UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY TECH

Escuela de Ciencias Químicas e Ingeniería

TÍTULO: Phytochemical characterization and antibacterial activity of bioactive components from methanolic extracts of *Eupatorium glutinosum* Lam. leaves.

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

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Urcuquí, marzo 2021

Urcuquí, 6 de mayo de 2021

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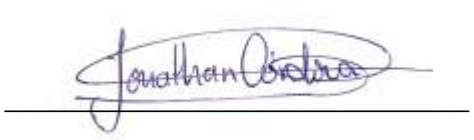

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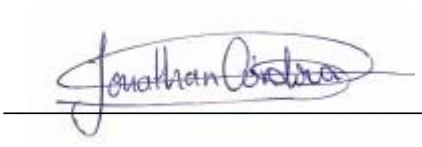

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Dedicatoria

Este largo camino no hubiese sido posible culminarlo sin el incondicional apoyo de mis queridos padres, Vinicio y Norma, quienes con su ayuda, motivación y gran amor me han impulsado a ser mejor cada día. Han sido un pilar a lo largo de toda mi vida, nunca me han limitado en mis sueños, ni mucho menos han cuestionado mis decisiones, además me han enseñado que la perseverancia, convicción, esfuerzo, respeto y honestidad son factores que me ayudarán a cumplir todas mis metas. Estoy eternamente agradecido con ellos por todo lo que me han podido dar. Por esta y muchas más razones, éste trabajo de investigación es dedicado a mis padres. Este logro no es solamente mío, es de todas las personas que confiaron y creyeron en mí.

Eternamente agradecido,

Jonathan Alexis Córdova Naranjo

Agradecimientos

Para lograr este sueño muchas personas han sido participes, y me gustaría agradecer a cada una de ellas por el cariño, ayuda y compresión que me han brindado a lo largo de la carrera.

Para empezar, me gustaría agradecer a mis padres por todo el apoyo y amor que me han brindado a lo largo de mi existencia, además que con todo su esfuerzo y lucha han logrado ayudarme a culminar mis estudios universitarios. También quiero agradecer a Sofía, mi hermana, que con cada locura y travesura me ha llenado de alegría y motivación para seguir luchando hasta conseguir este sueño. Agradezco también a mis abuelitos, Mentor, María, Sixto y Delia, que con sus consejos y anécdotas han hecho de mí un hombre que luche por sus sueños.

Quiero también agradecer a la familia que elegí, mis amigos, quienes estuvieron en los buenos y malos momentos a lo largo de estos 5 años, pero en especial quiero agradecer a Christian Redroban y Steven Brito, por ser como un hermano para mí, siempre estuviste dispuesto a ayudarme en cualquier momento; Katherine Hinojosa, por ser alguien que estuvo conmigo en las buenas y en las malas; Michael Jiménez y Emilia Calle, por esos días de estudio que entre risas y sanduchitos aprendimos cosas académicas y de la vida, los considero como mis mejores amigos.

A mi tutora, Hortensia Rodríguez, a quién yo considero como mi madre académica, por haber estado al pendiente de mi a lo largo de este proceso, también por ayudarme con muchos de los materiales que necesité para llevar a cabo esta investigación. Quiero agradecer también a mi co-tutor, Nelson Vispo, quien ha estado disponible para aclarar mis dudas a cualquier momento, además ha sido de gran ayuda al momento de llevar a cabo los experimentos para determinar las actividades biológicas.

A todos los profesores que tuve a lo largo de mi carrera por inculcar en mí el espíritu de la investigación y el amor a la química, pero en especial a los profesores Manuel Caetano, Lola De Lima y Juan Pablo Saucedo por ayudarme a realizar los análisis de HPLC, NMR y apoyarme en lo que más pudieron para lograr culminar este proyecto.

Finalmente, quiero agradecer a la Escuela de Ciencias Químicas e Ingeniería de la Universidad Yachay Tech, ya que éste es el lugar en donde formé mis conocimientos que aplicaré profesionalmente, y sobre todo por ser el lugar en donde conocí personas extraordinarias.

Jonathan Alexis Córdova Naranjo

Resumen

La medicina tradicional ha sido usada desde la prehistoria, el uso de plantas medicinales es una de sus herramientas principales. En este contexto, las comunidades indígenas usan productos naturales debido a sus propiedades curativas frente a un sinnúmero de enfermedades, agrupando todos estos usos en el conocimiento ancestral. Toda esta información adquirida por las comunidades indígenas acerca de medicina tradicional, se transmite de una generación a otra a través del tiempo. Algunas investigaciones desarrolladas en esta temática, han demostrado que la bioactividad de las plantas medicinales se debe a la presencia de metabolitos secundarios, los cuales en solitario o con sinergias, producen determinada actividad. *Eupatorium glutinosum* Lam, popularmente conocido como Matico, es una planta medicinal ampliamente utilizada en la medicina tradicional ecuatoriana. En este trabajo, se revisa el estado del arte acerca de *Eupatorium glutinosum* Lam y se profundiza en el estudio fitoquímico de extractos metanólicos y acuosos. Los extractos metanólicos se prepararon usando procedimientos de Soxhlet y Maceración, mientras el extracto acuoso se obtuvo mediante maceración. Posterior a la identificación de los metabolitos secundarios presentes en los extractos metanólicos, se sometieron a una separación mediante cromatografía de columna. Las fracciones obtenidas fueron analizadas mediante Cromatografia de Capa Fina (TLC) y Cromatografia liquida de alta resolución (HPLC), para determinar componentes y pureza de las fracciones. Además, se utilizaron la espectroscopía infrarroja (FTIR), y la Resonancia magnética nuclear (NMR) para establecer la naturaleza de los grupos funcionales presentes en los compuestos de cada fracción. Finalmente, se estudió la actividad antibacterial de los extractos y las fracciones aisladas mediante los métodos de difusión en agar y mediciones de densidad óptica con la cepa bacteriana *Escherichia coli* (DH5- α) obteniendo resultados prometedores, ya que varias de las muestras analizadas mostraron una actividad antimicrobiana similar a la observada para el control positivo (ampicilina). Un análisis de todos los resultados nos permitió sugerir la naturaleza de los componentes biológicamente activos de los extractos metanólicos. Los resultados no son definitivos, puesto que se necesita un estudio estructural exhaustivo para elucidar inequívocamente las moléculas presentes en los extractos estudiados.

Palabras Clave:

Medicina tradicional, productos naturales, *Eupatorium glutinosum* Lam, Matico, metabolitos secundarios, análisis fitoquímico, actividad antibacteriana.

Abstract

Traditional medicine has been used since prehistoric times. The use of medicinal plants is the principal tool of this type of therapy. Indigenous communities used medical plants owing to their excellent biological response in the human body. Due to the mentioned, the knowledge earned by the indigenous communities in traditional medicine is transmitted through time from generation to generation. Several investigations have demonstrated that the medicinal plants' successful biological response is due to the secondary metabolites. *Eupatorium glutinosum* Lam, popularly called Matico, is a medicinal plant used in traditional Ecuadorian medicine. In this work, state-of-the-art about *Eupatorium glutinosum* Lam is reviewed, and the phytochemical study of methanol and aqueous extracts is deepened. Methanolic extracts were prepared using Soxhlet and Maceration procedures, while aqueous extract was obtained by maceration. After identifying the secondary metabolites present in the methanolic extracts, they were subjected to separation by column chromatography. The obtained fractions were analyzed by Thin Layer Chromatography (TLC) and high-resolution liquid chromatography (HPLC), to determine components and purity of the fractions. Besides, infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) were used to establish the nature of the functional groups present in each fraction's compounds. Finally, the antibacterial activity of the extracts and isolated fractions was studied by means of agar diffusion methods and optical density measurements with the bacterial strain *Escherichia coli* (DH5-α), obtaining promising results, since several of the samples analyzed showed antimicrobial activity similar to that observed for the positive control (Ampicillin). An analysis of all the results allowed us to suggest the nature of the methanolic extracts' biologically active components. The results are not definitive since an exhaustive structural study is needed to elucidate the molecules present in the extracts studied unequivocally.

Keyword:

Traditional medicine, Natural products, *Eupatorium glutinosum* Lam, Matico, secondary metabolites, phytochemical analysis, antibacterial activity.

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Chapter 1.

1. Introduction-Justification

1.1. General introduction

Since the human being's apparition in the earth during prehistoric times, the necessity of taking them care from diseases has been one of the major problems to survive. Therefore, to solve this issue, human beings have occupied natural products, such as plants, animals, marine organisms, to alleviate and treat some diseases[1]. The ancestral procedure of using natural products to treat diseases has been preserved along the time in different civilizations despite not knowing the actual consequences of consuming natural products. Further, the ancestral procedure of using natural products is denominated as traditional medicine.

Advancing in time, many traditional indigenous communities had prolonged contact with plants having medicinal properties. Due to this relationship, one of the first approaches to medicine was made by indigenous communities when they used several plants from their contour to alleviate some illnesses[2]. Due to this, the knowledge and uses obtained from these plants had been transmitted from generation to generation.

Nowadays, traditional medicine is prevalent, and plants still present a vast source of structurally novel compounds that may serve as scaffolds for the development of new drugs[2].

As a specific case, the genus *Eupatorium* belongs to the Eupatorieae, one of the thirteen genera of the Asteraceae family[3,4]. Moreover, the mentioned genus has approximately 1200 species distributed throughout America, Europe, Africa, and Asia. Eupatorium is well known for its medicinal properties for many decades, mostly the *Eupatorium glutinosum* Lam. species is known for its antibacterial, astringent, antirheumatic activities. For that reason, this plant has been employed in folk medicine in Ecuador[5]. The indigenous communities that use the plant get it at an altitude of about 3000 m in the Ecuatorian Andes[6].

The presence of terpenes, flavonoids, phytosterols, and sesquiterpenes lactones[2,7] have been demonstrated by studies performed over this gender, which is responsible for the biological activities previously mentioned. Due to its beneficial bioactivity, the chemical compounds found in *Eupatorium glutinosum* L. are identified as a promising source for

developing new drugs. However, related to *Eupatorium glutinosum* Lam., few phytochemical, chemical, antibacterial studies are done over it [7].

1.2. Problem Statement

Starting from the taxonomic classification of the studied plant, **Kingdom:** Plantae, **Subkingdom:** Viridiplantae, **Infrakingdom:** Streptophyta, **Superdivision:** Embryophyta, **Division:** Tracheophyta, **Subdivision:** Spermatophytina, **Class:** Magnoliopsida, **Superorder:** Asteranae, **Order:** Asterales, **Family:** Asteraceae, **Genus:** Eupatorium, **Specie:** *Eupatorium glutinosum*[2,3,8,9], is possible to search chemical and biological information.

As a result of the investigation, the chemical composition and biological activity of *Eupatorium glutinosum* Lam. has not been thoroughly studied. However, based on the few analyses reported during the last decades by El-Seedi et al. [5,7,10], and de las Heras et al. [11], some biological activities have been reported for *Eupatorium glutinosum* Lam. Since its first description in 2001, where El-Seedi et al.[7] described two diterpenes in the Ethyl Acetate (EtOAc) extract of the leaves from the plant, the interest of knowing the biological response of the reported diterpenes were investigated. Interestingly in 2002, El-Seedi et al.[5] based on the reported structures, tested the isolated compounds for antibacterial activity, resulting in the expected biological response. Also, in 2006 El-Seedi [10] demonstrated that similar biological activity is present when the n-hexane extract is tested over antibacterial analysis. Hence, it is proved that the secondary metabolites present in the EtOAc and n-hexane extracts have antimicrobial activity. The investigation done by El-Seedi [10] in 2006 was not only focused on the biological activity of the essential oil from *Eupatorium glutinosum* Lam.; he also performed a phytochemical screening finding the presence of twenty three know compounds and traces of unkown compounds. Taking into account the little information related to biological properties of *Eupatorium glutinosum* Lam., the search of other type of extracts from this plant can give a better understanding about it use in tradicional medicine, as well as it can provide more scientific support for its medical use. In addition, complementary chemical studies over the plant extracts can give a complete screening of its phytochemical composition and properties.

Therefore, as the **hypothesis**, a phytochemical screening of the methanolic extract, and the chemical analysis of the isolated fractions of *Eupatorium glutinosum* Lam. leaves

could open the way in the search and identification for new bioactive principles while generating knowledge about its mode of action and potential bioactivity.

1.3. General and specific objectives

1.3.1. General objective

The present research aims to carry out a preliminary phytochemical screening of methanolic and aqueous extracts of *Eupatorium glutinosum* Lam. leaves and determine the antibacterial activity of isolated fractions of methanolic extracts to deepen the knowledge related to the properties of matico.

1.3.2. Specific objectives

- To perform the aqueous and methanolic extraction of the *Eupatorium glutinosum* Lam. leaves by both, Soxhlet and maceration methods.
- To carry out a phytochemical screening of aqueous and methanolic extracts from *Eupatorium glutinosum* Lam. leaves.
- To separate chemical entities present in the methanolic extracts of the *Eupatorium glutinosum* Lam. leaves using Column Chromatography.
- To characterize the isolated fractions from the methanolic extract of the *Eupatorium glutinosum* Lam. using Fourier Transform Infrared Spectroscopy (FT-IR), Nuclear Magnetic Resonance (NMR), High-Performance Liquid Chromatography (HPLC).
- To evaluate the crude methanolic extract's antibacterial activity and the isolated fractions from the methanolic extract of the *Eupatorium glutinosum* Lam. leaves.
- To propose the bioactive components or fractions from the methanolic extract of the *Eupatorium glutinosum* Lam. leaves.

Chapter 2.

2. Methodology

2.1. General information

2.1.1. Reagents

All of the reagents used during this research project's experimental work were purchased from commercial sources and used without further purification. Acetonitrile and toluene with HPLC grade, hydrochloric acid 12.1N, methanol, hexane and ethyl acetate with Certified ACS grade, sodium carbonate ,copper (II) sulphate pentahydrated and sodium citrate dihydrated with Granular Certified were purchased from Fisher Chemical; ammonia solution 25%, sulphuric acid for analysis (95-97%), sodium chloride and sodium hydroxide with Certified ACS grade, and magnesium powder particle size about 0.06 – 0.3 mm were purchased from Merk KGaA; ninhydrin for amino acid determination and ethylenediaminetetraacetic acid disodium salt dihydrate for molecular biology were purchased from SIGMA-Aldrich ® Life Science; chloroform 99.8%, citric acid anhydrous 99.5%, and dextrose anhydrous AR with Bacteriological grade were purchased from LOBA Chemie; glacial acetic acid with Certified ACS grade was purchased from J.T. Baker; iron (III) chloride hexahydrated with ≥ 97% for analysis was purchased from ISOLAB ® chemicals; potassium sodium tartrate tetrahydrate with Certified ACS grade for analysis was purchased from AppliChem®Panreac ITW Companies. silica 60 0.04-0-063 mm by Marcherey-Nagel were used for Column Chromatography, TLC silica gel 60 plates was purchased from Merk KGaA were used for thin-layer chromatography.

2.1.2. Equipment

High-Performance Liquid Chromatography was performed in an UltiMate 3000 UHPLC from ThermoFisher Scientific™, with a reversed-phase, C-18 column for HPLC Hypertensil GOLD™ (150 x 4.6 mm), with an UltiMate WPS-3000RS/TRS Rapid Separation Well Plate Autosamplers as injector, with a quaternary pump, and with a photodiode array detector (PAD). Fourier Transform Infrared Spectroscopy (FT-IR) was performed in a Cary 630 FTIR Spectrometer. The analysis is done by the Total Reflection Attenuated (ATR) method. Nuclear Magnetic Resonance, ^{1}H-NMR, and ^{13}C-NMR, was carried out in a NMReady60 benchtop spectrometer from Nanalysis Corp

SL 40R Centrifuge from ThermoFisher Scientific™, MaxQ™ 4450 Benchtop Orbital Shakers (Digital Shaker) from Thermo Scientific™ Company was used in the samples

preparation Biological analysis. Nanodrop Uv-Vis Spectrophotometer from Thermo Fisher Scientific™ Company and Heratherm Refrigerated incubator IMP-180 from Thermo Fisher Scientific™ Company was operated for Biological tests. All biological assays were realized in AV-30/70 vertical laminar Flow bech from Teslar.

2.2. Chemical section

2.2.1. Aqueous and Methanolic extraction of the leaves from *Eupatorium glutinosum* Lam.

The previously reported procedures for Soxhlet extraction and maceration was followed with minor modifications. [12,13]

The plant (*Eupatorium glutinosum* Lam.) was collected based on its use in folk medicine in Ibarra and Ambato-Ecuador's flowering season. The plant was sun-dried for ten days and pulverized. A total of 16 g of the plant leaves were obtained to subject them to the extraction process using methanol as solvent.

Soxhlet Method (SE)

Eight grams of the plant's pulverized leaves were put into a soxhlet extractor connected to a 500 mL flask containing 190 mL of methanol. The extraction was performed at the boiling temperature of methanol (64,7 °C) for four hours for 21 cycles of extraction. The extract was refrigerated for two days. After this time, a gray precipitate appears, collected, labeled as **F0JC**, and stored for further analysis. After the solid separation, the solvent was rotoevaporated until the complete removal of methanol, allowing for 700 mg of dry extract (Soxhlet extract (**SE**), a yield of 8,75% of the whole mass).

Maceration Method (ME)

The same mass of leaves (8 grams) previously use in the Soxhlet method was placed inside 500 mL beaker. Then, 200 mL of methanol was added to the beaker, and the mixture was putting to macerate for 14 days. After that time, the mixture was filtrated to separate the methanolic extract from the leaves. The extract was rotoevaporated, allowing to obtain 680 mg of dry extract (Maceration Extract (**ME**), a yield of 8,5% of the whole mass).

Maceration Method for aqueous extract

In a beaker of 500 mL, the same mass of leaves (8 grams) as in the **SE** and **ME** extracts was added. Then, 200 mL of distilled water was placed inside the beaker, and the mixture was putting to macerate for 14 days. After that time, the mixture was filtrated and left to evaporate at room temperature from the aqueous extract. Notice that, in this case, the yield was not determined because this extract was only used for phytochemical screening.

Thin Layer Chromatography (TLC)

The Thin Layer Chromatography was carried out on **SE,** and **ME** extracts and all fractions using TLC pre-coated plates (silica gel $60F_{254}$) and one ascending technique. The plates were cut and marked with a pencil about 1 cm from the bottom of the plate. Each sample was uniformly applied on the plates using capillary tubes and allowed to dry. The TLC was developed in a chromatographic tank using Tol/EtOAc 8:2 and Hex/Tol/EtOAc/MeOH (2:5:2:1) as solvent systems for the **SE** and **ME**, respectively. The plates were dried and visualized under daylight, UV light (254 and 325 nm), and I_2 steam.

2.2.2. Phytochemical Qualitative Analysis

The methanolic and aqueous extracts of leaves from *Eupatorium glutinosum* were assessed for the phytochemicals' existence by using standard procedures reported in Ref.[14–21] with minor modifications.

2.2.2.1 Test for phenols and tannins[17,21]

1 mL of crude methanolic extract of leaves from *Eupatorium glutinosum* Lam. was mixed with 1 mL of 2% solution of $FeCl_3$ in a test tube. A blue-green or black coloration indicated the presence of phenols and tannins.

2.2.2.2 Test for flavonoids (Alkaline reagent test)[21]

1 mL of crude methanolic extract of leaves from *Eupatorium glutinosum* Lam. was mixed with 1 mL of 2% NaOH solution in a test tube. An intense yellow color was formed with turned colorless after adding few drops of diluted hydrochloric acid indicated flavonoids.

2.2.2.3 Test for flavonoids (Shinoda test)[20]

A 1 mL of crude methanolic extract of the leaves from *Eupatorium glutinosum* Lam. and ten drops of diluted hydrochloric acid were added in a test tube, followed by 500 mg of

magnesium Pink, Reddish Pink, or Brown color was produced in the presence of flavonoids.

2.2.2.4 Test for proteins or amino acids (Ninhydrin test)[20,21]

A 1 mL of crude methanolic extract of the leaves from *Eupatorium glutinosum* Lam. and 1 mL of freshly prepared 0.2% ninhydrin reagent were added in a test tube heated to boiling for 1-2 min. A blue or violet color of the sample suggests the presence of amino acids and proteins.

2.2.2.5 Test for Reducing Sugars (Fehling test)[17,21]

Equal volumes of freshly Fehling A and Fehling B reagents were mixed in a test tube, and 1 mL of it was added to another test tube containing 1 mL crude methanolic extract of *Eupatorium glutinosum* Lam. and gently boiled. The solution was observed for a color change. The formation of a purple ring at the interphase of two liquids or the presence of a brick-red precipitate indicates the presence of reducing sugars.

2.2.2.6 Test for saponins [21]

In a test tube, 1 mL of crude methanolic extract of leaves from *Eupatorium glutinosum* Lam. was mixed with 5mL of distilled water in a test tube, and it was shaken vigorously. The formation of stable foam was taken as an indicator for the presence of saponins.

2.2.2.7 Test for Carbohydrates (Benedit test)[21]

1 mL of the crude methanolic extract was mixed with 1 mL of Benedict reagent in a test tube and then is boiled. If a reddish-brown precipitate is formed, it indicated the presence of carbohydrates.

2.2.2.8 Test for Steroids (Libermann test)[21]

1 mL of crude methanolic extract of leaves from *Eupatorium glutinosum* Lam. was mixed with 2 mL of chloroform and 2 mL of acetic acid in a test tube. The mixture was cooled in ice. Carefully, 0,5 mL of concentrated sulphuric acid was added drop by drop. A color change to violet, blue or green indicates the presence of steroidal molecules.

2.2.2.9 Test for Terpenoids (Salkowski test)[21]

1 mL of crude methanolic extract of leaves from *Eupatorium glutinosum* Lam. was mixed with 2 mL of chloroform in a test tube. Carefully, 0,5 mL concentrated sulphuric acid was added drop by drop and heated for 2 minutes. A color change to grayish indicates the presence of terpenoids.

2.2.2.10 Test for Cardiac Glycosides (Keller-kilani test)[20,21]

1 mL of crude methanolic extract of leaves from *Eupatorium glutinosum* Lam. was mixed with 1 mL of glacial acetic acid containing two drops of 2% solution $FeCl_3$ in a test tube. The mixture was poured into another test tube containing 1 mL of concentrated sulphuric acid. A Brown ring at the interphase or a blue coloration in the acetic acid layer and red color in the two acids' interphase indicated the presence of glycosides.

2.2.2.11 Test for Alkaloids (Wagner test)[16,20,21]

1 mL crude methanolic extract of *Eupatorium glutinosum* Lam. was acidified with 1 mL of 1% hydrochloric acid in a test tube, and then it was heated gently. After, 10 to 20 drops of Wagner reagent were added. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

All of the exposed tests were also done over the previously prepared aqueous extract of leaves from *Eupatorium glutinosum* Lam. (**Table 1**).

Table 1 Phytochemical screening of methanolic and aqueous extracts.

Qualitative Phytochemical Analysis			
Secondary Metabolite	**Performed Test**	**Results**	
		ME Extract	**Aqueos Extract**
Phenols and Tannins	Phenols and Tannins' tests[17,21]	+	+
Flavonoids	Alkaline reagent Test[21]	-	+
	Shinoda Test[20]	-	+
Proteins/Amino Acids	Ninhydrin Test[20,21]	+	/
Reducing Sugars	Fehling Test[17,21]	+	-
Saponins	Saponins' Test[21]	+	/
Carbohydrates	Benedit Test[21]	-	-
Steroids	Libermann Test[21]	+	+
Terpenoids	Salkowski Test[21]	+	+
Cardiac Glycosides	Keller-kilani Test[20,21]	+	+
Alkaloids	Wegner Test[16,20,21]	+	+

(+) means the presence of the secondary metabolite, (-) means the absence of the secondary metabolite, (/) means the possible presence of the secondary metabolite

2.2.3. Isolation by Column Chromatography

The procedure reported in Ref.[22] was followed with minor modifications. The two methanolic extracts (**SE** and **ME**) were fractionated by Column Chromatography using different elution systems. The height of each column chromatography was 25 cm.

The methanolic extract **SE** (400 mg of the dry extract) was fractionated by column chromatography using silica gel 60 and eluted by toluene-EtOAc of increasing polarity (10:0, 9:1, and 8:2), to give four fractions (**F1JC**, **F2JC**, **F3JC**, and **F4JC**, See **Table 2**). Then, methanol was used as the final eluent to give one fraction (**F5JC**, See **Table 2**). All fractions were rotoevaporated and dried in vacuum until constant weight. Then, all collected fractions were subjected to TLC silica gel 60 plates using Tol/EtOAc 8:2 as the solvent system and revealed using UV lamp (at λ of 254 and 365 nm), and iodine chamber was also used. The fractions were pooled according to their Rf values (**Table 2**).

Table 2 Separation by Column Chromatography of the Methanolic extract obtained from the Soxhlet method.

Eluent System	Rf of pooled fractions	Pooled Fractions	Mass (mg)	Yield* (%)
Solid precipitate	NA	**F0JC**	7,6	1,9
Toluene/EtOAc (10:0)	0,79	**F1JC**	19	4,8
	0,50			
Toluene/EtOAC (9:1)	0,54	**F2JC**	31,9	8,0
	0,50			
Toluene/EtOAC (8:2)	0,70	**F3JC**	13,8	3,5
	0,70	**F4JC**	42,1	10,5
	0,57			
	0,30			
100% Methanol	0,57	**F5JC**	19,7	4,9
	0,30			
	0,20			

Yield was calculated based on the 400 mg of starting extract **SE.*

The methanolic extract **ME** (400 mg of the dry extract) was also fractionated by column chromatography using silica gel 60 methods, and eluted by n-hexane-EtOAc of increasing polarity (10:0, 9.5:0.5, 9:1, 8.5:1.5, 8:2, 7:3, and 4:7), to give 18 fractions. All fractions were rotoevaporated and dried in vacuum until constant weight.

All collected fractions were subjected to TLC silica gel 60 plates using Tol/EtOAc (8:2) in the first seven fractions, and Hex/Tol/EtOAC/MeOH (2:5:2:1) for the rest ones as

solvent systems, and revealed using a UV lamp (at 254 and 365 nm), but also an iodine chamber was used. The fractions were pooled according to their Rf values **Table 3**.

Table 3 Separation by Column Chromatography of the Methanolic extract obtained from the Maceration method.

Eluent System	Rf of Pooled Fractions	Pooled Fractions	Mass (mg)	Yield* (%)
n- hexane/EtOAc (10:0)	0,97	**A01JC**	21,7	5,4
n- hexane/EtOAc (95:5)	0,79	**A02JC**	24,7	6,2
n- hexane/EtOAc (9:1)	0,5	**A03JC**	10,6	2,7
	0,54	**A04JC**	25,5	6,4
	0,48	**A05JC**	7,6	1,9
	0,48	**A06JC**	18,2	4,6
	0,7			
n- hexane/EtOAc (85:15)	0,7	**A07JC**	17	4,3
	0,42	**A08JC**	7,2	1,8
	0,42	**A09JC**	12,6	3,2
	0,4			
	0,4	**A10JC**	10,7	2,7
	0,38	**A11JC**	4,9	1,2
n- hexane/EtOAc (8:2)	0,38	**A12JC**	6,9	1,7
	0,36			
	0,36	**A13JC**	8,9	2,2
n- hexane/EtOAc (7:3)	0,34	**A14JC**	9,3	2,3
	0,32	**A15JC**	4,2	1,1
	0,3			
	0,3	**A16JC**	8,7	2,2
n- hexane/EtOAc (4:7)	0,3	**A17JC**	6,2	1,6
	0,2	**A18JC**	6,3	1,6

Yield was calculated based on the 400 mg of starting extract **ME.*

2.2.4. Preliminary Chemical Characterization

The methanolic extracts (**SE** and **ME**), and the fractions obtained from the column chromatographies were analyzed for chromatographic and spectroscopic techniques such as HPLC, FT-IR, and NMR.

2.2.4.1. HPLC analysis

SE, **ME**, and all pooled fractions were analyzed using HPLC. HPLC analysis was carried out using a 3000 UltiMate UHPLC instruments with a UV-Vis detector at a wavelength of 254 nm. Gradient elution was performed at room temperature with solution A (type 1

water) and solution B (Acetonitrile) in the following elution program: 0-1 min 100% of solution A; 1-2 min 30% of solution A; 2-9,5 min 0% of solution A; 9,5-10 100% of solution A. Flow rate was 1mL/min and the injection volume was 20 µL of the solution at a concentration of 1mg/mL.

2.2.4.2. FT-IR spectroscopy analysis

The FT-IR spectra of all pooled fractions were recorded in the middle infrared region, between 4000 cm^{-1} and 400 cm^{-1}, using a Cary 630 FTIR Spectrometer with an ATR accessory equipped with a diamond crystal. Spectra were generated at a resolution of 4 cm^{-1} with 32 scans. A background spectrum of air was scanned before each measurement. The sample does not need further preparation for recording the spectra of the different fractions. A minimum amount of each fraction was placed on the diamond crystal and traces of solvent was left to evaporate. Then, the spectrum was recorded.

2.2.4.3. NMR spectroscopy analysis

For ^{1}H-NMR analysis, the spectrums were obtained from NMReady60 spectrometer with a resonance frequency of 60 MHz in permanent magnet. The sample was dissolved in the minimum amount of deuterated chloroform ($CDCl_3$) and placed in standard 5 mm NMR tubes. The solvent peak was noticed at 7.25 ppm. The number of scans given for each sample is 128 scans, typically 0.06 sec per scan at room temperature, and the data were collected in the range 0-15 ppm as spectral with. Tetramethyl silane (TMS) was used as internal standard reference peak at 0.0 ppm. The analysis was done over selected samples that present biological activity (**A09JC**, and **A12JC**) or show a high degree of purity in the HPLC analysis (**A02JC**, **A07JC**, and **F2JC**).

Fractions Characterization:

- **SE fractions**

F0JC. Solid *ATR FT-IR v* (cm^{-1}): 3296, 2917, 2850, 1463, 1328, 718.

F1JC. *ATR FT-IR v* (cm^{-1}): 2922, 2853, 1738, 1458, 1377, 721; *RP-UHPLC* (H_2O/MeCN from 100:0 to 0:100 over 10 minutes): t_R 9.67 min (28.46% purity); *UV-vis* (λ max, nm): 229.7.

F2JC *ATR FT-IR v* (cm^{-1}): 3425, 2922, 1717, 1640, 1389; *RP-UHPLC* (H_2O/MeCN from 100:0 to 0:100 over 10 minutes): t_R 6.56 min (79.23% purity); *UV-vis* (λ max, nm): 229.6; *^{1}H-NMR* (60 MHz, $CDCl_3$) δ (ppm): 0.87, 1.23, 2.39, 4.36, 7.34.

F3JC *ATR FT-IR v* (cm^{-1}): 3388, 2922, 1688, 1458, 1377; *RP-UHPLC* (H_2O/MeCN from 100:0 to 0:100 over 10 minutes): t_R 8.24 min (26,5% purity); *UV-vis* (λ max, nm): 233.9nm.

F4JC *ATR FT-IR v* (cm^{-1}): 3361, 2922, 1686, 1458, 1386, 753.

F5JC *ATR FT-IR v* (cm^{-1}): 3359, 2922, 1686, 1458, 1380, 755.

- **ME fractions**

A01JC *ATR FT-IR v* (cm^{-1}): 2912, 1458, 1377, 969.

A02JC *ATR FT-IR v* (cm^{-1}): 2954, 1742, 1458, 1377, 968; *RP-UHPLC* (H_2O/MeCN from 100:0 to 0:100 over 10 minutes): t_R 9.68 min (40.56% purity); *UV-vis* (λ max, nm): 233.6; *^{1}H-NMR* (60 MHz, $CDCl_3$) δ (ppm): 0.87, 1.10, 1.23, 1.75.

A03JC *ATR FT-IR v* (cm^{-1}): 3843, 3449, 2953, 1741, 1458, 1377, 967.

A04JC *ATR FT-IR v* (cm^{-1}): 2954, 1720, 1641, 1458, 1377, 971; *RP-UHPLC* (H_2O/MeCN from 100:0 to 0:100 over 10 minutes): t_R 6.56 min (49.87% purity); *UV-vis* (λ max, nm): 231.3.

A05JC *ATR FT-IR v* (cm^{-1}): 3451, 2922, 1723, 1459, 1287, 731.

A06JC *ATR FT-IR v* (cm^{-1}): 3649, 3403, 2954, 1740, 1711, 1458, 1377, 968.

A07JC *ATR FT-IR v* (cm^{-1}): 3643, 3376, 2953, 1740, 1458, 1377, 967; *RP-UHPLC* (H_2O/MeCN from 100:0 to 0:100 over 10 minutes) : t_R 8.28 min (24.74% purity); *UV-vis* (λ max, nm): 234.2; *^{1}H-NMR* (60 MHz, $CDCl_3$) δ (ppm): 0.87, 1.10, 1.32, 1.66.

A08JC *ATR FT-IR v* (cm^{-1}): 3643, 3425, 2945, 1742, 1666, 1458, 1377, 966.

A09JC *ATR FT-IR v* (cm^{-1}): 3642, 3339, 2954, 1736, 1458, 1364, 968, 722; *^{1}H-NMR* (60 MHz, $CDCl_3$) δ (ppm): 0.85, 0.97, 1.26, 1.30, 1.63, 4.10.

A10JC *ATR FT-IR v* (cm^{-1}): 3642, 3332, 2954, 1739, 1457, 1363, 968.

A11JC *ATR FT-IR v* (cm^{-1}): 3645, 3379, 2958, 1718, 1458, 1363, 969.

A12JC *ATR FT-IR v* (cm^{-1}): 2954, 1742, 1458, 1377, 967; *^{1}H-NMR* (60 MHz, $CDCl_3$) δ (ppm): 0.86, 1.10, 1.25, 1.53, 2.01, 2.24, 4.10.

A13JC *ATR FT-IR v* (cm^{-1}): 3349, 2958, 1740, 1458, 1377, 967.

A14JC *ATR FT-IR v* (cm^{-1}): 3364, 2954, 1736, 1458, 1377, 967.

A15JC *ATR FT-IR v* (cm^{-1}): 3642, 3341, 2954, 1736, 1458, 1363, 968.

A16JC *ATR FT-IR v* (cm^{-1}): 3383, 2954, 1708, 1458, 1364, 968.

A17JC *ATR FT-IR v* (cm^{-1}): 3342, 2952, 1718, 1458, 1378, 969.

A18JC *ATR FT-IR v* (cm^{-1}): 3336, 2950, 1707, 1458, 1378, 970.

2.3.Biological activity

2.3.1. Preparation of inoculums

2.3.1.1. Culture media

The culture media was prepared for solid and liquid media. Solid media was prepared using 12 g of Luria Bertani (LB) Agar, Miller from TM MEDIA placed in a clean 1-liter flask and 300 mL of distilled water. Liquid media was prepared using 6 g of Luria Bertani (LB) Broth Base from Invitrogen placed in a clean 1-liter flask and add 300 mL of distilled water. It is relevant to note that containers used for media might have vented tops and should be capable of holding 20% more than the medium's intended volume, allowing for expansion during sterilization. Swirl the flask to mix the powder mixture into the water as homogeneously as possible. Cover the flask with aluminum foil and put it in the autoclave for 30 minutes at 1 atm and 120 °C. Once the sterilization is finished, take the liquid media flask out and let it cool down at room temperature for 50 to 60 minutes. The solid media flask was distributed in the necessary Petri dishes in a Laminar Flow Hood until the place's whole area is filled. Allow the plates to cool and the LB-Agar to completely solidifies.

2.3.1.2. Bacteria strains

Escherichia coli (DH5-α) was chosen for evaluating antibacterial activity. The bacterial stock cultures were incubated for 24 hours at 37°C and 124 rpm on Luria Bertani (LB) broth base, following by refrigeration storage at 4°C.

2.3.2. Antibacterial activity

The antibacterial activity of **SE**, **ME,** and the corresponding fractions were tested using agar diffusion and optical density measurements (OD), as qualitative and quantitative tests, respectively.

2.3.2.1. Agar diffusion method

A solution of each sample was prepared at 1 mg/mL in acetonitrile as solvent. All solutions were diluted in a proportion of 5:5 water/MeCN system, given a solution at 500 µg/mL. Once the dilutions were prepared, the agar plates containing the appropriate medium with the bacteria (*E. coli* DH5-α) strains were inoculated with 3 µL of each solution. The inoculated plates were incubated at 37 °C for 24 hours. After that time, the

inhibition zones were observed. Ampicillin (100 µg/mL) and the solvent system (H_2O/MeCN (5:5)) were used as positive control and blank, respectively.

2.3.2.2. Optical density measurements (OD)

A Thermo-Scientific Nanodrop Uv-Vis Spectrophotometer was used to perform the optical density measurements at 600 nm. Every 30 minutes have been measured the absorbance of the analyzed samples at room temperature and the wavelength mentioned. For this study, nine different tubes were prepared with 3 mL of Luria Bertani (LB) broth base and 31,95 µL of *E. coli* (DH5-α) strains to obtain an initial OD_{600} of 0.02.

LB with *E. coli*. was used as the negative control (Tube 1), while LB, *E. coli*, and Ampicillin (150 µL) as an antibiotic was a positive control (Tube 2). LB, with *E. coli* and H_2O/MeCN(150 µL) was also prepared and measured to consider the effect of the solvent used.

150 µL of **A04JC**, **A06JC**, **A09JC**, **A12JC**, **A16JC**, and **F5JC** previously prepared at 500 µg/mL were inoculated in each tube.

Chapter 3.

3. Results and discussion

Since prehistoric times the use of plants has been well known[1], the plant kingdom is a comprehensive source of potential drugs; for this reason, during the last years, there has been an increasing awareness about the importance of medicinal plants[21]. Medicinal plants contain several organic compounds that have a physiological action on the human body. The biologically active compounds are secondary metabolites called natural products such as tannins, alkaloids, carbohydrates, terpenoids, steroids, and flavonoids[21]. The main reason why natural products can have medicinal effects in the human body is due to the unique and complex chemical diversity of the secondary metabolites. Consequently, many phytochemicals belonging to many chemical classes have been shown to have inhibitory effects on microorganisms in vitro[21].

Medicinal plants are used worldwide in traditional medicine[1,5]; however, in Ecuador, the use of Matico came from many decades ago [6]. Matico is the common name of *Eupatorium glutinosum* Lam. belongs from the genus Eupatorium, which has been studied and demonstrates that its active principles possess a broad range of pharmacological activities cytotoxic[23–25], antifungal[26,27], antibacterial[26,28], and anti-inflammatory[11,24] effect[1,6,29–34]. Owing to the multiple biological activities presented for the different secondary metabolites of *Eupatorium glutinosum* Lam, they could serve as a scaffold for developing new drugs[1]. In the present work, we report a phytochemical screening of methanolic and aqueous extracts, and the preliminary chemical characterization of the methanolic extract from leaves of *Eupatorium glutinosum* Lam. The aqueous extract was prepared using the Maceration procedure. The methanolic extract was prepared through two different methods: Soxhlet and Maceration procedures. Both methanolic extracts were fractionated using column chromatography, and antibacterial activity against *E. coli* strains of the crudes and fractions was also studied.

3.1. Chemical section

3.1.1. Preparation of methanolic and aqueous extracts of the leaves from *Eupatorium glutinosum* Lam.

Soxhlet and Maceration procedures were used to prepare the methanolic extracts based on two different reported methodologies[12, 13] with minor modifications allowing to obtain two methanolic extracts (Soxhlet Extract (**SE**), and Maceration Extract (**ME**), and one aqueous extract (**MAE**). For both extraction methods, equal amounts of starting material was used (8 g); hence the expected amount of crude extract should be the same; nonetheless, the Soxhlet method (yield of 8.8%) had a slightly higher amount of crude extract than the Maceration method (yield of 8.5%).

The efficiency results were as expected due to the Soxhlet procedure allows the use of a large amount of sample, and no filtration is required after extraction. However, the applied temperature (at the boiling point of solvent), although it should accelerate the extraction, may improve the sample's decomposition. On the other hand, the maceration procedure is a simple extraction method that, although valuable, presents a long extraction time and generally lower efficiency. Therefore, and considering only those related to the efficiency process, the best extraction method was the Soxhlet method.

The Maceration procedure was also applied to prepare an aqueous extract of leaves from *Eupatorium glutinosum* Lam, whose phytochemical screening will be analyzed later.

Thin Layer Chromatography (TLC) was carried out on both methanolic extracts (**SE** and **ME**) as a first approximation to our extracts' content. After testing various solvent systems, toluene: EtOAc (8:2) was selected. The plates were revealed using the UV lamp (at λ 254 and 325 nm) and iodine chamber.

As expected, the TLC of **SE** and **ME** showed several points (**Figure 1**). This chromatography procedure was the first approach to the chemical content of natural crudes. Both **SE** and **ME** revealed complex mixtures, although apparently, the **ME** exhibited more complexity (**Figure 1**).

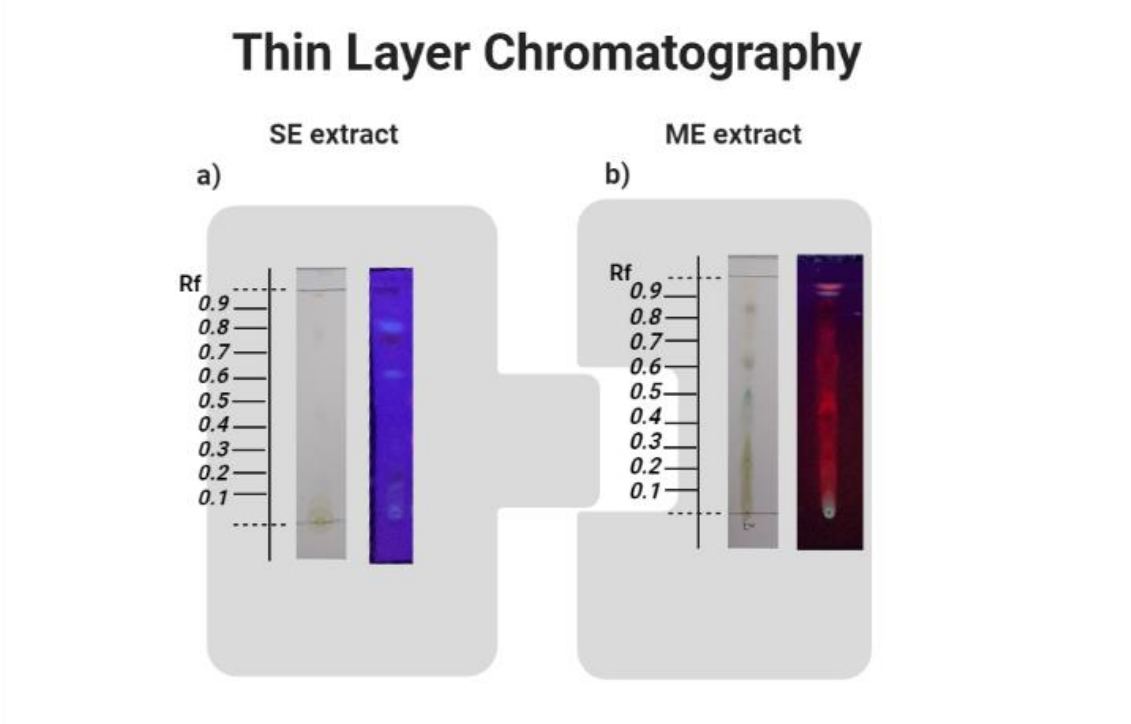

*Figure 1 TLC revealed at natural light and UV lamp at 365 nm of a) **SE** extract, and b) **ME** extract*

After two days of refrigeration, the colorless precipitate was observed in **SE**. The mixture was filtered to isolate the solid (**F0JC**) and then rotoevaporated to continue the process of analysis and separation.

HPLC was carried out to detect all susceptible UV/Vis compounds present in our two methanolic extracts (**SE** and **ME**) using a linear gradient H_2O:MeCN (100:0) to (0:100) over 10 minutes (**Figure 2**). Both extracts showed similar HPLC profiles, with the presence of four peaks at 6.5, 6.7, 8.2, and 9.7 minutes, highlighting the peak at 6.5 minutes as the majority fraction according to the percentage of the chromatographic peak area (23 %) (**Figure 2**).

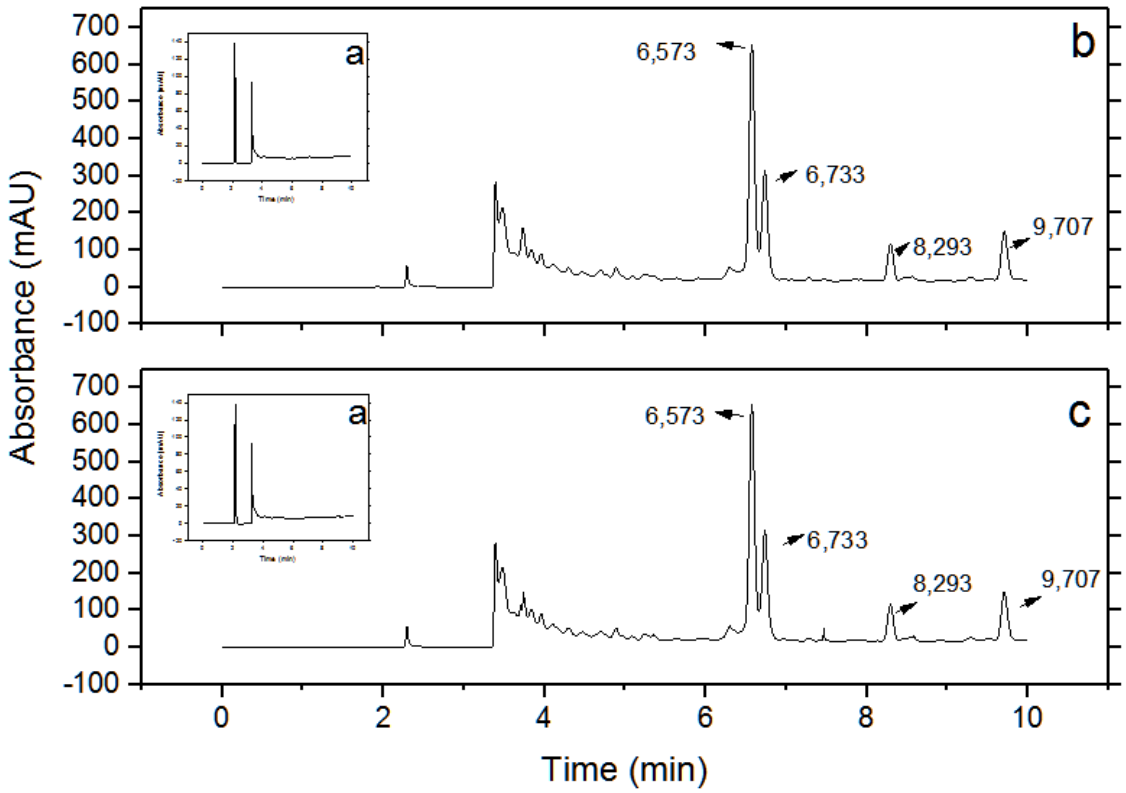

*Figure 2 HPLC chromatograms. a) Acetonitrile hplc grade (blank), b) Soxhlet Extract (**SE**), c) Macerationt Extract (**ME**)*

At this point, a preliminary phytochemical screening was carried out to define what kind of compounds would be present in **ME**. The same phytochemical studies were performed in the aqueous extract of leaves from *Eupatorium glutinosum* Lam. prepared through maceration procedure, and all results were compared.

3.1.2 Phytochemical Qualitative Analysis.

The phytochemical analysis is essential to evaluate the possible medicinal utilities and determine the active principle responsible for the plants' known biological activities. According to literature[35], in the methanolic extract, secondary metabolites such as anthocyanins, terpenoids, saponins, tannins, lactones, xanthoxylines, and totarol, quassinoids, flavones, phenones, and polyphenols could be present.

Phytochemical screening of methanolic and aqueous extracts was carried out using various chemical assays to identify either the presence or absence of secondary metabolites such as phenols and tannins, flavonoids, proteins, and amino acids, reducing sugars, saponins, carbohydrates, steroids, terpenoids, cardiac glycosides, and alkaloids (**Figure 3)**

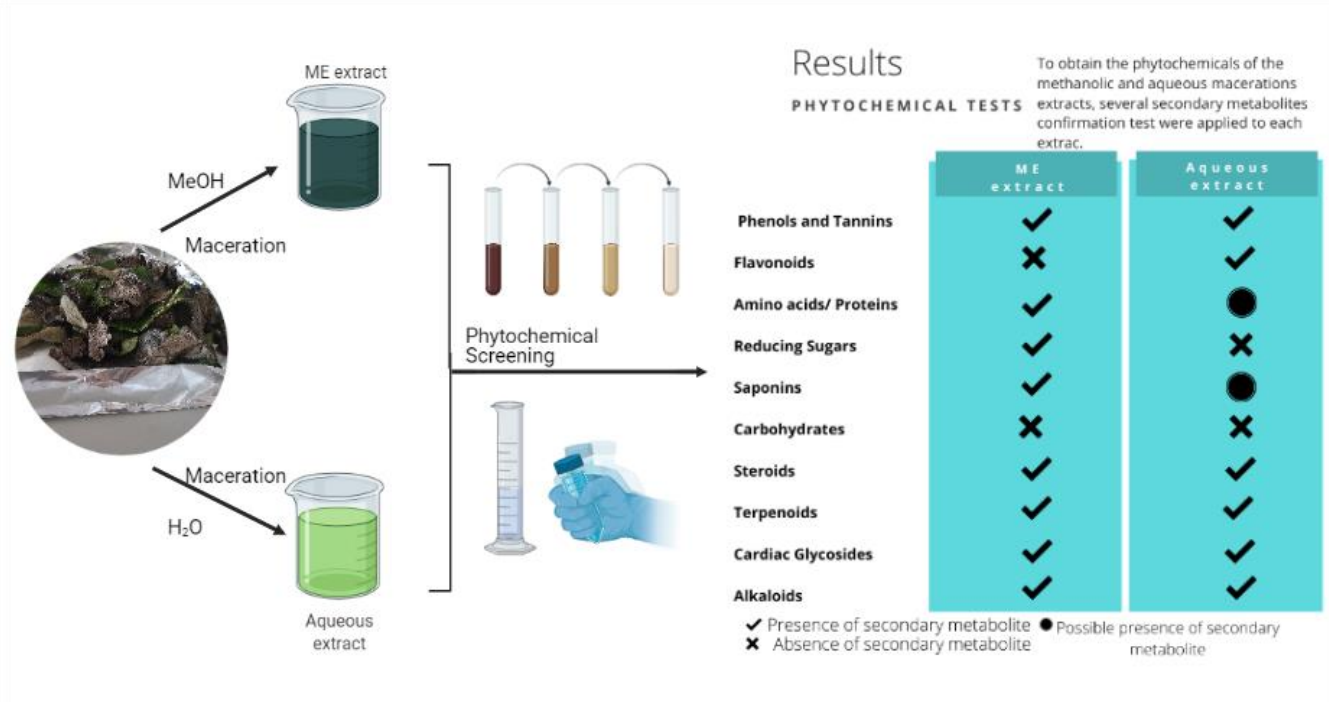

Figure 3 Result of the phytochemical screening

Both extracts revealed the presence of phenols and tannins, steroids, terpenoids, cardiac glycosides, and alkaloids, and both resulted negative to Benedict test demonstrating the absence of carbohydrates. Flavonoids were abundant in the aqueous extract while they were not present in the methanolic extract. Carbohydrates were not detected in any of the crudes. The tests to determine amino acids', proteins', and saponins' presence were not conclusive for the aqueous extract; besides, reducing sugars were not detected either in this extract (**Figure 3**).

The phytochemical screening allows us to shows the secondary metabolites present in the methanolic and aqueous extracts of leaves from *Eupatorium glutinosum* Lam., but how are those secondary metabolites distributed in the different extracts? Secondary metabolites in a specific extract depend on their affinity with the solvent used. Another critical aspect that influences natural product extraction is the solvent's ability to preserve such mentioned compounds [35].

Besides, several environmental factors, both biotic and abiotic, are responsible for plants' phytochemical composition. Further, the adaption mechanisms develop by the plant are determined by the stress level to which it is subjected. These mechanisms involve molecular responses that alter plants' metabolism, either by synthesis, degradation, and

storage of secondary metabolites. Hence, any species' phytochemicals shall directly depend on the environment and conditions they are developed[36].

Encourage by the preliminary phytochemical screening results, **SE** and **ME** were subjected to separation by column chromatography.

3.1.3 Chemical separation by Column Chromatography (CC)

To isolate phytochemicals of methanolic extracts, **SE** and **ME** were subjected to Column Chromatography on a silica gel pack. Therefore, two chromatographic columns were performed under slightly different solvent systems gradients.

SE extract was fractionated by CC with an apolar-to-polar gradient system using Toluene and Ethyl Acetate mixtures. The starting amount of crude extract added into the stationary face was 400 mg. The separation allowed to obtained thirty-three fractions pooled according to similar Rf values obtained from Thin-Layer Chromatography using Tol/EtOAc (8:2) as solvent system. Finally, five isolated fractions were obtained (**Figure 4**), where the two fractions **F2JC** (Rf 0.54), and **F4JC** (Rf 0.57) were the majority with 8 and 10.5 %, respectively. After the column chromatography, only 33.6 % was recovered from the starting **SE**.

Using the previous experience with **SE**, **ME** was also fractionated by column chromatography but with a much flatter gradient from apolar to polar media (Hexane: Ethyl acetate). The starting amount of crude extract was the same (400 mg) that for the first column. After the separation procedure, eighteen fractions were obtained, which were pooled using TLC analysis, where **A02JC** (Rf 0.79) and **A04JC** (Rf 0.54) were majority with 6.2 and 6.4 %, respectively. The overall recovered yield was 53.1% higher than those obtained for the **SE** separation (33.6%) (**Figure 4**).

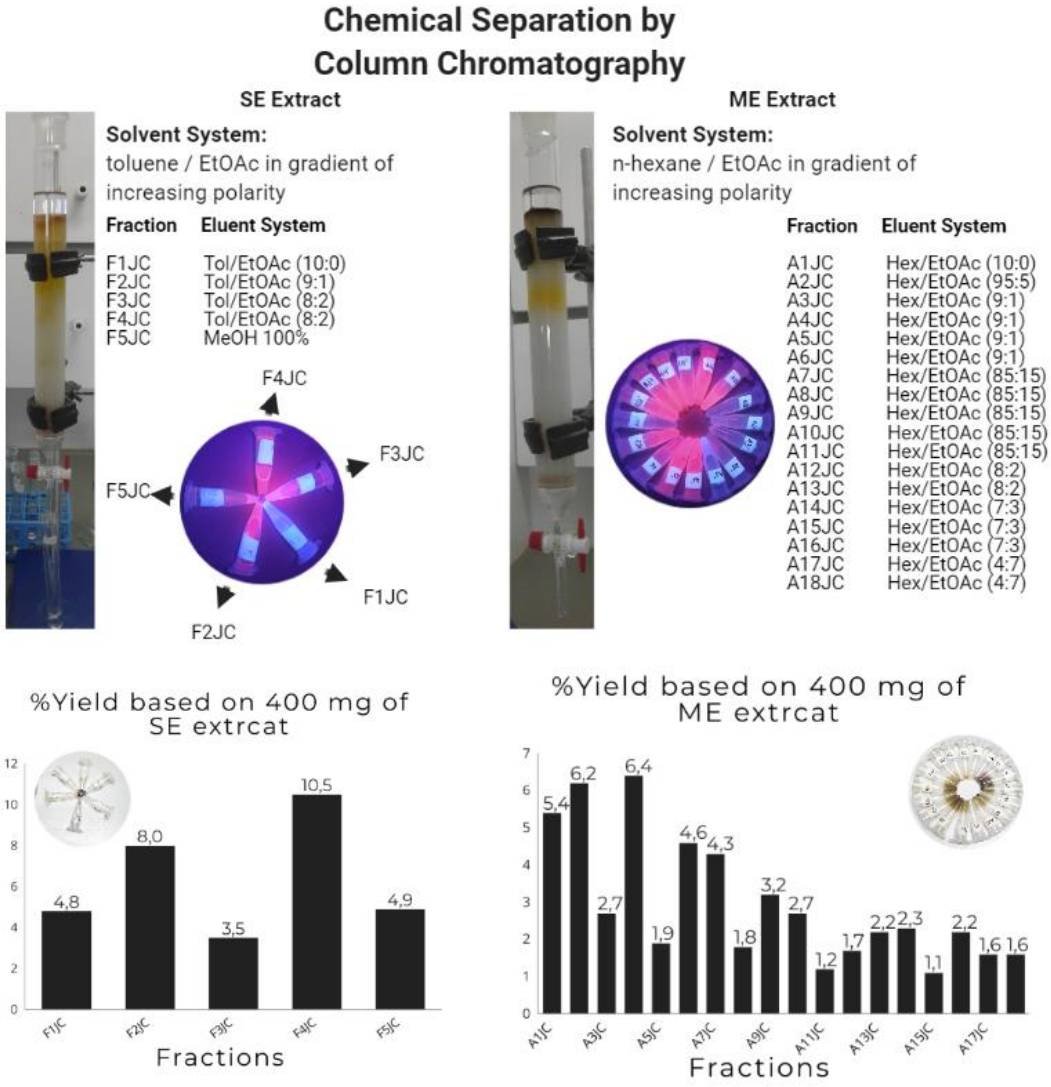

*Figure 4 Column Chromatographic separation of **SE** and **ME**.*

According to our results, the best eluent system corresponds to the apolar to polar gradient hexane/Ethyl Acetate used in the **ME** extract separation. The second column not only showed better overall yield (53.1 % > 33.6%) but allowed a more efficient separation of phytochemicals.

TLC of all isolated fractions allowed us a first approach to the content and purity of fractions. The Rf of each detected compound was determined for each fraction. Related to **SE** (**Figure 5**), toluene:ethyl acetate (8:2) was used as the mobile phase. Under the mentioned conditions, only **F3JC** showed a unique stain at Rf 0.70. The other fractions exhibited two marks for **F1JC** and **F2JC** and three stains for **F4JC** and **F5JC**. Besides, similar spots were present in more than one fraction. Some stains only were colored in Uv light. All results were consistent with an unsuccessful extract separation (**Figure 5**).

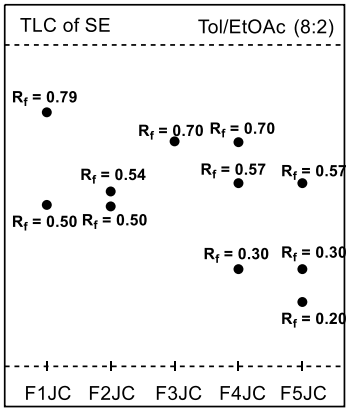

*Figure 5 TLC representation of the **SE** fractions.*

Regarding TLC of **ME**, the 18 isolated fractions were analyzed. The TLC plates were observed at daylight and revealed under UV (at λ of 365 nm) and using iodine vapors. Except for **A06JC**, **A09JC**, **A12JC**, and **A15JC**, they all showed a unique stain with a broad range of Rfs corroborated a more efficient separation than the **SE** separation (**Figure 6**).

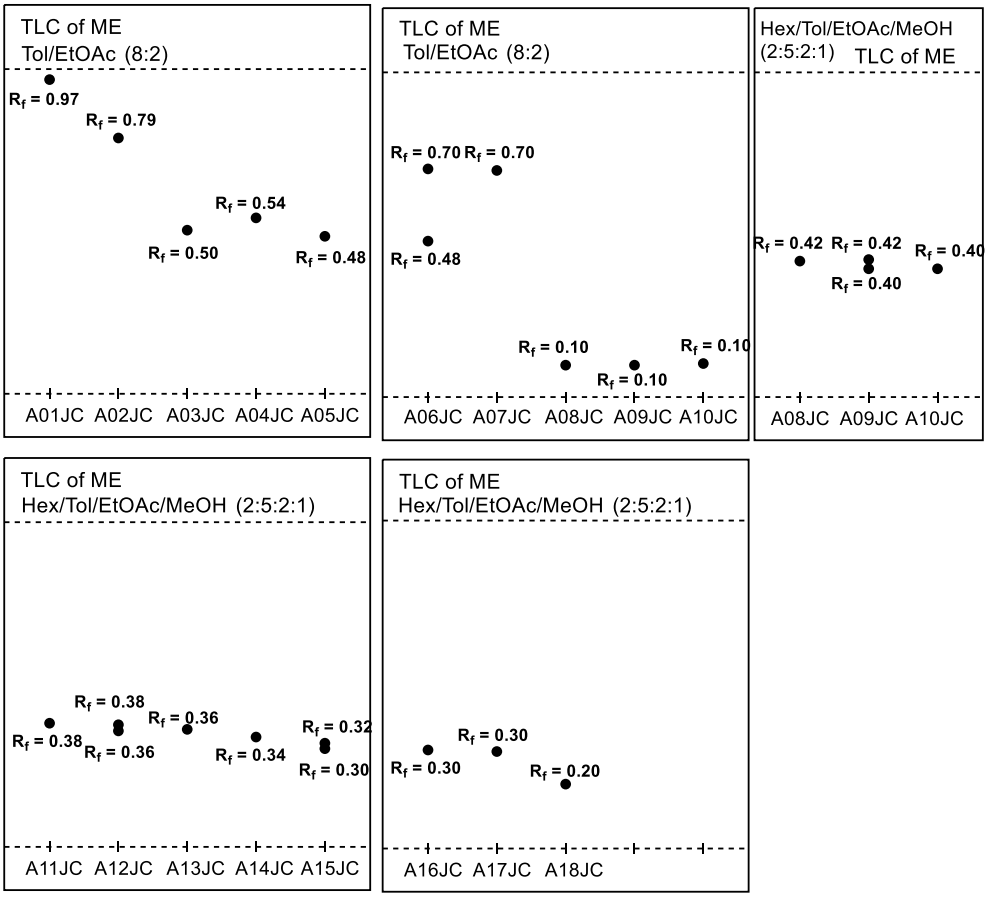

*Figure 6 TLC representation of the **ME** fractions.*

To summarizing, fractions from **ME** showed a better purity compared to **SE**. For **ME**, 14 fractions showed one stain, while for **SE**, only one fraction seems to have pure. In both TLC analyses (**SE** and **ME**), spots with similar Rfs were observed, allowed to conclude that both methanolic extracts have similar profiles (**Figure 5 and 6**). This conclusion is aligned to the similitude of methanolic extracts UHPLC profiles (**Figure 2**).

With TLC results in hand, each fraction's UHPLC analysis was carried out to corroborate the content, purity, and quantity of phytochemicals in the methanolic extracts.

3.1.3.1 Ultra-High-Performance Liquid Chromatography (UHPLC) analysis

As mentioned before, the UHPLC profile of crude methanolic extract **SE** and **ME** showed four main fractions (**Figure 2**). UHPLC allowed us to analyze all isolated fractions to identify and correlate each fraction with the peaks observed in the crude. In this regard, all isolated fractions were injected into the UHPLC to get its profiles using a linear gradient H_2O:MeCN (100:0) to (0:100) over 10 minutes, idem to those previously used for methanolic extract. The fractions not mentioned in the following discussion showed planar UHPLC profiles, demonstrating the absence of UV/Vis susceptible compounds in the isolated fractions.

The fractions obtained from the **SE**, all showed one majority peak corresponding to compounds with purities from 26.5% to 79.2% according to the chromatographic peak area (%) observed for each UHPLC profile. Using this analysis was possible to correlate the fraction **F2JC** (purity 79.2 %) with the crude's component eluted at a retention time (t_R) of 6.57 minutes (**Figure 7**).

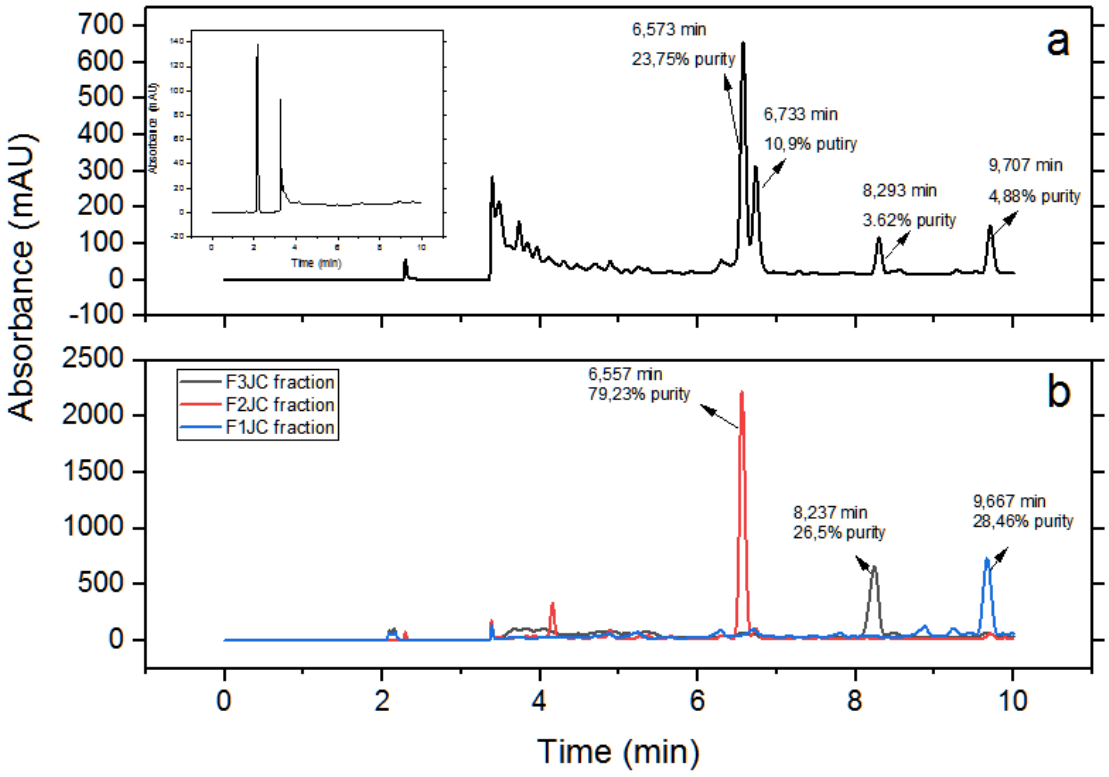

*Figure 7 UHPLC chromatogram of **a)** the crude methanolic **SE** extract, **b) F1JC** fraction in blue, **F2JC** fraction in red and **F3JC** fraction in gray.*

Moreover, it was possible to correlate the **F3JC** fraction (Retention time (t_R) 8.24 minutes, purity 26.5 %) with the third majority component of the **SE** crude, which elutes at 8.29 minutes of retention time (t_R) (**Figure 7**). Finally, the **F1JC** fraction (Retention time (t_R) 9.67 minutes, purity 28.5%) corresponds to the fourth majority component of the crude **SE** methanolic extract observed at retention time (t_R) 9.71 minutes (**Figure 7**).

Following the same methodology, the fractions obtained from **ME** column chromatography were injected in the UHPLC, searching correlation between isolated fractions and observed HPLC profile for the **ME** crude extract. Consequently, it was possible to conclude that the fractions **A02JC**, **A04JC**, and **A07JC** correspond to UV/Vis susceptible compounds eluted at 9.71, 6.57, and 8.30 minutes, respectively (**Figure 8**).

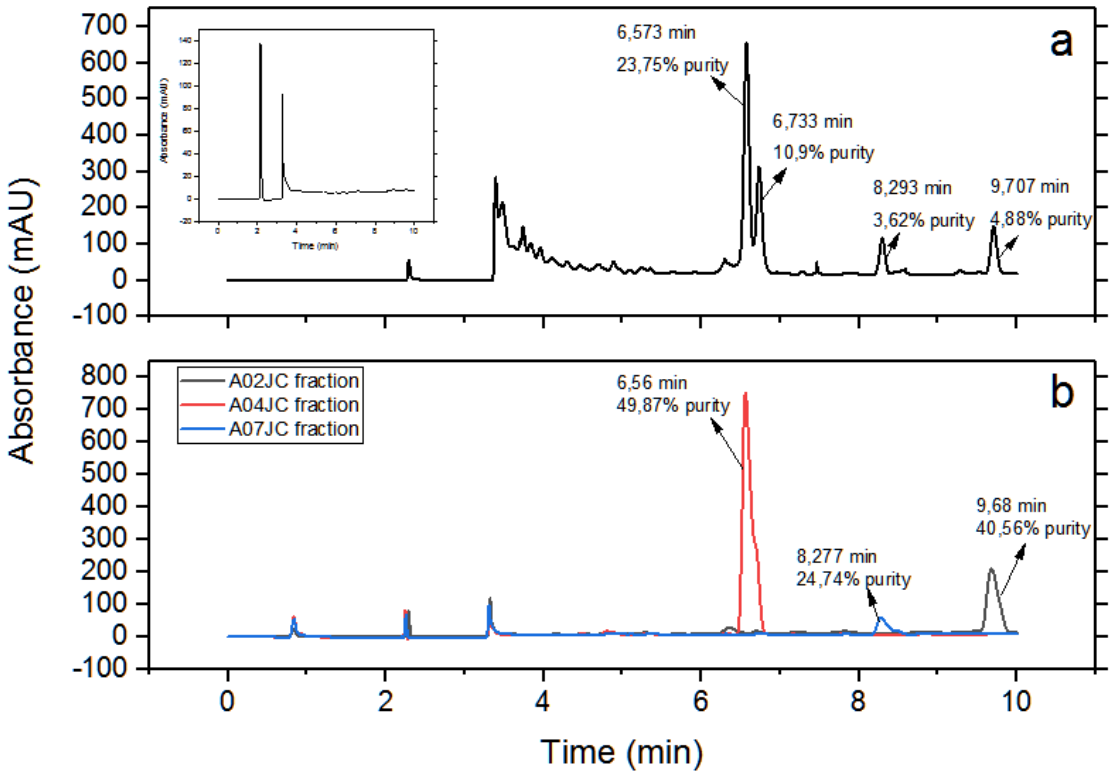

*Figure 8 HPLC chromatograms of a) the crude methanolic **ME** extract, b) the **A02JC** fraction in gray, **A04JC** fraction in red and **A07JC** fraction in blue*

Summarizing, the UHPLC profiles of the two fractions set from the **SE** and **ME** column chromatographies showed that the second one had a better chemical separation due to the better HPLC profiles and purities and the detection of more phytochemicals for the **ME** separation. Besides, UHPLC allowed us to establish the correlation between **SE** and **ME** extracts' isolated fractions and the corresponding crudes. Notwithstanding the differences in solvent systems uses in each CC, the fraction's behavior under UHPLC conditions was similar. The fractions that eluted at the beginning of the CC (lower polarity of solvent systems, toluene for **SE** extract separation, and hexane for **ME** extract separation) correspond to compound eluted at 9.6 minutes in the UHPLC, while when the polarity was slightly increased (toluene: ethyl acetate (8:2) for **SE** extract separation and hexane: ethyl acetate 85:15 for **ME** extract separation), the isolated compound eluted at 8.2 minutes under UHPLC conditions. The majority peak detected at 6.5 minutes of retention time (t_R) in the UHPLC for both crudes (**SE** and **ME** extracts), was correlated to the eluted fraction of CC at an intermediate polarity (toluene: ethyl acetate (9:1) for **SE** extract separation and hexane: ethyl acetate 9:1 for **ME** extract separation), of those previously mentioned (**Figure 9**).

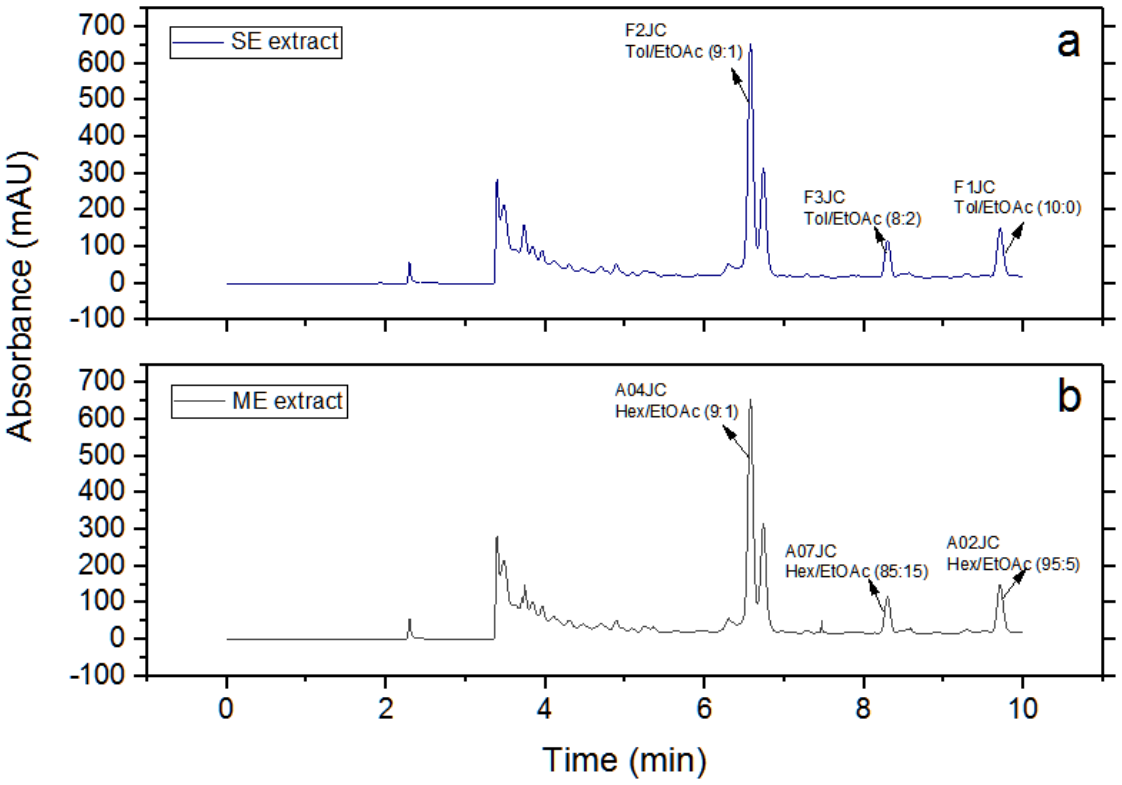

*Figure 9 UHPLC chromatograms labeled with the fractions that contain the majority components of a) **SE**, and b) **ME**.*

With several pure fractions in hand, we decided to face its chemical characterization using spectroscopic procedures such as UV/Vis, FT-IR, and NMR.

3.1.4 Chemical characterization of phytochemicals

One of this investigation's main objectives is to have a first approach to the structural characterization of the methanolic extract of leaves from *Eupatorium glutinosum* Lam. For this reason, after a chromatographic separation corroborated by TLC and UHPLC, different spectroscopic techniques such as UV/Vis, FT-IR, and NMR were performed over selected fractions.

To carry out the characterization analysis, the fractions' spectroscopic results were pooled according to its UHPLC retention time for those UV/Vis sensitive samples and the FTIR and NMR likeness.

Fractions analysis

F1JC, A02JC, and related fractions (A01JC, and A03JC)

Based on the UHPLC analysis results, the fractions **F1JC** and **A02JC** elute at similar retention times (t_R) (**Figure 9**) to be from the same component. For this reason, spectroscopic analysis was done over the mentioned fractions to establish the similarities

or differences of their components. UV-vis spectra allowed us to conclude that these fractions have similar λ max, at 232,95 nm and 233,59 nm for **F1JC** and **A02JC**, respectively, with a slight difference between them (**Figure 10a**).

Both fractions' FT-IR spectra from **F1JC** and **A02JC** have similar bands but with different intensities, possibly associated with the components' different concentrations in both analyzed fractions(**Figure 10b)**.

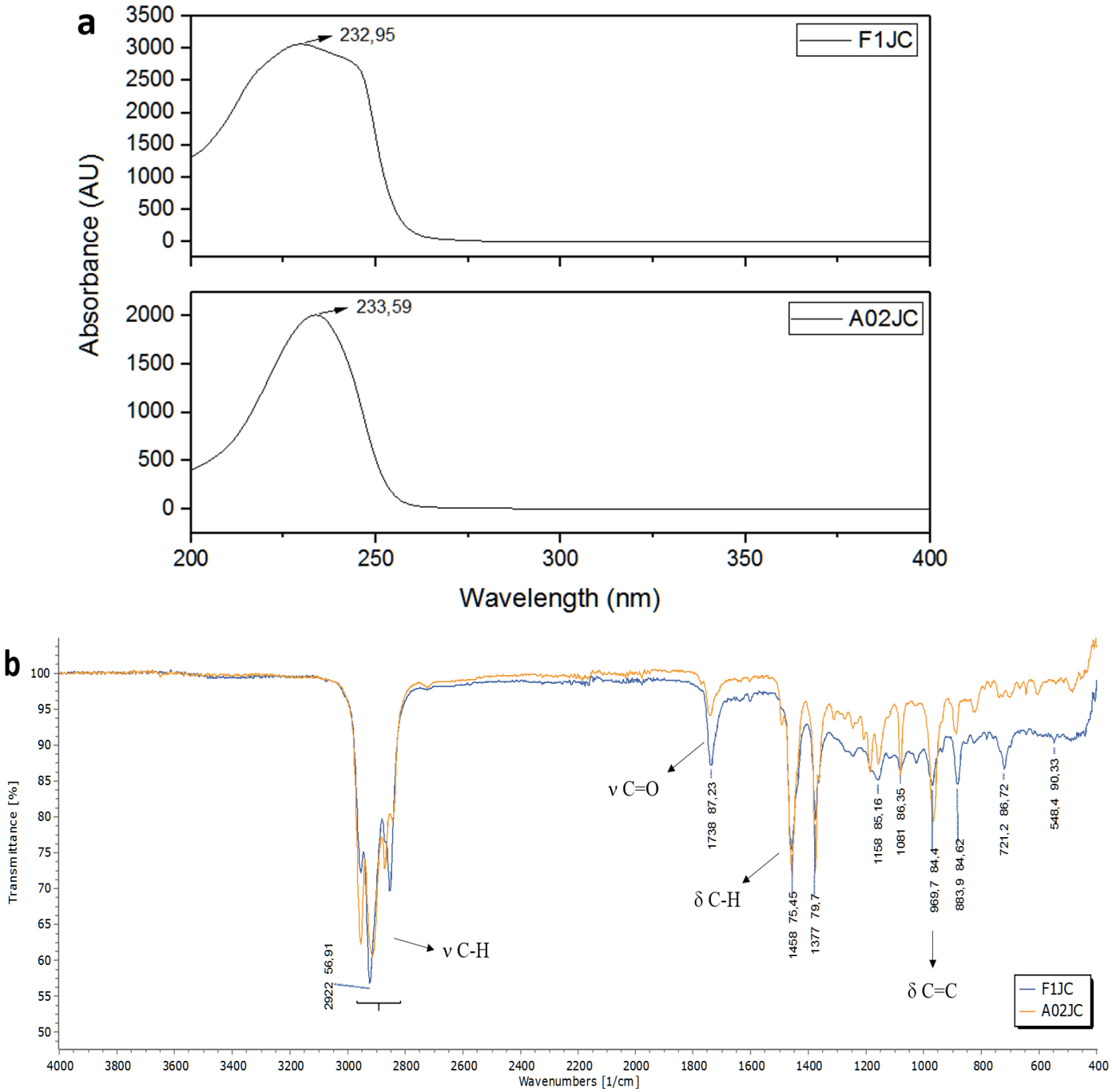

*Figure 10 a) UV-vis spectra of **F1JC** and **A02JC** fractions, b) FT-IR spectra of the fractions **F1JC**, **A02JC***

Moreover, looking for similarities between those fractions with others, it is possible to say that **A01JC** and **A03JC** share closeness similarities with **F1JC** and **A02JC**, according to the FTIR analysis results (**Figure 11**).

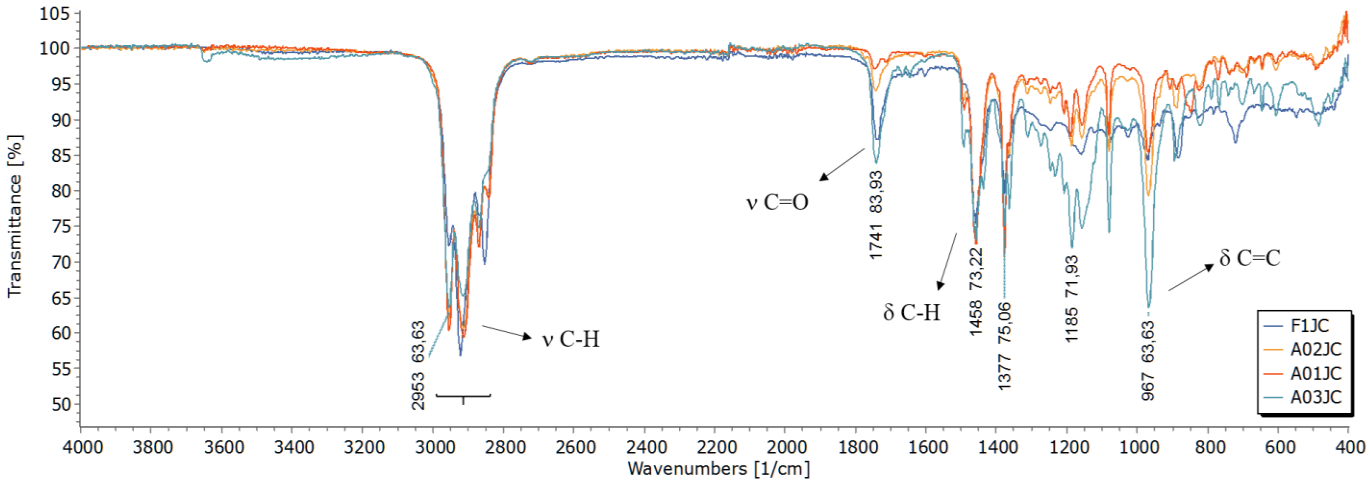

*Figure 11 FT-IR spectra of the fractions **F1JC, A01-A03JC***

On the other hand, the ^{1}H-NMR spectrum of **A02JC** showed several peaks in the aliphatic zone (0,7 - 1,7 ppm) (**Figure 12**), allowing us to propose that the molecules from the mentioned fractions **F1JC**, **A02JC**, **A01JC,** and **A03JC** are structures composed mainly by saturated bonds. With this crucial information, it is possible to assign the characteristic aliphatic bands of the IR spectra. In the range of 3000 to 2800 cm^{-1} appears strong bands corresponding to C-H stretching (ν C-H) of the aliphatic chain, and around 1458 cm^{-1} it is possible to observe the bands corresponding to C-H bending of methylene and methyl (δC-H). The strong signal at 967 cm^{-1} could be assigned to C=C bending (δC=C) of tetra-substitute alkene, taking into account that ^{1}H-NMR spectrum of **A02JC** doesn't show ^{1}H chemical shift of the protons directly bonded to carbons atoms involved in the double bonds, which are generally large, at 4.5 ppm to 7 ppm. Out of the IR spectra' fingerprint zone, the signal around 1740 cm^{-1} could be related to C=O stretching. Still, we do not have enough information to decide if it could be of aldehyde, cyclopentanones, lactones, esters, among other functional groups containing C=O. The absence of bands at 860 – 680 cm^{-1} corresponding to aromatic C-H bending and medium signals at 1700 – 1500 cm^{-1} from aromatic C=C bending confirms the compounds' non-aromatic nature of **F1JC**, **A02JC**, **A01JC,** and **A03JC**.

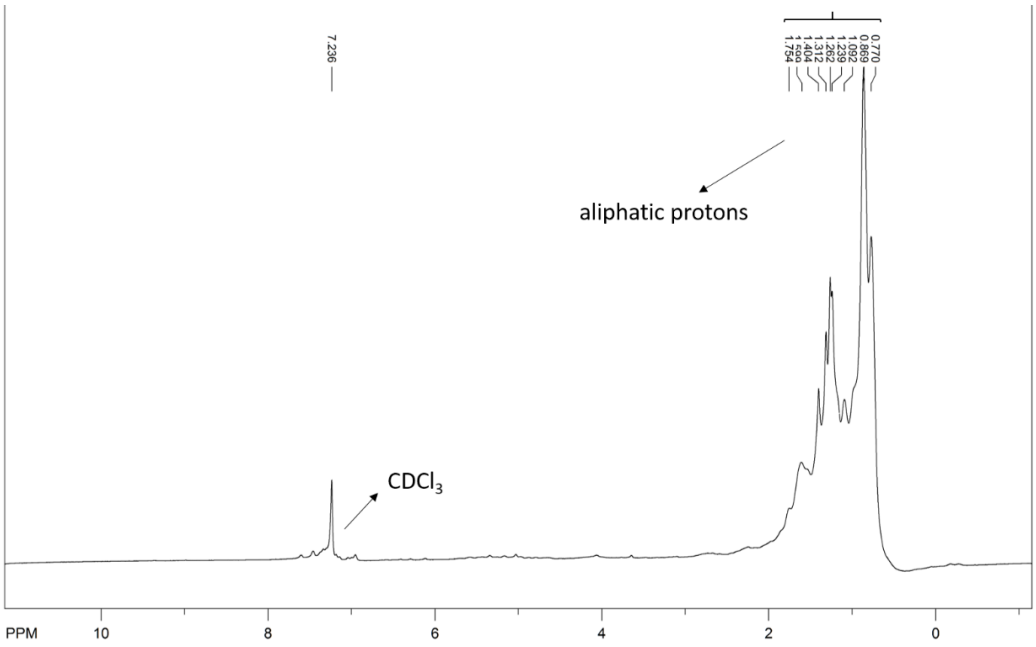

*Figure 12 [1]H-NMR spectra of **A02JC** fraction*

F2JC, A04JC, and a related fraction (A05JC)

Following the same reasoning, which is base on the preliminary coincidence of retention time of UHPLC profiles for fractions **F2JC** and **A04JC**(t_R=6.5 minutes, **Figure 9**), the integral spectroscopic analysis was carried out. The UV-vis spectra (**Figure 13a**) showed near λ max values at 233,65 nm and 230,68 nm for **F2JC** and **A04JC**, respectively. The FTIR spectra (**Figure 13b**) exhibited similar bands distribution with slight differences in intensities, suggesting that both components have similar chemical structures. The FTIR spectrum of **A05JC** also matched the profiles of **F2JC** and **A04JC**, structurally correlating them. The ^{1}H-NMR from **F2JC** (**Figure 14**) not only has signals corresponding to Hs in aliphatic scaffolds (0.7 ppm to 2.5 ppm), but peaks at middle fields (4,14 ppm to 4,61 ppm), and aromatic region (around 7.2 ppm) were also observed. This ^{1}H-NMR data was consistent with the FTIR spectra of **F2JC**, **A04JC**, and **A05JC**. The analyzed samples might have been composed of a structural skeleton of saturated bonds, with hydroxyl and carbonyl groups, based on the IR bands at 3425 cm^{-1} (ν O-H) and 1717 cm^{-1} (νC=O), respectively. The fingerprint region signals 860-680 cm^{-1}could be assigned to aromatic C-H bending (δC-H_{arom}), which, alongside the band at 1458 cm^{-1}, corresponding to aromatic C=C bending (δC=C $_{arom}$), suggest the presence of aromatic fragment in the molecules of the analyzed samples.

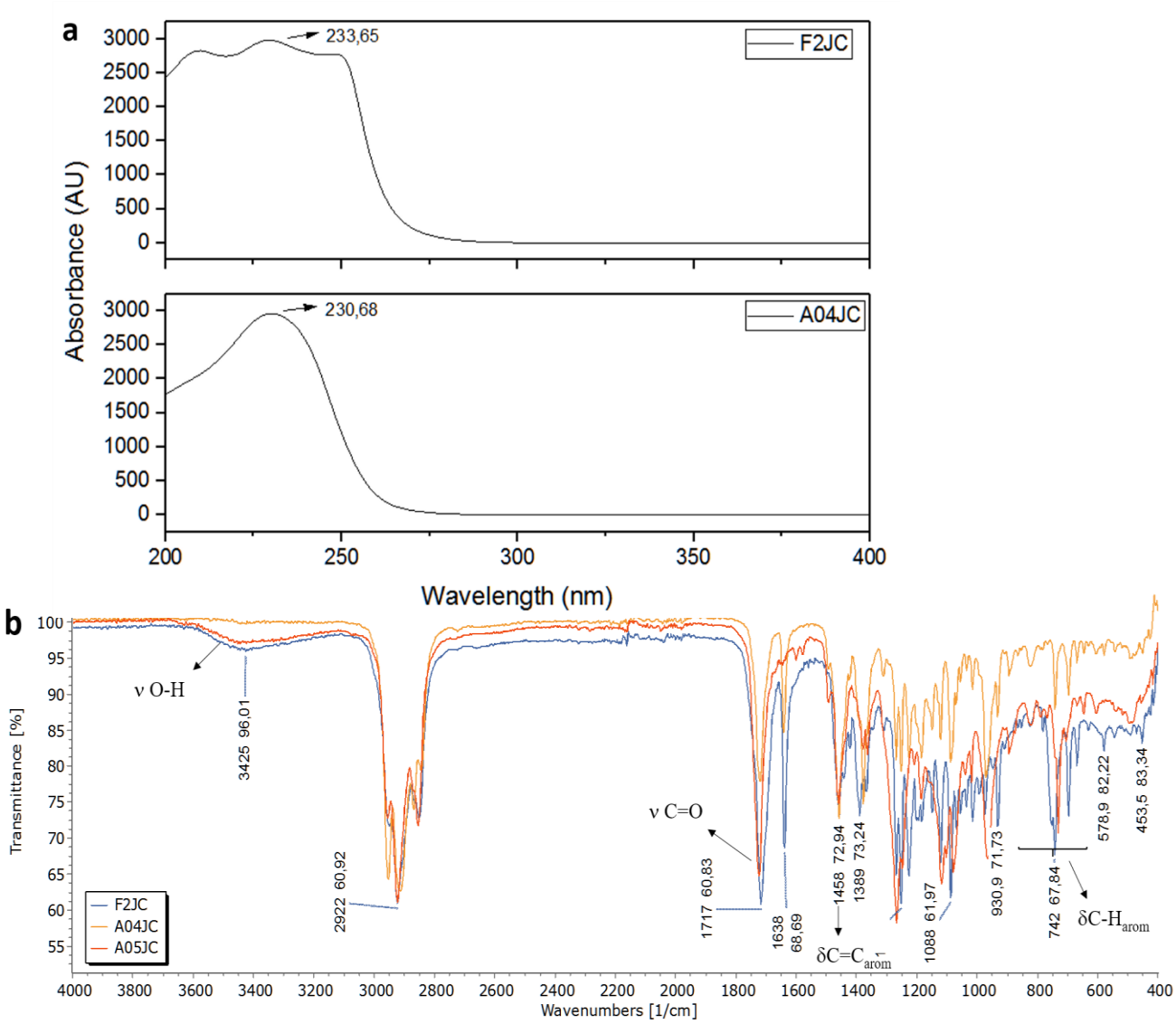

*Figure 13 a) UV-vis spectra of **F2JC** and **A04JC** fractions, b) FT-IR spectra of the fractions **F2JC**, **A04JC**, **A05JC***

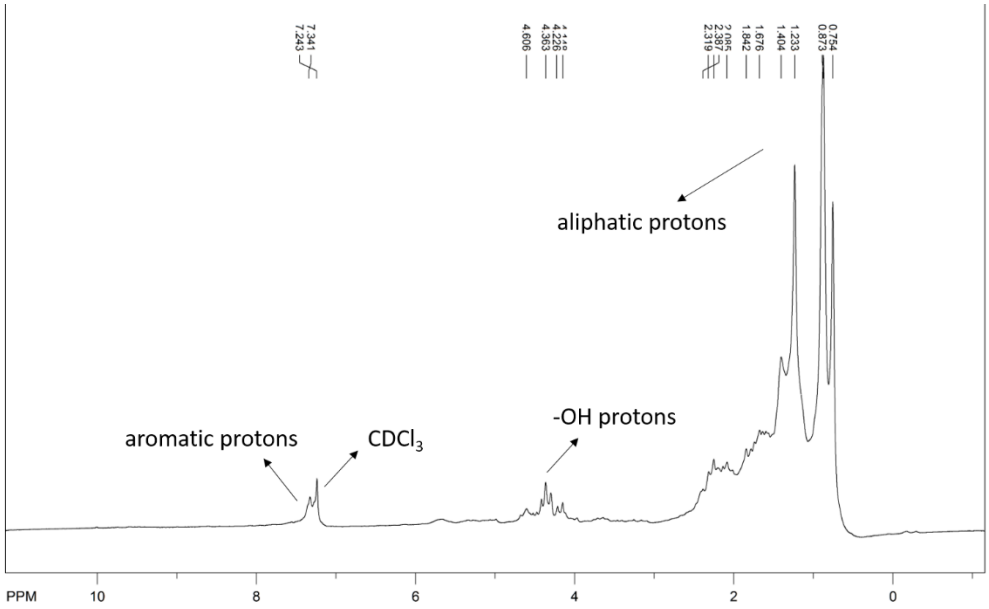

*Figure 14 ^{1}H-NMR spectra of **F2JC** fraction*

F3JC, A07JC, and related fractions (A06JC, A08JC, A09JC, A10JC, and A11JC)

The isolated fractions **F3JC** and **A07JC** eluted at 8.2 minutes according to UHPLC retention time advocated its structural similarities. The UV-vis spectra (**Figure 15a**) have similar shape and closer λ max values of 233,93 nm, and 234,16 nm for **F3JC** and **A07JC**, respectively. As was expected, the FTIR profiles were similar for analyzed fractions (**Figure 15b**), and samples **A06JC**, **A08JC**, **A09JC**, **A10JC**, and **A11JC** also showed some coincidences(**Figure 16**).

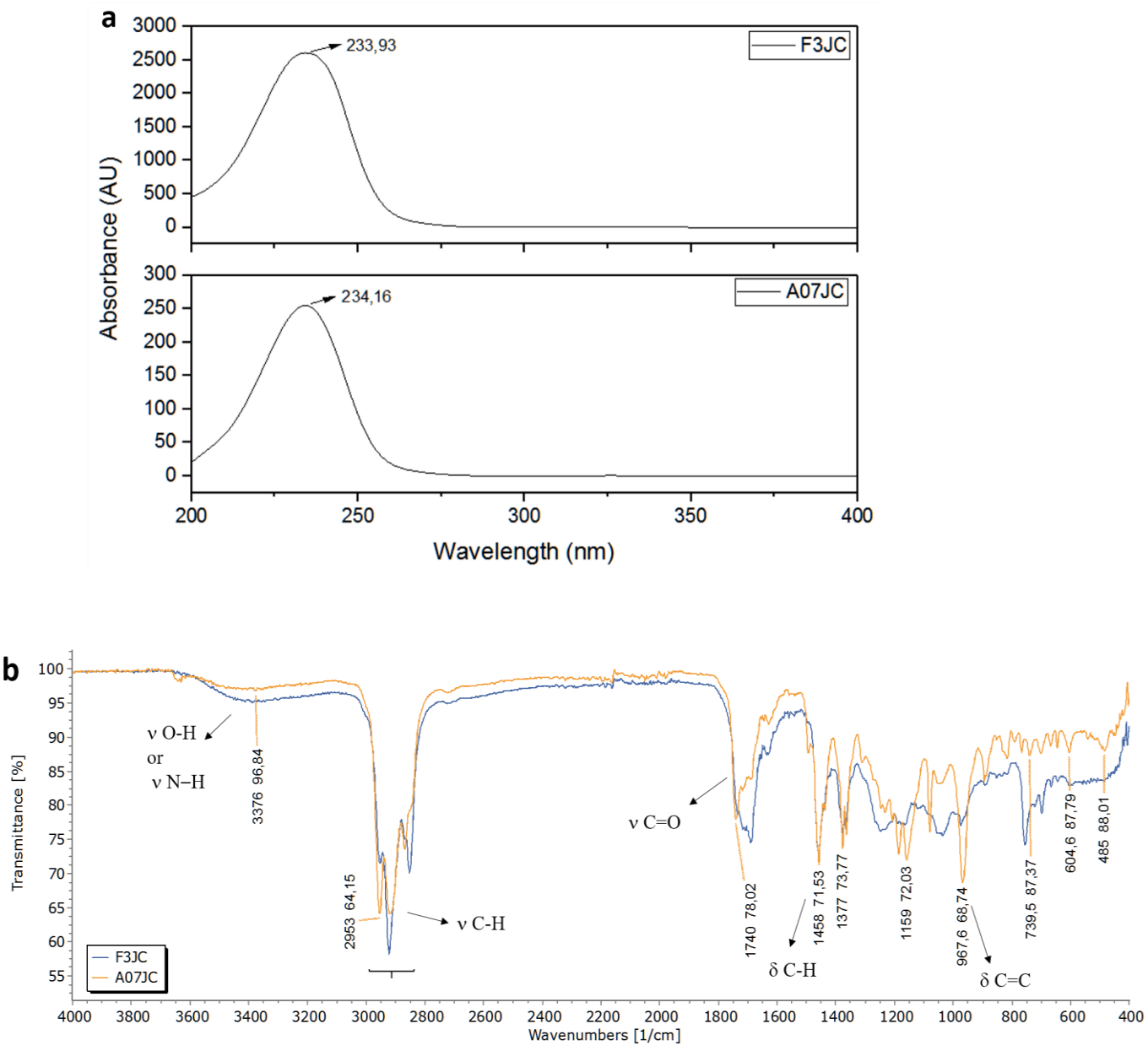

*Figure 15 a) UV-vis spectra of **F3JC** and **A07JC** fractions, b) FT-IR spectra of the fractions **F3JC**, **A07JC***

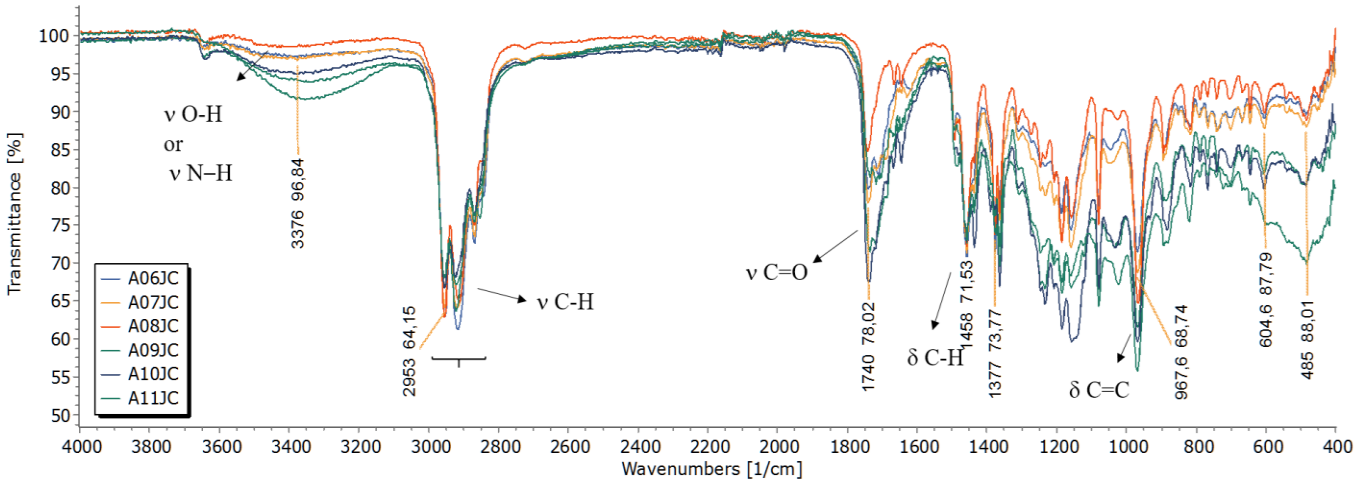

*Figure 16 FT-IR spectra of the fractions **A06-A11JC***

^{1}H and ^{13}C-NMR characterized A07JC, showing signals consistent with aliphatic protons (0.5 ppm to 2.0 ppm), and six peaks corresponding to saturated C at higher fields (19 ppm to 45 ppm) (**Figure 17** and **Figure 18**). Using the NMR information, it is possible to assign the FTIR signals. Centered around 2850 cm^{-1} appears strong bands corresponding to C-H stretching (ν C-H) of the aliphatic chain. At 1458 cm^{-1} it is possible to observe the bands corresponding to C-H bending of methylene and methyl (δC-H). Additionally, all analyzed fractions showed a broad signal centered at 3339 cm^{-1}, which should be assigned to O-H or N-H stretching; however, it is impossible to define with the obtained data unequivocally. Besides, at 1700 cm^{-1} appear a signal typical of carbonyl stretching (νC=O).

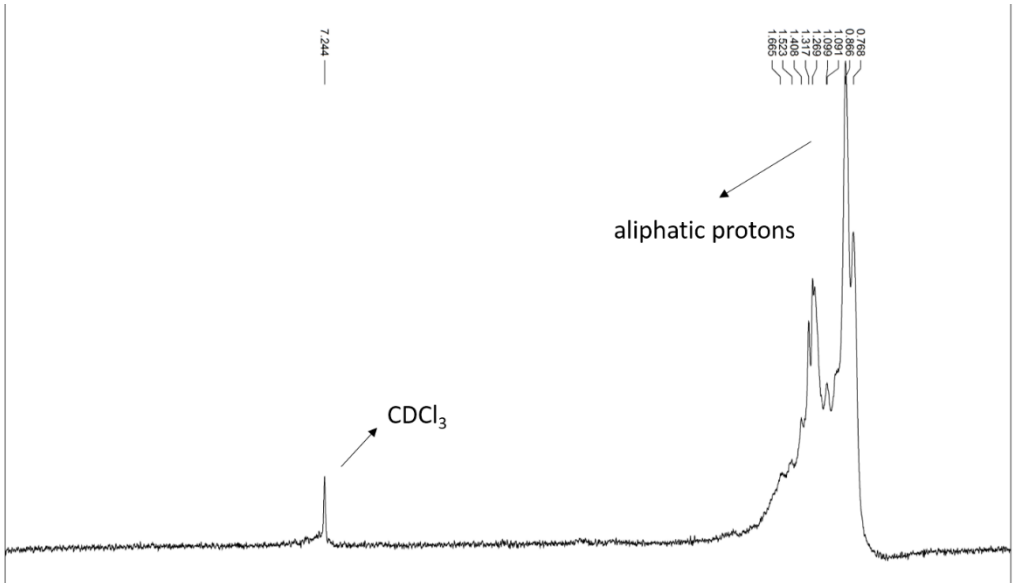

*Figure 17 ^{1}H-NMR spectra of **A07JC** fraction*

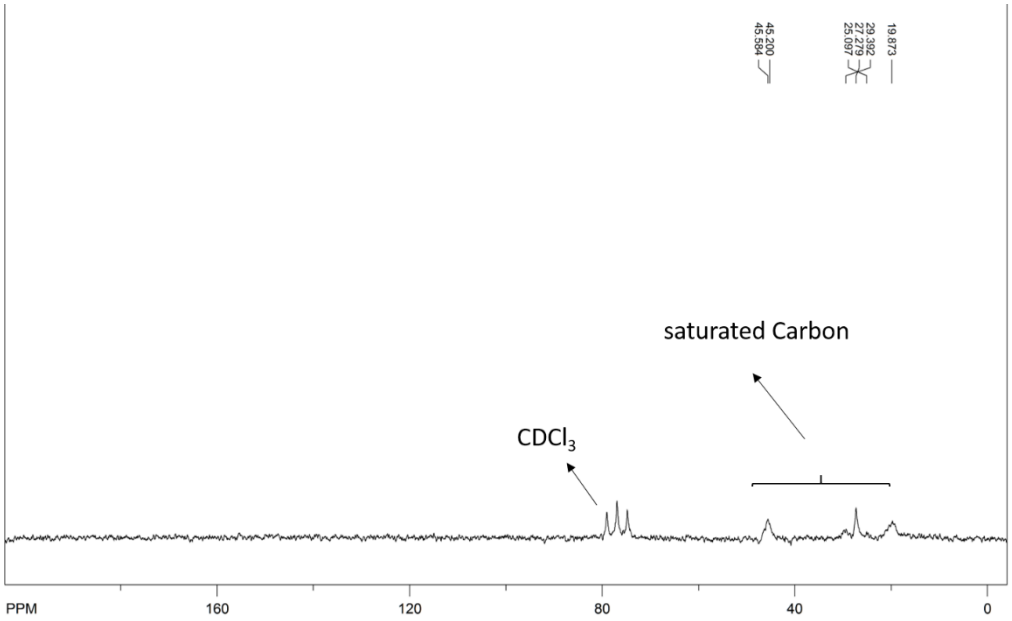

*Figure 18 ^{13}C-NMR spectra of **A07JC** fraction*

In the fingerprint zone of the spectra, it was interesting to note that all fractions **A07JC. A06JC**, **A08JC**, **A09JC**, **A10JC**, and **A11JC**, except **F3JC**, don't show bands in the range 860 cm^{-1} to 680 cm^{-1}, demonstrating the absence of signals corresponding to aromatic C-H bending (δC-H_{arom}). Besides, all fractions except for **F3JC**, exhibited the band at 967 cm^{-1} similar to those observed and assigned for **F1JC**, **A02JC**, and related fractions (**A01JC**, and **A03JC**) (**Figure 16**).

Despite the **F3JC** and **A07JC** having the same UHPLC retention time, FTIR profiles showed some differences, highlighting the possible aromatic nature of **F3JC**. In this point, we have several possibilities. On the one hand, we could conclude that **F3JC** and **A07JC** are not the same molecules, and on the other, a chemical transformation such as decomposition over time could explain our preliminary UHPLC results. Nevertheless, a more in-depth characterization must be carried out to elucidate these structures.

F4JC and F5JC fractions

The last eluted samples of **SE**, showed matched FTIR spectra (**Figure 19**), with some commun features in comparison with all analyzed samples. Saturated carbon skeleton (2922 cm^{-1} assigned to ν C-H, and 1458 cm^{-1} assigned to δC-H methyl $-CH_3$, and methylene $-CH_2-$). Besides, these FTIR spectra also exhibited a broad signal at 3361

cm^{-1} corresponding to –OH and/or –NH strechtching, but unlike the other samples, a carbonyl stretching (νC=O) at 1686 cm^{-1} in the typical range of carbonyl amides were observed. A definite signal in the IR fingerprint zone at 753 cm^{-1} could be assigned to aromatic C-H bending ($\delta C\text{-}H_{arom}$), which, alongside the band at 1458 cm^{-1}, corresponding to aromatic C=C bending ($\delta C{=}C_{arom}$), indicates the presence of aromatic fragments in the molecular structures. With the preliminary results for **F4JC** and **F5JC**, these samples could be associated with peptides or protein samples, taking into account the phytochemical analysis previously described in this research.

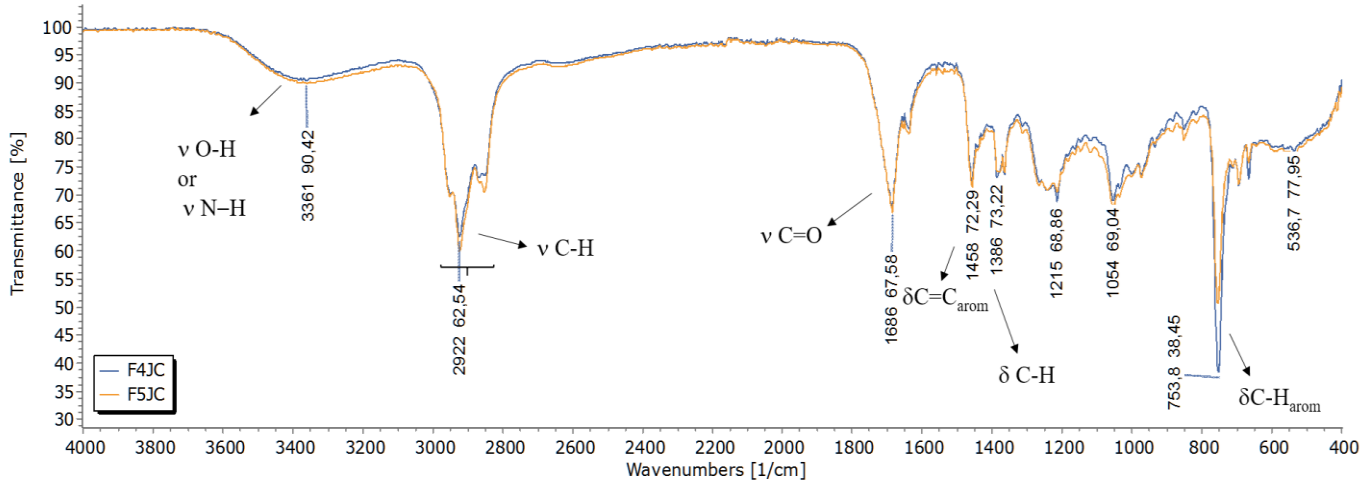

*Figure 19 FT-IR spectra of the fractions **F4JC** and **F5JC***

A12JC to A18JC fractions

From **A08JC** until the **A18JC**, all those fractions had planar UHPLC chromatograms at the analyzed wavelength. The FTIR spectra of the fractions **A12JC** to **A14JC** showed matching features (**Figure 20**), demonstrating that these samples are constituted by saturated and insaturated bonds scaffold (2922 cm^{-1} assigned to ν C-H, and 1458 cm^{-1} assigned to δC-H methyl $-CH_3$, and methylene $-CH_2-$). Related to **A15JC** to **A18JC** samples, its FTIR profiles were similar to previously analyzed fractions, highlighting a marked band at 3341 cm^{-1}, which would be assigned to O-H or N-H stretching. All analyzed fractions also exhibited signals around 1740 cm^{-1} and 967 cm^{-1}, corresponding to carbonyl stretching (νC=O) and C=C bending (δC=C) of tetra-substitute alkene, respectively. The saturated squeleton proposed for **A12JC** to **A18JC**, was confirmed by

^{1}H-NMR of **A12JC** (**Figure 21**), characterized by peaks only at higher fields (δ: 0.86, 1.10, 1.25, 1.53, 2.01, 2.24, and 4.10 ppm) (**Figure 22**).

To summarize, a preliminary characterization of methanolic fractions allowed us a first approximation to structures elucidation. Taking into account all compile data, the isolated samples **F1JC**, **A02JC**, **A03JC**, **A06JC** to **A18JC** are composed by saturated carbon skeleton, with some insaturations, where the polarity of molecules are due to carbonyls, or alcohols or amines, although NMR did not corroborate the presence of these last mentioned functional groups. This structural approach with phytochemical screening allow us to define the absence of phenols. Besides, only **F2JC**, **F3JC**, **F4JC**, **F5JC**, **A04JC,** and **A05JC** showed any hint related to the presence of aromatic fragments. The lower stretching frecuency of carbonyl signal for **F4JC**, and **F5JC**, typical values of carbonyl from amides, could be indicates that these fractions are peptides or proteins. Unfortunately, it is not possible to propose any specific structure, but the collected data provides the basis for a more in-depth phytochemical analysis.

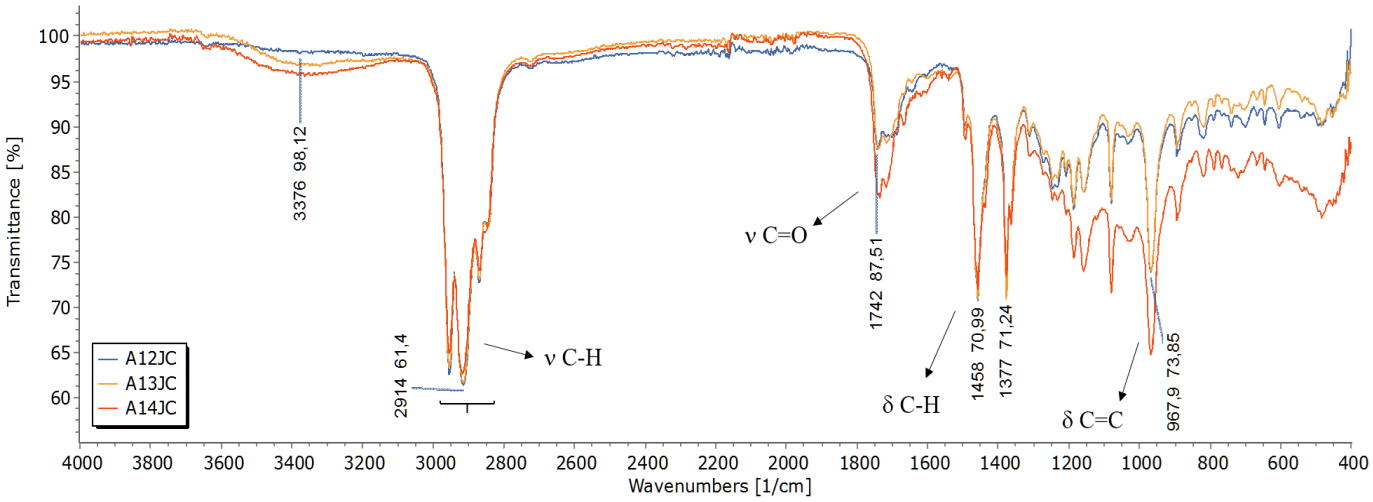

*Figure 20 FT-IR spectra of the fractions **A12JC**. **A13JC** and **A14JC***

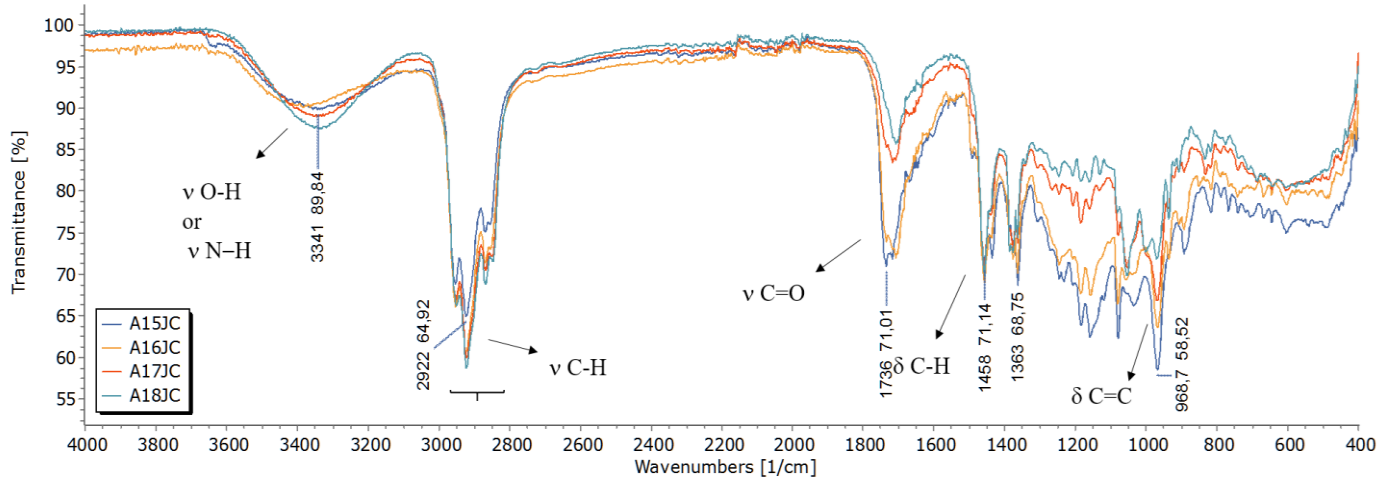

*Figure 21 FT-IR spectra of the fractions **A15JC**. to **A18JC***

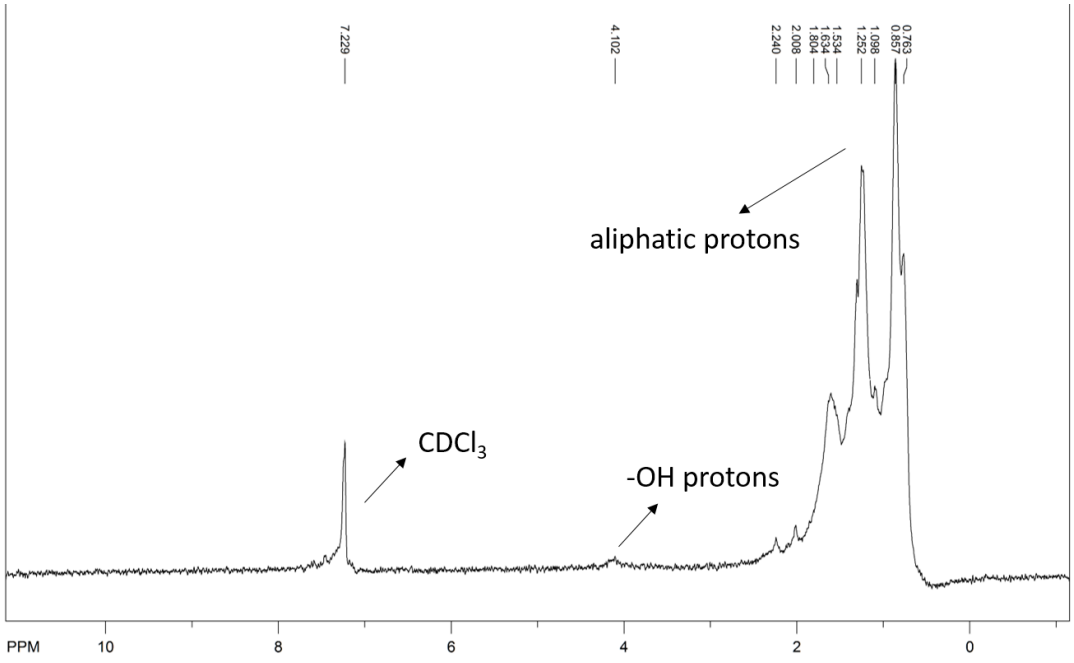

*Figure 22 [1]H-NMR spectra of **A12JC** fraction*

3.2 Biological activity

3.2.1 Antibacterial activity

Another challenge of this investigation is analyzing the antibacterial activity of the separated fraction and methanolic extract of the leaves from *Eupatorium glutinosum* Lam. The antibacterial activity was tested for each fraction of the two sets of samples from the column chromatographies performed to **SE** and **ME** extracts. In this regard, two techniques were used to study the antibacterial efficacy of the isolated fractions, the agar diffusion method and the optical density measurements (OD). The chosen bacteria was *E. coli* because it is one of the most frequent bacteria found in wound infections[37].

3.2.1.1 Agar diffusion method

After the agar diffusion method's performance, the results showed that several fractions tested had inhibitory zones (**Table 4**, **Figure 23** and **24**), confirming its antibacterial activity at a qualitative level. Ampicillin (AMP) was used as a positive control of the experiment. Besides, to rule out any potential activity derived from the solvents used to prepare the samples, an additional control using water:acetonitrile (H_2O:MeCN (5:5)) was included in the procedure. The solvent system showed a little, almost negligible antibacterial activity, not comparable in intensity to that observed for other tested fractions. Furthermore, methanolic and aqueous extracts presented antibacterial activity, highlighting that **ME** showed better defined *E. coli* bacterial inhibition (defined inhibition) than aqueous extract (low defined inhibition) (**Table 4**, **Figure 23**).

Related to **SE**, **F2JC**, **F4JC** and **F5JC** showed well-defined inhibition zones, while **F1JC** and **F3JC** exhibited low defined inhibition zones, confirmed its antibacterial activity at 500 μg/mL. **F2JC**, **F4JC**, and **F5JC** had higher antibacterial activity in front of *E. coli* than those observed to our positive control (AMP) (**Table 4**, **Figure 23**).

Table 4 Agar diffusion method results over E.coli (DH5- alpha)

Antibacterial activity					
Samples		**Results**	**Samples**		**Results**
ME		++	**ME extract separation**	**A07JC**	-
Aqueous Extract		+		**A08JC**	+
SE extract separation	**F1JC**	+		**A09JC**	+++
	F2JC	+++		**A10JC**	++
	F3JC	+		**A11JC**	+++
	F4JC	+++		**A12JC**	+++
	F5JC	+++		**A13JC**	-
ME extract separation	**A01JC**	+		**A14JC**	++
	A02JC	-		**A15JC**	+
	A03JC	-		**A16JC**	+++
	A04JC	++		**A17JC**	++
	A05JC	-		**A18JC**	++
	A06JC	+++			

+++ well defined; ++ defined; + low defined; - no response

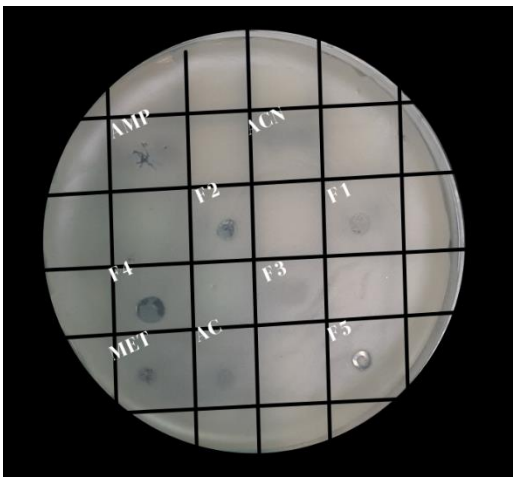

*Figure 23 Antibacterial activity test performed in E. coli strains for **SE** isolated fractions, and Aqueos (AC) and Methanolic extracts (MET). Ampicillin (AMP) was used as a positive control. H_2O:MeCN (5:5) was tested as blank.*

On the other hand, **ME** fractions also showed promising results. From its 18 isolated fractions, only five of them (**A02JC**, **A03JC**, **A05JC**, **A07JC**, and **A13JC**) do not show the inhibitory activity of *E. coli*. Well-defined antibacterial activity was corroborated to five fractions (**A06JC**, **A09JC**, **A11JC**, **A12JC**, and **A16JC**), while seven fractions displayed defined or low defined antibacterial activity (**Table 4**, **Figure 24**).

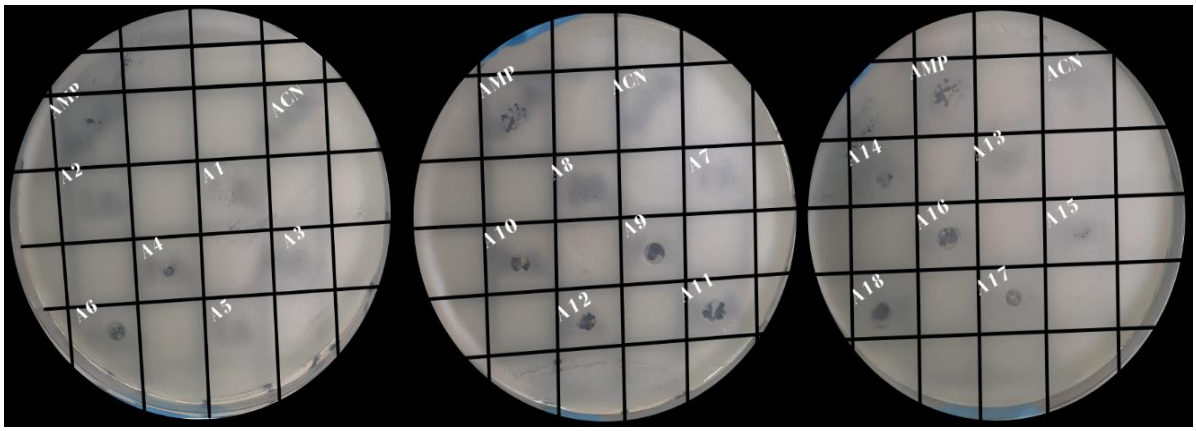

*Figure 24 Antibacterial activity test performed in E. coli strains for **ME** isolated fractions. Ampicillin (AMP) was used as a positive control. H_2O:MeCN (5:5) was tested as blank.*

The agar diffusion method results showed that methanolic extract has a better antimicrobial response than the aqueous extract of leaves from *Eupatorium glutinosum* Lam. This study has also shown that the extracts' antimicrobial activity results from the antagonistic interaction of the extract's phytochemicals. On the other hand, it is possible to confirm at least five phytochemicals with well-defined inhibitory activity in front of *E. coli* strain. Related to the nature of these phytochemicals, antimicrobial activity has been reported for phenolic compounds such as tannins and flavonoids by inhibiting bacterial growth [38]. Besides, examples of terpenoids and alkaloids with the mentioned biological response have also been reported[28,39]. All mentioned secondary metabolites were detected in the phytochemical screening previously described. However, with compile results, it is impossible to establish more specific assignments of compounds class to the isolated fractions. Further studies are necessary to identify the active compound and understand the action mechanism responsible for the biological activity.

Encourage by the excellent qualitative results of the agar diffusion method, Optical Density measurements at 600 nm (OD_{600}) were carried out to quantify the antibacterial activity using

3.2.1.2 Optical density measurements (OD_{600})

Based on previous agar diffusion results, the samples **F5JC**, **A04JC**, **A06JC**, **A09JC**, **A12JC**, **A16JC** were selected for OD_{600} measurements against *E. coli* strain.

The OD_{600} measurements were taken every 30 minutes for 4.5 hours, expecting to reach the steady-state in the growth curve (**Figure 25**). The OD_{600} results demonstrated that

each tested sample presented antibacterial activity with a behavior similar to the positive control (AMP), corroborated previously analyzed agar diffusion results (**Figure 25**). The growth curve did not reach the steady-state due to the assay time was not enough[40]. Nevertheless, this issue does not affect the interpretation of the OD_{600} studies. Water:acetonitrile sample (5:5) was also included in this study, demonstrated that the solvent system used to prepare our biological studies does not influence the final results (**Figure 25**).

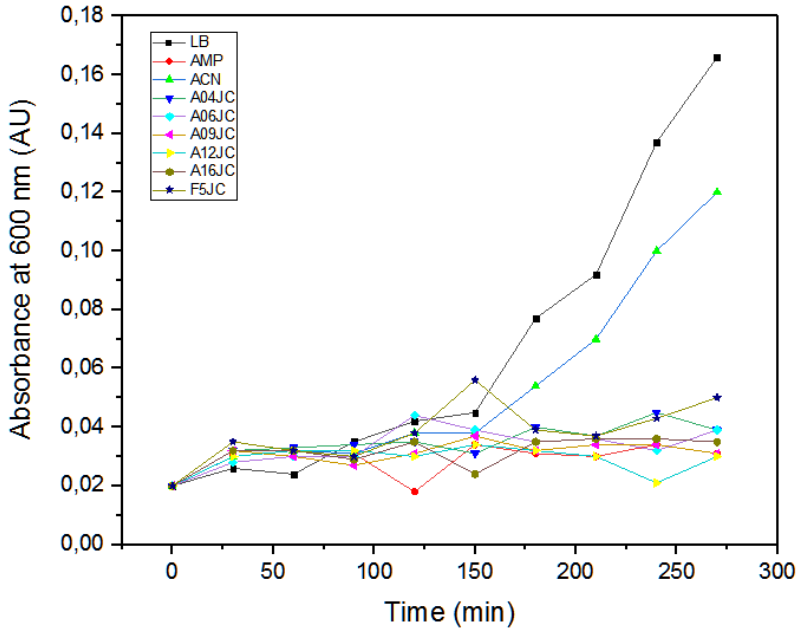

Figure 25 Growth curve for the Antibacterial activity of all fractions tested.

An estimated growth inhibition percentage of the tested fractions was also analyzed. It is reported that *E. coli* culture incubated in LB broth presents an OD_{600} of 0.1 and has a concentration of $8x10^8$ cell/mL [41]. Using this value, it is possible to estimate the number of cells per milliliter and the percentage of inhibition that corresponds to the final OD_{600} measured values of the tested fractions (**Table 5**).

Table 5 Final OD_{600} values and the estimated number of cells per mL for each growth curve performed.

	OD_{600}	cells/mL	% of cells in LB medium	% of inhibition
Standard	0,1	$8{,}00x10^8$		
LB	0,166	$1{,}33x10^9$	100	0
AMP	0,031	$2{,}48x10^8$	18,6746988	81,3
ACN	0,12	$9{,}60x10^8$	72,2891566	27,7
A04JC	0,039	$3{,}12x10^8$	23,4939759	76,5
A06JC	0,039	$3{,}12x10^8$	23,4939759	76,5
A09JC	0,031	$2{,}48x10^8$	18,6746988	81,3
A12JC	0,03	$2{,}40x10^8$	18,0722892	81,9
A16JC	0,035	$2{,}80x10^8$	21,0843373	78,9
F5JC	0,05	$4{,}00x10^8$	30,1204819	69,9

The OD_{600} studies allowed to have a quantitative approximation about each fraction antibacterial response's efficiency. Focused on the Ampicillin's value (AMP) as the positive control with 81,3% of efficacy, we can compare it with the results to the analyzed fractions. Firstly, the antibiotic's value should be the maximum growth inhibition power against *E. coli* strain. The obtained results were consistent with those described in the Agar diffusion study. All tested samples showed an excellent % of inhibition from 69.9 to 81.9%. Even **A09JC**, **A12JC**, and **A16JC** had similar behavior to the positive control. Besides, the solvent system H_2O:MeCN (5:5) used to prepare the samples shows a low efficacy with 27,7% inhibition capacity, confirmed that the antimicrobial activity detected is mainly because of the isolated phytochemicals. However, a potential additive/non-interactive or a positive synergy interaction between acetonitrile and secondary metabolites would also be possible.

The isolated fractions of the extracts of leaves from *Eupatorium glutinosum* Lam. showed promising antibacterial activity against *E. coli* (DH5-α). Almost all phytochemicals demonstrated a high grade of growth inhibition of *E. coli* strain, which was confirmed by quantitative OD_{600} measurements.

Conclusions

- Two methanolic extracts (**SE** and **ME**) of the *Eupatorium glutinosum* Lam. leaves were successfully prepared using Soxhlet and Maceration procedures and reaching 8,75 and 8,5 % of yield, respectively. Besides, Maceration method was also used to prepare an aqueous extract.
- Methanolic extracts (**SE** and **ME**) were analyzed through PDA-UHPLC, and as expected, both crudes showed similar profiles with four majority peaks at 6,5, 6.7, 8.3, and 9.7 minutes.
- Phytochemical screening was performed on the methanolic extract **ME** and on the aqueous extract of leaves from *Eupatorium glutinosum* Lam. For both extracts, phenols, tannins, steroids, terpenoids, cardiac glycosides, and alkaloids were detected, and the presence of carbohydrates was discarded. Flavonoids were identified only in aqueous extract, and reducing sugars were only found in methanolic extract. Amino acids, proteins, and saponins were disclosed in the methanolic extract, while the corresponding test results in the aqueous extract were not conclusive.
- **SE** and **ME** were fractionated using Column Chromatography (CC) with Toluene:EtOAc and Hexane:EtOAc, respectively. **SE** was separated into five fractions (overall yield 33.6%), while **ME** showed 18 isolated fractions (overall yield 52.8%). Hexane:EtOAc worked better as the mobile phase, but the gradient's greater planarity also influenced the more efficient separation.
- A first approach to the content and purity of the different isolated fractions was carried out by TLC and UHPLC. The fractions that eluted at the beginning of the CC (lower polarity of solvent systems, toluene for **SE** extract separation, and hexane for **ME** extract separation) corresponded to compound eluted at 9.6 minutes in the HPLC, while when the polarity was slightly increased (toluene: ethyl acetate (8:2) for **SE** extract separation and hexane: ethyl acetate 85:15 for **ME** extract separation), the isolated compound eluted at 8.2 minutes under HPLC conditions. The majority peak detected at 6.5 minutes of retention time in the HPLC for both crudes (**SE** and **ME** extracts), was correlated to the eluted fraction of CC at an intermediate polarity (toluene: ethyl acetate (9:1) for **SE** extract separation and hexane: ethyl acetate (9:1) for **ME** extract separation), of those previously mentioned.

- The analysis of characterization data obtained through UV/Vis, FTIR and NMR, allowed to have a first approximation to structural characterization of methanolic extracts from *Eupatorium glutinosum* Lam. leaves. All isolated samples seem to have a saturated carbon scaffold with presence of some polar functional groups such as alcohol, amines, or carbonyls. Besides, **F2JC**, **F3JC**, **F4JC**, **F5JC**, **A04JC,** and **A05JC** present aromatic fragments.
- Antibacterial activity of all samples (crude extracts and fractions) were established through Agar diffusion technique. **F2JC**, **F4JC**, **F5JC**, **A06JC**, **A09JC**, **A11JC**, **A12JC**,and **A16JC** exhibited well defined bioactivity in front of *E.coli*, similar to those observed to the positive control ampicillin (AMP). Only five samples (**A02JC**, **A03JC**, **A05JC**, **A07JC**,and **A13JC**) did not respond decreasing bacterial growth.
- Antibacterial activity of **F5JC**, **A04JC**, **A06JC**, **A09JC**, **A12JC**, **A16JC** were determined by OD_{600} measurements against *E. coli* strain. All tested samples showed an excellent % of inhibition from 69.9 to 81.9%, even **A09JC**, **A12JC**, and **A16JC** exhibited similar behavior to the positive control.

Recommendations

- To carry out the phytochemical screening of isolated fractions to match this information with the chemical characterization.
- To apply other characterization techniques such as Mass spectroscopy, Elemental analysis, among others, to unequivocally establish a molecular structure for the samples.
- To extend the search for bioactivity to Gram positive bacteria

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