/mL in DMSO) during 24 hours, and after the optical density was measured. If the growth of bacteria is interrupted by the extract, the optical density is lower than control. We had some technical issue that could not been resolved before the end of this thesis; however, we can observe some tendency of activity of our samples. The malfunction of nanodrop produce unexpected high absorbance reading from 150-200 min (Figure 34) and it seems is due the equipment heating. If we omit this range of time we can deduce de that fraction E2C1F2 (in yellow) produce slightly bacteria inhibition. However, this is very preliminary result and test should be repeated in future.

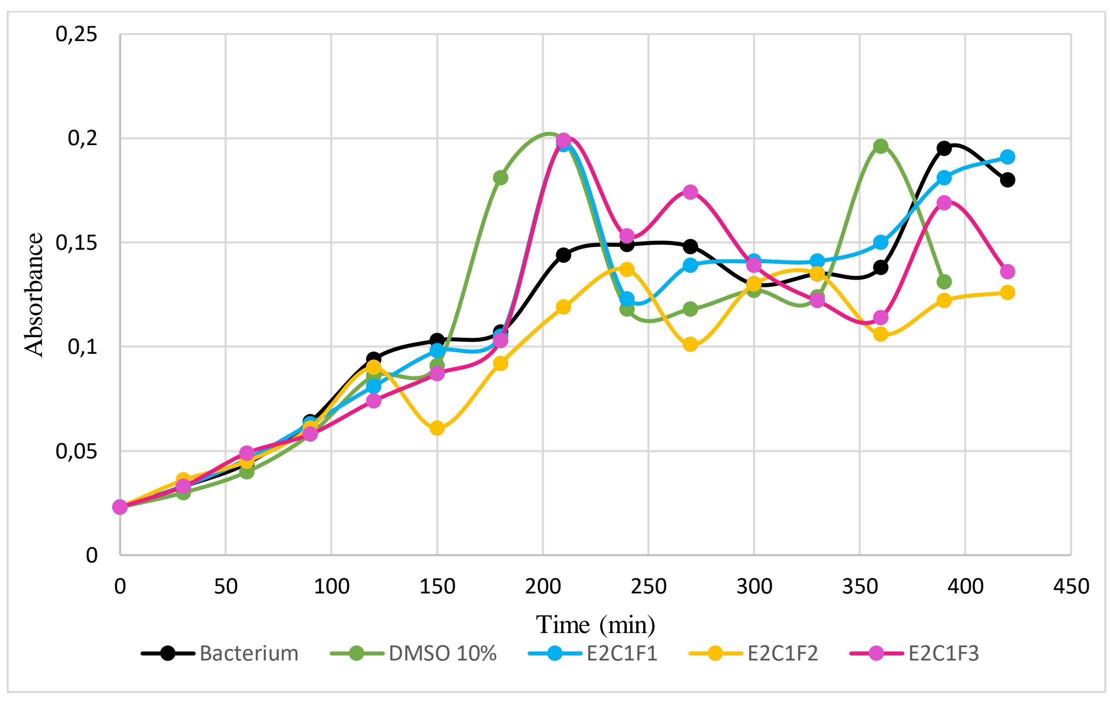


Figure 34. Growth curve of E. coli bacteria against fractions E2C1F1, E2C1F2, E2C1F3. Absorbance data detailed in Annex 1.

CHAPTER 3

5. METHODOLOGY

5.1. Collection and drying of plant material under study

Coriaria thymifolia leaves were used as study material for the present work. They were collected in August in the afternoon, specifically at 4 pm, on the slopes of Imbabura hill with the following coordinates:

Shanshi: a altitude of 2785 masl with latitude 0°17'54.8"N and longitude 78°11'01.7"W.

Figure 35. Locationt of Coriaria thymifolia plant on Imbabura hill.

..

The leaves were collected manually from shrubs approximately 4 m in size. The collected plant material was placed in clean plastic covers ensuring ventilation and was transferred to the Chemistry Laboratory of the Yachay Tech Experimental Technology Research University.

Figure 36. Vegetable material collected.

The fresh leaves collected were placed in aluminum foil and dried in the oven at a temperature of 45 ° C for four days. The weight of the leaves was taken at the beginning of its drying and this was monitored every day until obtaining a constant weight and thus ensure that the leaves are completely dry. Then, it was allowed five more days to dry in the desiccator using magnesium sulfate ($MgSO_4$). The total weight of the leaves was 1129.43 g.

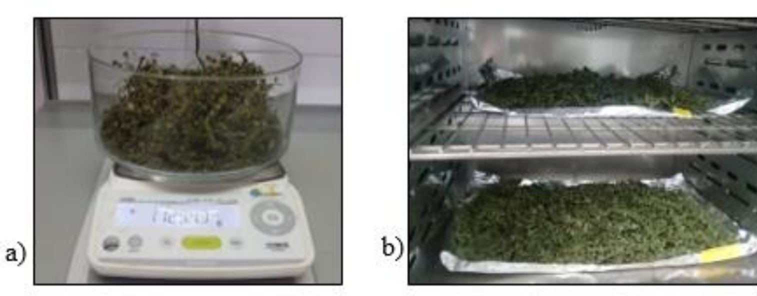


Figure 37 a) Shanshi leaf weight. b) Drying the leaves on the stove at 40 degrees.

5.2. Extraction of plant material

Two extractions were carried out each started with 30 g of plant material. The leaves were crushed using a porcelain mortar with pistil until a fine powder was obtained.

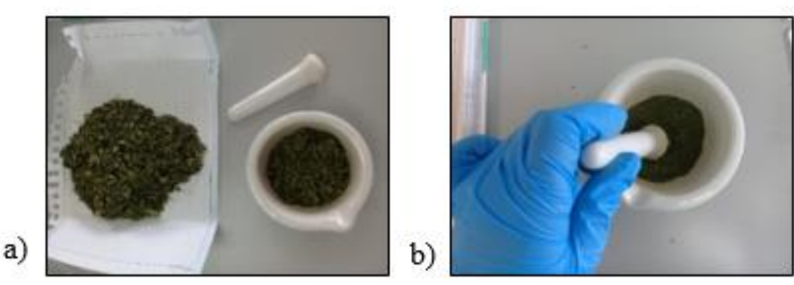


Figure 38. a) Leaves of Coriaria thymifolia, b) Crushing of plant material.

5.2.1. Ultrasound assisted bath extraction

30 g of the powdered leaves were placed together with 100 mL of 96% EtOH in a 250 mL Erlenmeyer. The extraction was carried out in a sonicator for 1h 15 min at 36 ° C. The solution obtained was filtered under vacuum using a Buchner funnel and filter paper, the filtrate was deposited in a urine sample container. The remaining solid from the filtration was reextracted with 40 mL of 96% ethanol in the sonicator for 30 min at the same temperature, then it was filtered and the resulting filtrates was combined with the previous one. The extracts were stored in the freezer for four days.

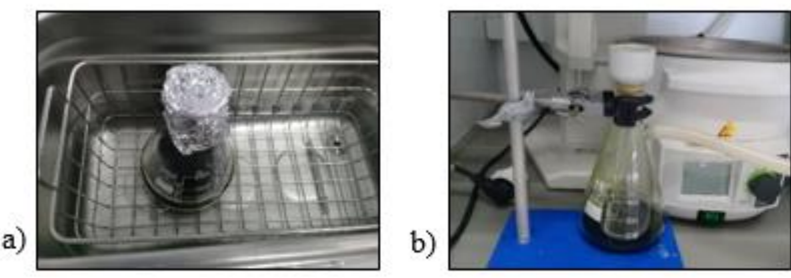


Figure 39. a) Ultrasound bath extraction, b) Filtration of extract of Coriaria thymifolia.

The ethanol extract was removed from the freezer and passed to a 250 mL balloon, this was concentrated in a rotary evaporator at 40 ° C for 2h. The concentrated extract was taken to a desiccator containing magnesium sulfate ($MgSO_4$) and kept under vacuum, for three days, until it is completely dry. The extract obtained again was stored in the freezer for further analysis.

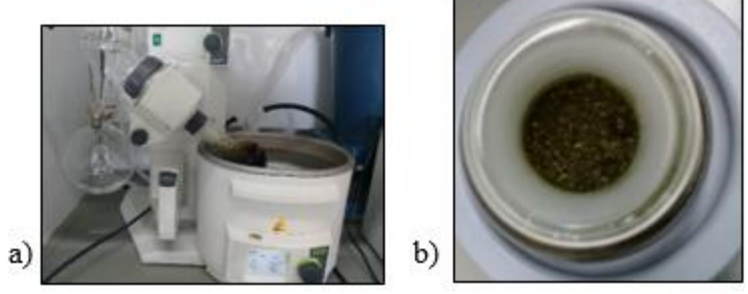


Figure 40. a) Evaporation of Shanshi extract, b) Solid extract.

5.2.2. Maceration Extraction

30g of previously ground plant material was weighed, this was placed in a glass jar covered with aluminum foil, to avoid light, along with 110 mL of 96% EtOH. It was left to macerate for eight days. The extract obtained was filtered in vacuum using a Buchner funnel, then the extract was concentrated in a rotary evaporator at 40 ° C using a 250 mL balloon. The resulting solid extract was stored in a vial in the freezer. A part of the solid remained in the ball and was removed with petroleum ether.

5.3. Chemical characterization of extracts and fractions of *Coriaria thymifolia*, by thin layer chromatography (TLC)

5.3.1. Thin Layer Chromatography (TLC)

Once the polar extracts were obtained, taking into account both types of extraction, a thin layer chromatography was performed. This procedure was performed as a previous step to column chromatography, to monitor the separation of the compounds according to their polarity, for this, different eluent systems in the mobile phase were tested.

For TLC a sample of the polar extract was taken using a glass microcapillary. A point of this sample was placed on the baseline drawn approximately 1 cm from the edge of the plate. The applied sample was allowed to dry and taken to UV light to verify the concentration of the spot. In a chromatographic chamber, 10 mL of different solvent systems were placed. Later, the plate containing silica gel and the sample was placed inside the chamber. The chamber was covered and the mobile phase was allowed to rise to the front line of the solvent, at a distance of 0.9 mm from the top of the plate, allowing separation of the extract components. Then, the plate was removed from the chromatographic chamber and the solvent was allowed to dry to visualize it under UV light allowing to detect the presence of some compounds not visible under normal light. Finally, the plates were submerged in developer solutions and dried with a heat gun to detect the presence of compounds based on color changes given by the reaction with the developer. This same procedure was used for the two types of polar extracts obtained.

	Ultrasound assited Extract	Macerated Extract
Stationary Phase	TLC Silica Gel 60 F_{254}	
Mobile Phase 1	Ethyl acetate: Petroleum ether (1:1) v/v	

Staining	UV light 254 nm and UV light 366 nm

Table 4. Data of mobile phase used in Ultrasound Extract and Macerated Extract.

TLC was also performed for each of the fractions obtained from column chromatography, this will be indicated in section 5.4.1.1, 5.4.1.2 and 5.4.1.3.

5.4. Purification and Fractionation of Extracts

5.4.1. Column Chromatography of Extracts

Once the extracts were obtained, they were purified through the normal phase column chromatography technique. For this technique 60 MESH silica gel was used as stationary phase and different solvents in ascending polarity as the mobile phase. The eluents chosen vary in each of the extracts mentioned in 5.4.1.1 and 5.4.1.2.

To carry out this type of chromatography, a glass column was used, at the bottom of this, a 1.5 cm cotton layer was placed. Next, silica gel, previously dissolved in the solvent to be used to separate the compounds from the extracts, was introduced. Subsequently, the eluent was added so that the adsorbent is hydrated and completely packaged. Then, another 1 cm cotton layer was placed (Figure 41). Once the column was prepared, the extract of interest was placed and the samples were collected in test tubes.

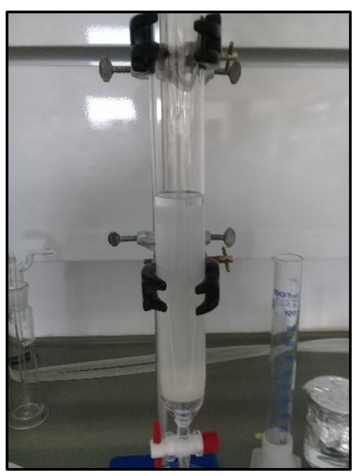


Figure 41. Chromatographic column preparation.

5.4.1.1. Fractionation of Ultrasonic assisted Extract

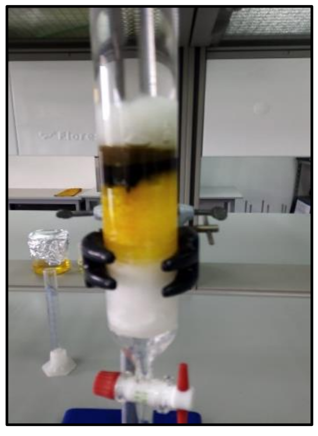
For the purification of the ultrasonic extract, a silica gel layer of approximately 8 cm was used, in which 5.7 grams of the extract was purified using a total of 480 mL of solvents distributed in 192 mL of petroleum ether, 279 mL of ethyl acetate and 9 mL of

ethanol. Different solvent systems with ascending polarity were used as petroleum ether: ethyl acetate starting with (90:10), (80:20), (60:40), (50:50), (30:70), (10:90) and ethyl acetate: ethanol (57: 3), (54: 6).

At the end of column chromatography, a TLC was performed with the samples of each of the tubes obtained in the CC. For this, fractions 1-13 were taken and analyzed with mobile phase 2, fractions 18-41 with mobile phase 3 and fractions 44-63 with mobile phase 4. Table 5 shows the eluent system and proportions used for the TLC.

	Ultrasound assisted Extract	Proportions
Stationary Phase	TLC Silica Gel 60 F_{254}	
Mobile Phase 1	$CHCl_3$: AcOEt: AcOH	(1:8:1) v/v/v
Mobile Phase 2	Benzene : AcOEt	(9:1) v/v
Mobile Phase 3	Benzene : AcOEt	(7:3) v/v
Mobile Phase 4	AcOEt : EtOH	(1:1) v/v
Staining	UV light 254 nm and UV light 366 nm, Vanillin Anisaldehyde.	

Table 5. Data of Mobile Phase used in the fractionation of Ultrasound extract.



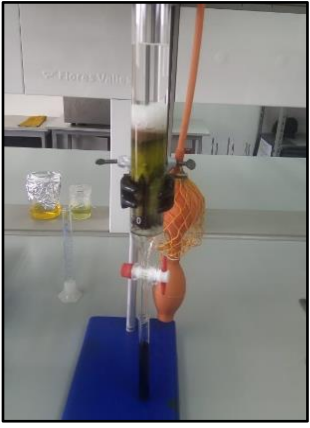


Figure 42. In the left the column with the Ultrasonic assisted extract of Shanshi; In the right the purification.

5.4.1.2. Fractionation of the Macerated Extract (Column 1)

In this case, the silica gel layer was approximately 7 cm, in which 2.3 grams of the extract were separated using a total of 300 mL of solvents distributed in 120 mL of chloroform, 180 mL of ethanol. The solvent system used was CHCl3: EtOH in the following order of polarity (8:2), (6:4), (4:6), (2:8) and (0:100).

Each of the test tubes obtained was analyzed by TLC to determine the fractions obtained from the column. In Table 6 the mobile phase 1,2, 3 and 5 were used to visualize the separation of compounds from Column 1 from the macerated extract.

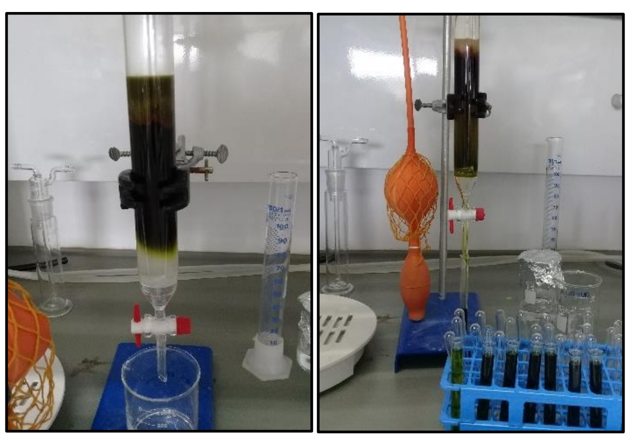


Figure 43. In the left the column with the Macerated extract of Shanshi; In the right the purification.

5.4.1.3. Re-Purification of Macerated Extract (Column 2)

From this extract, one of the fractions obtained from Column 1 was purified, for this purpose 600 milligrams of extract fraction were purified on a column containing 7 cm of silica gel. A mixture of chloroform: ethanol was used in the order that follows (8: 2) two volumes, (6: 4) three volumes, (4: 6) one volume, (2: 8) two volumes and (1: 9). Each volume was 50 mL, in total 450 mL of solvents were used. In Table 6, mobile phases 4 and 5 served for the TLC analysis of Column 2 of the Macerated Extract fraction.

	Macerated Extract	Proportions
Stationary Phase	TLC Silica Gel 60 F_{254}	
Mobile Phase 1	$CHCl_3$: EtOH	(8:2) v/v
Mobile Phase 2	$CHCl_3$: EtOH	(1:9) v/v
Mobile Phase 3	EtOH	10 v
Mobile Phase 4	Benzene : AcOEt	(9:1) v/v
Mobile Phase 5	$CHCl_3$: EtOH	(4:6) v/v
Staining	UV light 254 nm and UV light 366 nm, Anisaldehyde.	

Table 6. Mobile phase 1,2,3 and 5 where used to analyze the samples obtained from Column 1 and mobile phase 4 and 5 where used to analyze the fractions of column 2.

5.5. Phytochemical Characterization

For the chemical characterization of the different functional groups, characteristic coloring reactions were used, which will be explained later. The test procedure was based on the Sheel protocol (2014) [31]. For the application of each qualitative test, 1 mL of the extract of fractions E1C1F8, E1C1F10 and E1C1F12 obtained from the fractionation of the Ultrasonic extract and fractions E2C1F1, E2C1F2 and E2C1F3 obtained from the fractionation of the Macerated extract were used. Each of the fractions was placed in different test tubes, where the solutions prepared for each test were applied.

5.5.1. Test for tannins and phenolic compounds

* Ferric Chloride Test.- In 1 mL of each fraction four drops of 5% $FeCl_3$ were added. The color change from green to black indicates the presence of Tannins.

* Acetic Acid Solution Test.- In each fraction three drops of this solution were added. The red coloration in the sample indicates a positive result.

* Iodine Solution Test.-Three drops of this solution were added, this test shows transient red color.

* HNO3 Test.- Six drops of this solution were added in the fractions. A positive result in this test shows the change from reddish to yellow.

5.5.2. Test for flavonoids

For flavonoid tests, samples must be prepared beforehand. To 1 mL of the extract of each fraction is added 2 mL of 2M HCl and heated in test tubes in a water bath for 40 min at 100 ° C. Then, the extract is cooled and 2 mL of ethyl acetate is added to each tube.

* Ferric Chloride Test. - To 2 mL of the prepared solution some drops of $FeCl_3$ are added, which in case of being positive shows an intense green color.

* Alkaline Reagent Test.- 2 mL of the extract is treated with 2 mL of a NaOH solution, which generates a deep yellow color that will discolor with the addition of three drops of dilute HCl.

5.5.3. Test for Glycosides

* Keller-Kiliani Test.- In 2 mL of extract of the fractions, 2 mL of glacial acetic acid, a drop of 5% $FeCl_3$ and a few drops of concentrated H_2SO_4 are added. In this test two layers are formed, a reddish-brown color will appear right at the junction of the two liquid layers. The top layer will appear in bluish-green.

5.5.4. Test for Sterols

* Salkowski Test.- In 2 mL of extract, 1 mL of chloroform and six drops of concentrated H_2SO_4 are added. The chloroform layer will appear red, while the acidic layer will have a fluorescent greenish-yellow color.

5.5.5. Test for terpenoids

* Liebermann-Burchard Test.- 2 mL of extract was mixed with chloroform, 2 mL acetic anhydride and two drops of concentrated H_2SO_4. A positive result for sterols will show a red color, then blue and finally a green color.

5.6. UV-VIS Spectrum analysis

Ultraviolet-visible spectrophotometry (UV-Vis) is a widely used technique since it helps determine the presence of phytoconstituents in plants. This technique is based on photon spectroscopy in the UV-visible region. The molecules undergo electronic transitions in the region of the electromagnetic spectrum [38 39].

The extracts were scanned at a wavelength ranging from 250 to 900 nm using a spectrophotometer, in a quartz cuvette 10 mm thick. The samples measured were fractions F8, F10 and F12 obtained from the ultrasonic extract, and fractions F1, F2 and F3 obtained from the macerated extract. The samples were diluted to 1:10 using ethanol. The UV-Vis data were recorded.

5.7. Biological Tests- Antioxidant Activity

To prove the antioxidant power of the extracts of *Coriaria thymifolia* was carry out a test based on the transfer of hydrogen atoms (HAT) called beta carotene bleaching. This method, first tested in 1968 [25], has been modified by several researchers to date, it measures the ability of an antioxidant to inhibit lipid peroxidation.

To carry out the antioxidant test it is necessary to prepare a beta carotene emulsion, where a beta carotene solution is essential. Thus, before starting the protocol used to determine the antioxidant capacity of the extract of *Coriaria thymifolia*, the extraction process of Beta carotene is presented.

5.7.1. Beta-Carotene Extraction

Thin pieces of carrot were cut, of these 95 grams were used to extract the pigments. The carrot was placed in a 1000 mL Erlenmeyer containing 100 ml of 96% hot EtOH (Figure 44 a). The extraction was carried out for 2 h and 30 min at a temperature of 65 ° C until the carrots were colorless, then it was passed the extract obtained to a 500 mL Kitasate. The pigments were re-extracted with 25 mL more of EtOH for 30 min and both extracts were combined in the Kitasate. The solution was cooled and filtered under vacuum. Next, the filtrate was heated and 40 mL of distilled water was added to lower the concentration of ethanol. Then, the extract was allowed to cool to room temperature and was taken to a separatory funnel where 70 mL of petroleum ether was added. There, the solution was stirred and allowed to stand until they form two layers, the upper ether layer carries the carotenes and the lower one the xanthophylls (Figure 44 b). The upper layer was collected and stored, while the lower layer was taken back to the funnel to wash with 3 successive portions of 25 mL each of petroleum ether, to make sure we collect all the carotene pigments. To complete the extraction, the petroleum ether extracts were deposited in a 250 mL balloon and the solvent was evaporated using a rotary evaporator at 30 ° C. The solid obtained from Beta carotene was placed in a vial covered with aluminum foil and stored in the freezer [26, 40, 41, 34].

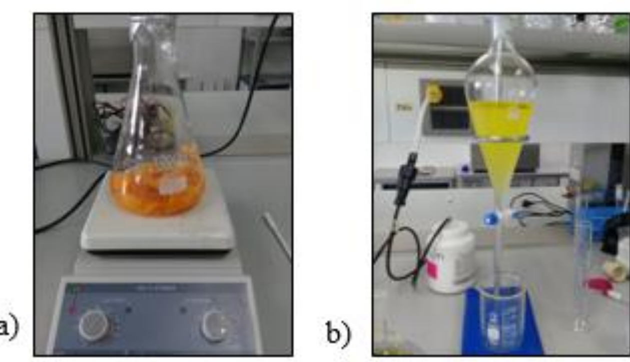


Figure 44. a) Extraction of beta carotene, b) Separation of layers in the settling funnel.

5.7.1.1. β-Carotene Purification

To obtain only the beta-carotene pigment, 0.15 g of carotene extract obtained from the carrot was purified (Figure 45). The mobile phase used was petroleum ether: acetone (95:

5) 2 volumes, in total 120 mL of solvent was needed to collect the fractions where the carotene of interest was present.

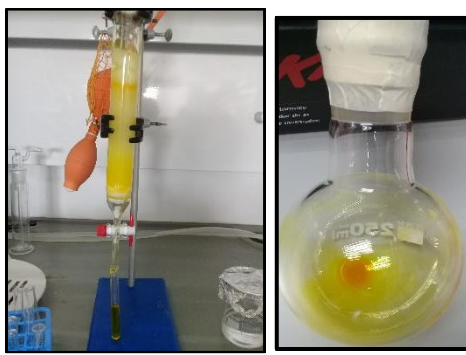


Figure 45. In the left chromatographic column of carotenes; In the right obtention of beta-carotene.

5.7.2. β-carotene Bleaching assay

The beta-carotene bleaching protocol is based on the test described by Miller (1970) and modified by several authors [42, 43, 44].

To carry out the test, a beta carotene emulsion is prepared, where 1 mg of beta carotene is dissolved in 10 mL of chloroform. Of this solution 1 mL is placed in a 100 mL round bottom balloon, 50 ul of linoleic acid and 400 ul of tween 20 surfactant are added there. The chloroform is evaporated in the rotary evaporator for 3 min. Then, 50 mL of saturated oxygen water is added, add 5 aliquots of 10 mL to vigorous stirring. 5 mL of emulsion is transferred to each of the test tubes containing a known sample concentration (plant extracts of 1mg/mL) and standard antioxidant in this case Ascorbic Acid, used as a positive control. A negative control is also prepared consisting of the emulsion with the solvent of the extracts, ethanol [42, 45, 46, 47, 24, 48, 49, 25].

5.8. Antibacterial Activity

The optical density (OD) technique was used to test the antibacterial activity of Coriaria thymifolia. The bacterial strain used in this test was *Escherichia coli* (DH5-alpha). Before starting the test, the bacteria must be inoculated one day before, for this, 50 uL of the bacterial strain was placed in a falcon tube together with 2 mL of the Luria Bertani broth base (LB). It was allowed to incubate for 24 hours at 37 ° C and with stirring. The next day, 3 mL of the prepared inoculate was used and placed in a falcon tube (tube a) together with 45 mL of LB to obtain an optical density measurement

(OD600) of 0.02. Eight different test tubes were prepared, each of the tubes in common containing 3 μL of the solution from tube a together with 2 mL of LB. Tube 1 only had the two solutions mentioned above, in tube 2 additional to the first one, 100 μL of 10% DMSO solvent was increased. In tubes 3, 4, 5, 6, 7, 8, 100 μL of fractions F1, F2, F3, F8, F10 and F12 respectively were added (fractions at 1mg/mL). After having prepared all the falcon tubes, they were taken to an incubator with constant agitation at 37 ° C during the 7 and a half hours in which the measurements were taken. The optical density measurements were taken using a Thermo-Scientific Nanodrop UV-Vis spectrophotometer, every 30 minutes at a wavelength of 600 nm.

5.9. UPLC-MS and HPLC

UPLC-MS spectrometry was performed on a Waters instrument comprising a binary system manager (ACQUITY UPLC® I-Class) with a reversed-phase column SunFire™ C18 3.5 μm (2.1×100 mm) and an automatic injector and Waters® SYNAPT® G2-S/Si as mass spectrometer. Linear gradients of MeCN (0.01% formic acid) into H_2O (0.01% formic acid) were run at flow rate of 0.3 mL/min. The solvents for UPLC were H_2O (Type I), and MeCN (HPLC quality) with a gradient 5 to 95% MeCN to H_2O.

HPLC analysis was performed in a DIONEX UHPLC 3000

6. CONCLUSIONS

- The results from the preliminary phytochemical screening showed that the *Coriaria thymifolia* plant extract contain a mixture of phytochemicals as tannins and phenolic compounds, flavonoids, glycosides and sterols. This suggests that the leaves of *Coriaria thymifolia* may have biological activities.
- Using the UV-VIS spectroscopic technique, characteristic absorption bands of flavonoids were found. This allows concluding that *Shanshi* extract contains this metabolite whose percentage and identity are unknown.
- UPLC-MS analysis tandem Progenesis software led to possible identification of batch of compounds presents in the plant.
- The β-carotene antioxidant bleaching test was developed using self extracted and purified β-carotene and isomeric mixture of linoleic acid.
- The Fraction E2C2F6 of the *Coriaria thymifolia* extracts exhibited significant antioxidant activity, leading to the conclusion that the extract of the *Shanshi* leaves has a rich source of natural antioxidant compounds, which can be justified by some secondary metabolite found in the qualitative tests. Therefore, an additional characterization is needed to know the identity of the compound responsible for this activity.
- The antibacterial activity test regarding technical issues gave positive results for Fraction E2C1F2 against the growth of the *E. Coli* bacteria strain

7. RECOMMENDATIONS

- In the antioxidant test, there was an additional step that was the extraction of β-carotene, it is important to use extraction processes and analysis techniques that do not cause isomerization and oxidation of this compound. Also, it is recommended to use it in the shortest possible time and store it in solvents with good affinity for this compound.
- In the growth curve of the antibacterial test, the graph did not have a fixed exponential growth, however, there were strong results regarding this

biological activity of the extract of *Coriaria thymifolia.* Therefore, it is recommended to perform this test again with a properly functioning device.

- The fraction E2C1F2 that gave positive result in both biological test should be re-purify using reverse phase cartridge due the separation of polar compounds in mixture using flash chromatography and silica gel in normal phase is very difficult. Posterior UPLC-MS, IR and NMR analysis should be performed to identify the active species.

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9. ANNEX

			Fractions						
Time	**Bacterium**	**DMSO 10%**	**E2C1 F1**	**E2C1 F2**	**E2C1 F3**	**E1C1 F8**	**E1C1F 10**	**E1C1F 12**	
0	0,023	0,023	0,023	0,023	0,023	0,023	0,023	0,023	**OD600**
30	0,033	0,03	0,033	0,036	0,033	0,039	0,034	0,034	
60	0,044	0,04	0,046	0,045	0,049	0,054	0,05	0,042	
90	0,064	0,058	0,063	0,061	0,058	0,064	0,057	0,063	
120	0,094	0,086	0,081	0,09	0,074	0,091	0,074	0,071	
150	0,103	0,091	0,098	0,061	0,087	0,107	0,097	0,085	
180	0,107	0,181	0,105	0,092	0,103	0,112	0,123	0,088	
210	0,144	0,199	0,197	0,119	0,199	0,246	0,201	0,101	
240	0,149	0,118	0,123	0,137	0,153	0,132	0,173	0,105	
270	0,148	0,118	0,139	0,101	0,174	0,153	0,107	0,108	
300	0,13	0,127	0,141	0,13	0,139	0,134	0,144	0,115	
330	0,135	0,124	0,141	0,135	0,122	0,134	0,125	0,115	

360	0,138	0,196	0,15	0,106	0,114	0,139	0,144	0,132	
390	0,195	0,131	0,181	0,122	0,169	0,175	0,204	0,162	
420	0,18		0,191	0,126	0,136	0,149	0,186	0,211	

Annex 1. Data about the measurements of OD600 for each one of the fractions used in the antibacterial test.

	Absorbance				
Time	E2C1F1	E2C1F2	E2C1F3	Ascorbic Acid	Control
0	0,685	0,647	0,714	0,657	0,591
30	0,552	0,502	0,463	0,61	0,55
60	0,503	0,468	0,399	0,561	0,543
90	0,469	0,449	0,366	0,525	0,53
120	0,442	0,429	0,337	0,464	0,506
150	0,426	0,414	0,315	0,48	0,468

Annex 2. Absorbance of fractions of the macerated extract against Beta-carotene bleaching.

	Absorbance								
Time	E2C2 F1	E2C2 F2	E2C2 F3	E2C2 F4	E2C2 F5	E2C2 F6	E2C1F2 Pattern	Control	Ascorbic Acid
0	1,41	1,361	1,385	1,4	1,344	1,439	1,439	1,359	1,419
30	1,178	1,15	1,15	1,16	1,13	1,216	1,27	0,732	1,188
60	0,858	0,857	0,877	0,862	0,837	0,985	0,982	0,462	0,962
90	0,655	0,618	0,642	0,7	0,632	0,79	0,77	0,367	0,738
120	0,519	0,481	0,537	0,547	0,555	0,631	0,621	0,338	0,576

Annex 3. Absorbance of fractionsE2C2F1, E2C2F2, E2C2F3, E2C2F4, E2C2F5 and E2C2F6 obtained from the fraction of Colum 1 against Beta-carotene bleaching.

One hundred entries – results from Progenesis Q1 software

Compound	m/z	Retention time (min)	Compound ID	Adducts	Formula	Description
1	333.0320	0.46	CSID14466524	M+H-H2O	C16H11N2NaO4S	4-(4-Hydroxy-1-naphthylazo)benzenesulfonic acid sodium salt
2	395.1449	1.68	CSID391155	M+Na, M+K	C21H24O6	fargesone A
3	395.1449	1.68	CSID391193	M+Na, M+K	C21H24O6	Kadsurin A
4	395.1449	1.68	CSID58506	M+Na, M+K	C21H24O6	(−)-Arctigenin
5	275.1313	0.9	CSID2624	M+H	C16H19ClN2	chlorphenamine
6	275.1313	0.9	CSID30576	M+H	C16H19ClN2	dexchlorpheniramine
7	324.0501	5.78	CSID2149	M+H-H2O	C17H11NO7	Aristolochic acid
8	324.0501	5.78	CSID571392	M+K	C10H12FN5O4	Fludarabine
9	715.2424	5.47	CSID5750	M+H	C32H52Br2N4O4	Demecarium bromide
10	292.9411	5.13	CSID4588899	M+H-H2O, M+NH4	C8H3Cl2N3O4	licostinel
11	561.1542	5.34	CSID30791012	M+NH4	C25H22N5NaO6S	Sodium (2S,5R,6R)-6-{[(2S)-2-{[(4-hydroxy-1,5-naphthyridin-3-yl)carbonyl]amino}-2-phenylacetyl]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate

12	561.1542	5.34	CSID8030195	M+Na	C27H29F3O6S	Fluticasone furoate
13	196.9810	9.12	CSID66716	M+K	C7H4F2O2	2,4-Difluorobenzoic acid
14	573.3523	7.30	CSID10631	M+H-H2O	C32H55BrN4O	Thonzonium Bromide
15	573.3523	7.30	CSID30791445	M+H	C30H53O8P	1-O-(Hydroxy{[(6Z,10E,14E)-3,7,11,15,19-pentamethyl-6,10,14,18-icosatetraen-1-yl]oxy}phosphoryl)-beta-D-xylopyranose
16	573.3523	7.30	CSID4588502	M+2Na-H	C33H52O5	(3beta,16alpha)-3-Acetoxy-16-hydroxy-24-methylenelanost-8-en-21-oic acid
17	640.5593	8.85	5283574	M+2Na-H	C38H77NO3	C20DH Cer; N-(Eicosanoyl)-Dihydroceramide; N-(Eicosanoyl)-Dihydroceramide :: Sphingolipids :: Lipids :: N-[(2S,3R)-1,3-dihydroxyoctadecan-2-yl]icosanamide
18	795.3100	5.42	CSID143441	M+K	C36H52N8O6S2	ascidiacyclamide
19	575.2441	8.21	CSID135759	M+K	C33H36N4O3	mozenavir
20	575.2441	8.21	CSID32524	M+NH4	C29H35NO8S	Bitolterol Mesylate
21	575.2441	8.21	CSID64519	M+NH4	C32H31ClN3O4	difenoximide hydrochloride
22	338.9269	6.37	CSID215802	M+Na	C14H8Cl4	1977640
23	338.9269	6.37	CSID2927	M+Na	C14H8Cl4	dichlorodiphenyldichloroethylene
24	691.3327	6.37	CSID24785666	M+NH4	C37H43N3O9	N1,N5,N10-Triferuloyl spermidine
25	331.0593	5.18	CSID31000	M+H-H2O, M+H	C17H12Cl2N2O	triarimol
26	331.0593	5.18	CSID39394	M+H-H2O, M+H	C17H12Cl2N2O	Fenarimol
27	465.0651	5.35	CSID20418	M+H-2H2O	C20H28Cl4N2O4	Teclozan
28	465.0651	5.35	CSID570933	M+H	C16H20N2O10S2	1-S-[2-(4-Hydroxy-1H-indol-3-yl)-N-(sulfooxy)ethanimidoyl]-1-thio-beta-D-glucopyranose
29	555.2226	0.6	CSID30791595	M+H, M+Na	C30H34O10	LAPPAOL C
30	619.2836	5.56	CSID4444692	M+2Na-H	C32H46O9	Cucurbitacin A
31	619.2836	5.56	CSID8051183	M+2Na-H	C33H50O4S2	Camobucol
32	469.1077	3.70	CSID8472911	M+Na, M+K	C23H23Cl2N2O3	3'-((2-(((2R)-2-(3-Chlorophenyl)-2-hydroxyethyl)amino)ethyl)amino)biphenyl-3-carboxylic acid hydrochloride
33	294.1913	8.49	CSID4444279	M+NH4	C14H20N4O2	p-coumaroylagmatine
34	294.1913	8.49	CSID90169	M+NH4	C13H24O6	Diethyl 2-(diethoxymethyl)succinate
35	294.1913	8.49	CSID9646210	M+NH4	C13H24O6	ascr#1
36	323.0110	4.08	CSID39727	M+H, M+K	C16H12O3S	tiopinac
37	471.0856	2.56	CSID29272772	M+2Na-H	C24H18N4O2S	4,7-Bis(4-methoxyphenyl)-6-(4-pyridinyl)[1,2,5]thiadiazolo[3,4-c]pyridine
38	471.0856	2.56	CSID391076	M+H-2H2O	C30H18O8	Cassiamin C
39	789.3904	2.73	52928344	M+K	C37H67O13P	PI 28:2 :: Phosphtatidylinositols :: Lipids :: [(2R)-3-[hydroxy-[(5R)-2,3,4,5,6-pentahydroxycyclohexyl]oxyphosphoryl]oxy-2-[(Z)-tetradec-9-enoyl]oxypropyl] (Z)-tetradec-9-enoate
40	789.3904	2.73	CSID24606166	M+H	C38H60O17	dulcoside B
41	789.3904	2.73	CSID24606167	M+H	C38H60O17	dulcoside A
42	562.8564	6.21	CSID6001	M+2Na-H	C17H12I2O3	Benziodarone

43	602.9272	8.94	CSID69649	M+K	C11HF21O2	PFUnDA
44	166.0178	8.95	CSID391411	M+NH4	C6H6Cl2	5,6-Dichloro-1,3-cyclohexadiene
45	232.9465	8.92	CSID391845	M+H-H2O	C7H7IO2	2-Iodo-6-methoxyphenol
46	271.9967	8.61	CSID184723	M+Na	C6H8ClN5O2S	Clothianidin
47	640.5592	8.52	5283574	M+2Na-H	C38H77NO3	C20DH Cer; N-(Eicosanoyl)-Dihydroceramide; N-(Eicosanoyl)-Dihydroceramide :: Sphingolipids :: Lipids :: N-[(2S,3R)-1,3-dihydroxyoctadecan-2-yl]icosanamide
48	291.0827	5.16	CSID165782	M+Na	C13H16O6	(1S,3R,4S)-1,6,8-Trihydroxy-3,4,5-trimethyl-3,4-dihydro-1H-isochromene-7-carboxylic acid
49	291.0827	5.16	CSID25677794	M+Na	C13H16O6	Dimethyl (3,4-dimethoxyphenyl)malonate
50	291.0827	5.16	CSID2688	M+H-H2O	C14H17ClN4S	Clobenpropit
51	553.2686	2.65	CSID135920	M+Na	C32H38N2O5	Bazedoxifene Acetate
52	553.2686	2.65	CSID59633	M+Na	C32H38N2O5	Cortivazol
53	344.8824	5.06	CSID15822	M+Na	C12H6Cl4S	1884271
54	500.9889	8.90	CSID28651810	M+K	C18H17Cl3FN4OS	Afuresertib hydrochloride
55	599.2421	3.58	CSID143847	M+H	C28H38Cl2N4O6	IGANIDIPINE DIHYDROCHLORIDE
56	599.2421	3.58	CSID4952942	M+2Na-H	C32H43ClN2O2S	Zuclopenthixol decanoate
57	291.9841	9.09	CSID30791295	M+K	C10H7NO7	5-[(1Z)-3-Carboxy-3-oxo-1-propen-1-yl]-4-hydroxy-6-oxo-1,6-dihydro-2-pyridinecarboxylic acid
58	291.9841	9.09	CSID4444363	M+K	C10H7NO7	5-[(1Z)-3-Carboxy-3-oxo-1-propen-1-yl]-4,6-dioxo-1,4,5,6-tetrahydro-2-pyridinecarboxylic acid
59	291.9841	9.09	CSID4710689	M+K	C10H7NO7	3-[(1E)-3-Carboxy-3-oxo-1-propen-1-yl]-6-hydroxy-4-oxo-1,4-dihydro-2-pyridinecarboxylic acid
60	579.1431	9.37	CSID391109	M+2Na-H	C26H30O12	Dalpanin
61	579.1431	9.37	CSID4981055	M+2Na-H	C25H25F3N4O6	Delamanid
62	699.2477	6.35	CSID24606866	M+2Na-H	C32H55BrO5Si2	2-(4-Bromophenyl)-2-oxoethyl (9Z)-12,13-bis[(trimethylsilyl)oxy]-9-octadecenoate
63	699.2477	6.35	CSID24606867	M+2Na-H	C32H55BrO5Si2	2-(4-Bromophenyl)-2-oxoethyl (12Z)-9,10-bis[(trimethylsilyl)oxy]-12-octadecenoate
64	526.1126	5.13	CSID39831	M+H	C18H23N9O4S3	Cefotiam
65	387.2786	6.15	CSID76416	M+Na	C19H42NO3S+	N,N-Dimethyl-N-(3-sulfopropyl)-1-tetradecanaminium
66	473.1910	0.6	CSID4939322	M+H-2H2O, M+NH4	C24H32N2O10	cinepazet maleate
67	473.1910	0.6	CSID5293745	M+H-2H2O, M+NH4	C28H29F5O3	lonaprisan
68	425.3024	8.11	CSID390320	M+NH4	C23H37NO5	Cammaconine
69	425.3024	8.11	CSID4444677	M+NH4	C23H37NO5	Norerythrostachaldine
70	425.3024	8.11	CSID61837	M+NH4	C23H38ClN3O	disobutamide
71	672.3974	8.61	CSID22913871	M+H-H2O	C34H59NO13	Fumonisin B4
72	455.0627	5.16	CSID168435	M+K	C15H20N4O8S	O-Carbamoyl-deacetylcephalosporin C
73	455.0627	5.16	CSID30791384	M+Na	C20H17ClN2O7	4-Aminoanhydrochlortetracycline
74	455.0627	5.16	CSID49630	M+2Na-H	C16H18N4O7S	Bensulfuron-methyl [ANSI, WSSA]
75	143.0116	5.44	11671	M+K	C4H8O3	Alpha-Hydroxyisobutyric Acid :: Carboxylic Acids :: Polar Metabolites :: 2-hydroxy-2-methylpropanoic acid
76	143.0116	5.44	440864	M+K	C4H8O3	2-Hydroxybutyric Acid :: Carboxylic Acids :: Polar Metabolites :: (2S)-2-hydroxybutanoic acid

77	143.0116	5.44	92135	M+K	C4H8O3	3-Hydroxybutanoic Acid :: Carboxylic Acids :: Polar Metabolites :: (3R)-3-hydroxybutanoic acid
78	376.2752	8.18	CSID10128289	M+H-H2O	C25H35N3O	2-{(E)-[(5Z)-3-Methoxy-5-(2H-pyrrol-2-ylidene)-1,5-dihydro-2H-pyrrol-2-ylidene]methyl}-5-undecyl-1H-pyrrole
79	376.2752	8.18	CSID10570899	M+H-H2O	C25H35N3O	Undecylprodigiosin
80	376.2752	8.18	CSID7997700	M+H-H2O	C25H35N3O	amesergide
81	579.2184	3.06	CSID571019	M+Na	C22H40N2O12S	UNII:925FX3X776
82	579.2184	3.06	CSID8069005	M+2Na-H	C28H38ClN2O6	GX 1296B
83	377.2034	4.77	91451	M+2Na-H	C21H32O3	17-hydroxypregnenolone :: Steroids :: Lipids :: 1-[(3S,8R,9S,10R,13S,14S,17R)-3,17-dihydroxy-10,13-dimethyl-1,2,3,4,7,8,9,11,12,14,15,16-dodecahydrocyclopenta[a]phenanthren-17-yl]ethanone
84	377.2034	4.77	CSID135707	M+NH4	C21H23F2NO2	etoxazole
85	272.1305	3.39	CSID23628	M+H-H2O	C16H20ClN3	chloropyramine
86	272.1305	3.39	CSID453423	M+NH4	C12H18O4Si	Trimethylsilyl 3,4-dimethoxybenzoate
87	272.1305	3.39	CSID77680	M+H-2H2O	C16H22ClN3O	tebuconazole
88	250.9174	5.13	CSID229709	M+2Na-H	C8H5Cl3	1-Chloro-4-(2,2-dichlorovinyl)benzene
89	774.2831	7.87	CSID4445097	M+NH4	C34H44O19	Myricoside
90	407.3380	7.65	CSID391273	M+Na	C26H44N2	Buxamine E
91	481.1096	3.99	CSID54853	M+Na, M+K	C23H19N2NaO4S	DARGLITAZONE SODIUM
92	.	.	CSID4445282	M+2Na-H	C30H46N4O16	(2E)-N-[(2R,3R,4R,5R,6R)-2-{[(2R,3R,4R,5S,6R)-3-Acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]oxy}-6-{(2S)-2-[(2R,3S,4R,5R)-5-(2,4-dioxo-3,4-dihydro-1(2H)-pyrimidinyl)-3,4-dihydrox
95	484.9435	7.04	CSID4444084	M+Na	C15H10O13S2	quercetin 3,4'-bissulfate
96	469.1300	5.47	CSID391614	M+H-2H2O, M+H	C18H34ClN2O8PS	clindamycin-2-phosphate
97	469.1300	5.47	CSID5293534	M+H-2H2O, M+H	C21H23Cl2F3N6O	Vofopitant dihydrochloride
98	469.1300	5.47	CSID8521497	M+H-2H2O, M+H	C18H34ClN2O8PS	Clindamycin phosphate
99	629.3990	5.44	CSID140040	M+2Na-H	C33H60O8	PM-Toxin A
100	629.3990	5.44	CSID4446657	M+K	C39H58O4	ubiquinone-6

Annex 4. List of 100 hundred compound detected and identified by Progenesis together with ChemSpider database