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Présentée par

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Presented by:

Zohra TOUATI-NAIT CHABANE

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Suppor Exploitation of *Aloysia triphylla* leaves for food and pharmacological application

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Dedicace

I dedicate this work:

To my dear parents,

To my dear husband

To my dear children Anis, Yani and Mira

To my dear brotherAissa and his wife Yasmine

To my dear sisters Nora, Myriam and, to their husbands and children

To my family in law

May God keep you in good health

Zohra

Scientific publications

ZOHRA TOUATI1, MENANA GUEMGHAR1, KENZA BEDJAOUI1, NABILA DJERRADA1, KAHINA DJAOUD1, NAWEL ADJEROUD1, KHODIR MADANI1,2, LILA BOULEKBACHEMAKHLOUF1*. OPTIMIZATION OF THE MICROWAVE ASSISTED EXTRACTION AND BIOLOGICAL ACTIVITIES OF POLYPHENOLS FROM LEMON VERBENA LEAVES. *The Annals of the University Dunarea de Jos of Galati Fascicle VI – Food Technology (2021), 45(1), 157-177.*

Communications

1- **Zohra TOUATI-NAIT CHABANE,** NABILA DJERRADA, KAHINA DJAOUD, NAWEL ADJEROUD, KHODIR MADANI, LILA BOULEKBACHE-MAKHLOUF. Evaluation de l'activité antioxidante de la verveine citronnée (Lemon verbena). Univérsité de Bejaia, 20 et 21 Décembre, 2017.

2- **Zohra TOUATI-NAIT CHABANE,** MENANA GUEMGHAR, KENZA BEDJAOUI, NABILA DJERRADA, KAHINA DJAOUD, NAWEL ADJEROUD, KHODIR MADANI, LILA BOULEKBACHE-MAKHLOUF. Optimisation d'extraction des composés phénoliques par microonde, à partir de Lemon verbena et dosage des polyphénols. Université de Bejaia, 20 et 21 décembre 2018.

3- **Zohra TOUATI-NAIT CHABANE,** MENANA GUEMGHAR, KENZA BEDJAOUI, NABILA DJERRADA, KAHINA DJAOUD, NAWEL ADJEROUD, KHODIR MADANI, LILA BOULEKBACHE-MAKHLOUF. Evaluation de l'activité antioxidante des feuilles de la verveine citronnée (Lemon verbena). 20, 21 décembre 2017. Université de Bejaia.

4- **Zohra TOUATI-NAIT CHABANE,** MENANA GUEMGHAR, KENZA BEDJAOUI, NABILA DJERRADA, KAHINA DJAOUD, NAWEL ADJEROUD, KHODIR MADANI, LILA BOULEKBACHE-MAKHLOUF. Optimization by microwave assisted extraction of phenolic compounds from Lemon verbena (*Aloysia triphylla*). 29éme congrès international des sciences biologiques et de la biotechnologie de l'ATSB, Hotel Mouradi Palace, 26th to 29th of Mars 2018, Sousse, Tunisie.

5- **Zohra TOUATI-NAIT CHABANE,** MENANA GUEMGHAR, NABILA DJERRADA, KAHINA DJAOUD, KHODIR MADANI, LILA BOULEKBACHE-MAKHLOUF. Study of the antioxidant activities of Lemon verbena leaves and optimization of polyphenolic compounds extraction using the microwave. 15, 16 December 2018. Chemini, Bejaia. Algeria.

6- **Zohra TOUATI-NAIT CHABANE,** MENANA GUEMGHAR, NABILA DJERRADA, KHODIR MADANI, LILA BOULEKBACHE-MAKHLOUF. Optimization by microwave assisted extraction of phenolic compounds from Lemon verbena (*Aloysia triphylla*).15, 16 January 2018. University of Bejaia.

Liste of abbreviations

AchE: Acetylcholinesterase acrtivity

ABTS: 2,2-9-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid

ANOVA: Analysis Of Variance

BC: Before Christ

CCD: Central Composite Design

C.V.: Coefficient of Variation

CE: Conventional extraction

DPPH: 2,2-Diphenyl-picrylhydrazyl

DW: dry weight

FRAP: Ferric Reducing Antioxidant Power

F-Value: F-value Fisher value

GAE: Gallic acid equivalent

HPLC-DAD-ESI-MS/MS: High Performance Liquid Chromatography diode array detector-electrospray-MS/MS

HOSC: Hydroxyl radical scavenging capacity

JMP: John's Macintosh Project, pronounced, "jump"

MAE: Microwave Assisted Extraction

ME: Maceration extraction

ORAC: Oxygen Radical Absorbance Capacity

RSM: Response Surface Methodology

$R^{2:}$ Coefficient of determination

R^2 ***ajusted:*** Adjusted coefficient of determination

ROS: reactive oxygen species

TPCs: Total Phenolic Compounds

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General introduction

I General introduction

Since 60 000 years ago Neanderthal man was the first to use plants for therapeutic purposes (**Jamshidi-Kia *et al.*, 2018**). This practice has continued throughout antiquity (**Guidi and Landi, 2014; Hassan, 2015; Jamshidi-Kia *et al.*, 2018**), and middle ages (**Guidi and Landi, 2014**). This continued interest in plants was due to the therapeutic compounds they contain; they represent an inexhaustible source of bioactive compounds such as polyphenols (**Marmouzi *et al.*, 2019; Piwowarski *et al.*, 2020; Guemghar *et al.*, 2020**).

In a country as rich in flora as Algeria, it is essential to be interested in the sphere of aromatic and medicinal plants. Effectively, according to **Petrovska. (2012),** medicinal and aromatic plants are used mainly by the elderly in the isolated regions. In the Hoggar and in some remote areas, such as among the Tuareg, plants are used according to their availability as a source of food, medicine and fodder. The secrets of their knowledge are passed along from father to son (**Reguieg, 2011**; **Miara *et al.*, 2018)**. According to **Benarba. (2016),** 83 medicinal plants species are used by traditional healers from South-west of Algeria (Bechar and Adrar) to treat several diseases. Some of these plants are *Lawsonia inermis* L. (to treat cystitis), *Parietaria officinalis* L. (to treat kidney stones), Roots of *Anacyclus pyrethrum* (L.) Lag., *Berberis vulgaris* L., and leaves of *Prunus persica* (L.) Batsch (used to treat different cancers). In Kabylia, mountain people use 98 medicinal and aromatic plants species to treat different diseases (**Bounar *et al.*, 2018**), like *Ballota nigra* L., *Mentha pulegium* L., (to treat indigestion, constipation, stomach ulcer, hyperacidity, vomiting), *Urtica dioica* L., *Dioscorea communis* (L.) (to treat eczema, scabies, buttons, frostbite, freckles, allergic rashes) (**Meddour and Meddour-Sahar, 2015**). According to **Jamshidi-Kia *et al.* (2018),** traditional therapeutic knowledge, transmitted from generation to generation among rural populations, is an oral family heritage. It is dominant especially among older women. In the present time, the preservation of this endangered ancestral heritage becomes really essential.

Many potent drugs and therapeutic leads as well as many new pharmacologically active constituents have been developed from herbal medicines through the sustained efforts of researchers. There is a promising future of medicinal plants as there are about half million plants around the world. It is noticed that most of them are not investigated yet for their medical activities and their hidden potential of medical activities could be decisive in the treatment of present and future deseases (**Singh, 2015; Srivastava and Singh, 2020**).

Medicinal plants are rich sources of bioactive compounds, which are widely studied for their proven efficacy in health, cosmetics and food fields (**Bhramsoltani *et al.*, 2018**). Nowadays, despite the enormous discoveries made by medicine in the treatment of diseases with various synthetic drugs, phytotherapy represents a key issue in the treatment of chronic diseases like inflammatory, hepatic, steatosis, cancer, oxidative stress and diabetes diseases, that are mostly due to the presence of free radicals in the body (**Telli *et al.*, 2016; Ma *et al.*, 2018; Deepika *et al.*, 2019; Bouzabata and Mahomoodally, 2020**). In addition to the medical field, secondary metabolites of plants are used in the cosmetic and food fields (**Fongnzossie *et al.*, 2017; Hughes *et al.*, 2019; Tripodo and Mandracchia, 2019; Guemghar *et al.*, 2020**). These bioactive compounds are less aggressive, have fewer side effects and interactions, and they are better tolerated (**Câmara *et al.*, 2020**), knowing that recently, many serious side impacts in humans have been reported by synthetic preservatives such as cancer and cardiovascular disorders (**Atta *et al.*, 2017; Mohammadzadeh-Aghdash *et al.*, 2019**).

Several categories of phytochemicals have been identified in the different parts of plants such as terpenes, phenylethanoids, flavonoids and miscellaneous compounds (**Bahramsoltani *et al.*, 2018)** and phenolic acids (**Kumara and Goel *et al.*, 2019; Rattan *et al.*, 2020**). Polyphenols, as phytochemicals, have gained great importance due to their numerous health benefits with respect to lifestyle diseases and oxidative stress; they are the most compounds, which confer bioactive potential to plants (**Moshari - Nasirkandi *et al.*, 2020**). Indeed, phenolic compounds are known by their biological activities, like antioxidant, antibacterial, enzymatic and anti-inflammatory activities (**Cheurfa and Allam, 2016; Bahramsoltani *et al.*, 2018; Marques Jucá *et al.*, 2018; Djaoud *et al.*, 2020; Dirar *et al.*, 2019; Guemghar *et al.*, 2020**). This is why recent research has focused on improving the methods of their extraction, in order to optimize their recovery in terms of quantity and quality (**Djaoud *et al.*, 2020; Leyva-Jiménez *et al.*, 2020**).

The development of a single standard method for the efficient and rapid extraction of polyphenols from plant matrices has remained a challenge due to the inherent limitations of various conventional extraction methods (**Ameer *et al.*, 2017**). The exploitation of polyphenols as bioactive compounds at various commercial levels has motivated scientists to explore more eco-friendlier, efficient, and cost-effective extraction techniques. They are mainly based on a green extraction approach, such as supercritical fluid extraction, ultrasonic assisted extraction, microwave assisted extraction (MAE), pressurized liquid extraction, pressurized hot water

extraction, high voltage electrical discharges (HVED) and pulsed electric field (PEF). These methods are used as alternatives to conventional extraction methods for the extraction of polyphenols, such as maceration, Soxhlet extraction and others usually involve large amounts of solvent, are time consuming, and can causes degradation of some of the desired compounds. That is why such methods are considered non-green (**Oreopoulou *et al.*, 2019**). Green techniques show promise for the extraction of thermolabile phenolic compounds due to their advantages over time-consuming and laborious conventional extraction techniques, such as reduced solvent use, reduced time and energy consumption, and higher recoveries at lower operational costs. The growing interest in plant-based polyphenols is leading to a continuous search for modern, environmentally friendly and economically viable extraction techniques. Modern green extraction techniques represent promising approaches to overcoming current limitations in the exploitation of polyphenols as bioactive compounds in order to explore their broad applications on an industrial scale and in emerging global markets (**Lončarić *et al.*, 2020).**

Microwave assisted extraction is one of the most efficient eco-extraction methods (**Dahmoune *et al.*, 2015; Ekezie *et al.*, 2017**). In fact, it allows high extraction efficiency of total phenolic compunds, which can generally be attributed to its heating effect, which occurs due to the dipole rotation of the solvent in the microwave field. This causes the solvent temperature to rise, which then increases the solubility of the compound of interest (**Hayat *et al.*, 2009**). Its effectiveness is also due to the special microwave/matter interactions and the very rapid extraction time (**Setyaningsih *et al.*, 2015**). This process is used for the extraction of phytochemicals for the pharmaceutical and food industries (**Hayat *et al.*, 2009; Dahmoune *et al.*, 2015**).

Traditionally, optimization is achieved by monitoring the influence of one factor at a time. However, by using the response surface methodology (RSM), optimization is done simultaneously and in a multivariable form; the interaction effects between the factors can be assessed allowing a much more precise identification of the optimal conditions. The RSM, by means of mathematical equations, can describe the behaviour of the various variables and forecast the results for the system (**Simić *et al.*, 2016**). Several recent works had studied the optimization of response (s) from *Aloysia triphylla* leaves using RSM (**Ivanović *et al.*, 2018; Leyva-Jiménez *et al.*, 2020 a, Leyva-Jiménez *et al.*, 2020 b; Villegas-Aguilar *et al.*, 2020; Valiyan *et al.*, 2021**).

Aloysia triphylla belongs to the *Verbenaceae* family, is well known as one of the most important accumulating secondary metabolites in both vegetative and reproductive organs (**Shahhoseini *et al.*, 2013**), Lippia genus has more than 200 Species (**Boustani *et al.*, 2016**) which *Aloysia triphylla* is the most important species (**Khani *et al.*, 2012**). Limited informations are available on the production and economic impact of *Aloysia triphylla* in the world and in Algeria. According to **Bukero and Philipos. (2018)**, this plant, which is indigenous to Central and South America, is cultivated in Latin America, France, China and in North Africa. Indeed, in Ethiopia, the selected herbal plant provides the net return, net present value (NPV) and Benefit-Cost Ratio (BCR) of 12828.5$/ha, 10043.6 and 0.2 at fresh bio mass price of 0.4$/kg respectively.

Aloysia triphylla is commonly consumed as infusion, for the treatment of colic, diarrhea, indigestion diabetes, cancer, cardiovascular diseases and viral diseases like coronavirus (MERS-CoV) (**Bahramsoltani *et al.*, 2018; Brahmi *et al.*, 2022**)., due to the significant amounts of polyphenols (**Bilia *et al.*, 2008; Abderrahim *et al.*, 2011; Bahramsoltani *et al.*, 2018; Rocha *et al.*, 2019).**

Although the term "tea" refers to infusions made from leaves of *Camellia sinensis* (L.) Kuntze, it also refers to the wide variety of infusions prepared from dried aromatic plants or parts of plants, such as roots, rootstocks, shoots, leaves, flowers, barks, fruits or seeds other than the leaves of *C. sinensis*. The popularity of these herbal and fruit beverages prepared as infusions reflects the increasing consumer appreciation for their wide range of natural and refreshing tastes and other sensory properties (**Rocha *et al.*, 2020**). In addition, herbal teas help to supplement good hydration, which is essential for maintaining body water balance, and are part of the Mediterranean diet (**Bach-Faig *et al.*, 2011**). They also contribute to a balanced diet, as they contain no sugar and are almost calorie-free (**Schulzki *et al.*, 2017**). *Aloysia triphylla* infusions are rich in polyphenols, such as phenylpropanoid glycosides, especially verbascoside and flavone diglucuronides such as luteoline-7-diglucuronide, which has high antioxidant activity and other functional constituents that have relatively high antioxidant activities (**Felgines *et al.*, 2014**). In addition, the tea is consumed for many purposes such cold and fever, influenza, nerve problem, acne, and as an insecticide, bactericide, tonic, antispasmodic, carminative, and stimulant (**Bahramsoltani *et al.*, 2018**).

To our knowledge, there are no reports in the literature on the optimization of microwave-assisted extraction (MAE) of total phenolic compounds (TPC) from *Aloysia triphylla* leaves.

Research problem

The present work carried out on the leaves of *Aloysia triphylla* aims to:

- Optimize the extraction of TPC from *Aloysia triphylla* leaves by MAE and to compare this method to a reference procedure which is maceration (conventional extraction).

- Identify by HPLC-DAD-ESI-MS/MS the phenolic compounds contained in the two extracts of *Aloysia triphylla*, in the positive and negative ionization modes.

- Study the antioxidant, antibacterial and anti-acetylcholinesterase activities of the optimized extract and compare these activities with those of the conventional extract.

To this end, the present work will be presented in two parts:

The first part, concerns the bibliographical study of the *Aloysia triphylla* plant, it is divides into two parts:

- Part I.A. Aromatic, medicinal and ethnobotanical study of *Aloysia triphylla*, this part deal with 4 points:

❖ Aromatic and medicinal plants.
❖ *Aloysia triphylla* phytochemistry.
❖ Biological activities of *Aloysia triphylla*.
❖ Ethnobotanical study of *Aloysia triphylla* leaves.

- Part I.B. Plant sample preparation and extraction method. This part deals with three main points:

❖ Extraction methods.
❖ Microwave Assisted Extraction (MAE): principle.
❖ Factors affecting MAE.
❖ Optimization of Microwave Assisted Extraction by experimental design.

The seconde part concerns the experimental study, it is subdivided into four principale parts:

❖ Optimization of microwave phenolic compounds extraction by experimental design.
❖ Spectrophotometric determination of optimized and conventional extracts.
❖ Identification of phenolic compounds by LC-DAD-ESI-MS/MS.
❖ Biological activities of optimized and conventional extracts.

Theoretical part

PART. I. Aromatic and medicinal plants and Ethnobotanical study of Aloysia triphylla

I.1. Aromatic and medicinal plants

Medicinal plants have been the mainstay of traditional herbal medicine among rural people around the world for tens of thousands of years. They have been a resource to meet therapeutic needs. The first written records on medicinal application of plants date back to 2600 BC, which report the existence of a sophisticated medicinal system in Mesopotamia, comprising about 1000 plant-derived medicines (**Borchardt, 2002; Petrovska, 2012**). The therapeutic use of plants was demonstrated by the Sumerian and Akkadian civilizations around the third millennium BC. Egyptian medicine dates back to about 2900 BC, however, their most useful preserved record is the "Ebers Papyrus"from about 1550 BC, containing more than 700 drugs, mainly of plant origin (**Cragg and Newman., 2013**). Hippocrates (460-377 BC), one of the ancient authors who described medicinal natural products of plant and animal origin, listed about 400 different plant species for medicinal purposes (**Doughari, 2012**). Traditional Chinese medicine has been extensively documented over thousands of years (**Unschuld, 1987**), and the documentation of the Indian Ayurveda system dates back to the 1st millennium BC (**Patwardhan, 2005**).

Nowadays, synthetic drugs gained popularity against green remedies because of their fast-acting effects. However, people have begun to realize the benefits associated with natural remedies. Chemically prepared drugs may act quickly, but they have side effects which affect human body negatively in the long term, whereas, medicinal plants work in an integrated or probiotic with little or no adverse effects on the body (**Idu, 2009**).

Over the years, aromatic and medicinal plants have become a very important part of modern civilization as a natural source of therapy and are of particular interest to scientists looking for alternative sources of medicines. This great interest to medicinal plants is due to their pharmaceutical, cosmetic and nutritional values (**Jamshidi-Kia *et al.*, 2018)**.

Indeed, in recent years there has been a growing interest in the use of natural products, both for their medicinal properties and for their flavor characteristics. Despite the enormous discoveries made by medicine in the treatment of diseases with various synthetic drugs, phytotherapy currently represents a key issue in their treatment (**Li *et al.*, 2020**), thanks to the active compounds of plants such as alkaloids, phenols, tannins, glycocides and terpenoids (**Akinyemi *et al.*, 2018**). Therapeutic use of plants is omnipresent in some countries, especially in developing countries (**Sharifi-Rad *et al.*, 2016**). Indeed, about 3.4 billion people in the

developing world depends on traditional herbal medicines, which represents about 88 % of the world's population, who rely primarily on traditional medicine for their primary health care. According to the World Health Organization, a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemical-pharmaceutical semi-synthesis. Parts of such plant, including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds are used in the control or treatment of a disease, thanks to their therapeutic, tonic, purgative or other pharmacological properties due to the different chemical compounds they contain (**Doughari, 2012; Al-Janae'e *et al.*, 2017; Ates and Culpan, 2018; Anand *et al.*, 2019**).

These non-nutritive plant chemical compounds or bioactive components are often referred to as phytochemicals ("phyto-" from the Greek - phyto meaning "plant") or phytoconstituents and are responsible for protecting the plant against microbial infections or pest infestations (**Nweze *et al.*, 2004; Doughari *et al.*, 2012**). These phytochemicals have been extracted, isolated and identified from leaves, flowers, roots, seeds and spices of several plants, for their antioxidant, antibacterial and antifungal potential, treatment of indigestion, constipation, arthritis, skin diseases, liver and intestinal problems, treatment of diabetes, cancer, cardiovascular diseases and viral diseases like coronavirus (MERS-CoV) (**Delesa, 2018; Khan *et al.*, 2019; Adhikari *et al.*, 2020; Rolta *et al.*, 2020; Ardalani *et al.*, 2021; Jeyaraj *et al.*, 2021; Khanal *et al.*, 2021; Tiji *et al.*, 2021**).

Primary and secondary metabolites of phytochemicals are used in cosmetics, perfumes, food and other purposes (**Tripodo and Mandracchia, 2019**). They are added to food to preserve its freshness or to improve its colour, flavour or texture. According to **Delesa *et al.* (2018**), there is a growing interest of researchers in the application of natural compounds as a safe replacement for chemical and physical food preservatives, which have many side effects and cause health risks for consumers. Some additives have been used for centuries, for example, the preservation of food by pickling (with vinegar), salting, as with meat, and the preservation of sweets. With the advent of processed foods in the second half of the twentieth century, many other plant-based additives were introduced into the food industry. The importance of medicinal, aromatic and spice plants in food additives is due to their antimicrobial (bactericidal and fungicidal) properties, which make them popular preservatives for fresh vegetable or meat preparations as well as for canned products (**Davidson *et al.*, 2006)**. Given the increased consumer demand for natural preservatives and their distrust of chemical compounds, it is

imperative to increase research on the application of plant antimicrobials to food safety. (**Ghorbanpour *et al.*, 2018**).

In the other hand, plants have always been the main source of active cosmetic ingredients, with proven beneficial effects on human health, such as anti-ageing, antioxidant, anti-inflammatory, UV protection, anti-cancer, anti-wrinkle, skin soothing, whitening and moisturising. Aromatic and medicinal plant extracts have been widely used as effective active ingredients in cosmetic or nutricosmetic products, especially in topical application products and skin care formulations. Over the last decade, there has been a growing interest in cosmetic active ingredients derived from plant cell cultures. These are a "new generation" of high quality natural products, produced by modern global biotechnology methods, which generally exhibit stronger activities than plant extracts obtained by conventional methods (**Georgiev *et al.*, 2018**).

It is estimated that up to 70000 species are used in folk medicine, but it is not known how many species are used in other areas, such as cosmetics or flavors. However, it can be stated that at least one in four plants is used, a calculation based on the estimated total number of 300-350000 flowering plants (**Ates and Culpan, 2018**).

Algeria, with its 237.639.100 ha, its different bioclimatic and ecological regions and its remarkable diversity of species, is moderately ranked among the countries known for their taxonomic, ecosystemic, landscape and cultural diversity. Its privileged biogeographic position between the Mediterranean and Sub-Saharan Africa has enriched a floristic potential composed of Mediterranean elements, Ethiopian and endemic species (**Souilah *et al.*, 2018; Hamza *et al.*, 2019)**. In Algeria, as in all the Maghreb countries, medicinal and aromatic plants are used mainly in rural areas by the elderly, who still know some herbal recipes. In the Hoggar (Great Desert) and in the absence of doctors in certain isolated areas, the Tuaregs can treat themselves with medicinal and aromatic plants whose secret has been passed down from father to son (**Reguieg, 2011**). Species of spontaneous flora constitute a significant part of local genetic resources with pastoral, forage, food, aromatic and/or medicinal value (**Nazim *et al.*, 2020**).

A remarkably rich ancestral knowledge of traditional medicine still exists in the forest and mountain region of the province of Tizi Ouzou (Djurdjura Park) (Algeria). There, mainly illiterate and unemployed women of over 50 years old, have a good knowledge of this traditional medicinal practice. A total of 80 plant species have been identified, belonging to 73 genera and 43 families, most of which belong to the *Lamiaceae* family. These aromatic and

medicinal plants are mostly spontaneous (81.25%). They are used in the preparation of 239 therapeutic recipes to treat a range of 70 diseases and symptoms. Utmost medicinal plants (88.75%) are non-toxic, furthermore, they are often multipurpose plants, thanks to their medicinal, food and fodder utilities. These plants are used with caution in traditional medicine, and only for external use (**Meddour *et al.*, 2020**).

Helali *et al.* (2019) carried out a recent study, on the use of medicinal plants by the Algerian population, during the epidemic due to the coronavirus. 57 species were used, 62% of the participants declared having resorted to medicinal plants from the beginning of the epidemic, either to clean the air or to relieve certain symptoms linked to the respiratory infection caused by this virus. Among the plants used, Eucalyptus, Oregano, Cloves, Thyme, *Aloysia triphylla*, Spearmint and Lemon. These plants are known for their richness in essential oils whose anti-infectious and antibacterial activities as well as their marked tropism to the bronchopulmonary sphere have been demonstrated by numerous in *vitro* and in *vivo* studies. Nevertheless, it should be noted that none of these plants cited has a proven antiviral activity against 2019-nCoV.

Brahmi *et al.* (2022) conducted an ethnobotanical survey in the department of Bejaia, Algeria, to identify plants used for the treatment of coronavirus (COVID-19). The local population, to prevent and treat COVID-19 infection, has adopted 23 medicinal plants. Among these plants were *Aloysia triphylla*, *Mentha spicata* L., *Citrus limon* (L.) Osbeck, *Thymus vulgaris* L., *Zingiber officinalis* Roscoe, *Artemisia herba-alba* Asso and *Eucalyptus globules* labill, most of these plants were used as infusions.

Indeed, several authors have published an ethnobotanical research about many plants in Algeria, (**Lakhdari *et al.*, 2016; Ouelbani *et al.*, 2016; Hamza *et al.*, 2019; Ammar *et al.*, 2020; Benaissa *et al.*, 2020**). Among the medicinal plants with significant therapeutic interest, include *Aloysia triphylla* (**Reguieg, 2011; Cheurfa and Allem, 2016**).

I.2. *Aloysia triphylla* phytochemistry

Several categories of phytochemicals have been identified in different parts of this plant, such as terpenes, phenylethanoids and phenylpropanoids, flavonoids, miscellaneous compounds (**Bahramsoltani *et al.*, 2018)** and phenolics (**Pereira *et al.*, 2017; Bekara *et al.*, 2020**). These compounds are identified in the aqueous and organic extracts of this plant like dihydrocaffeic acid , 4-hydroxycinnamic acid, luteolin 7-0-glycoside, they are responsible for antimicrobial, anti-inflammatory and antioxidant activities **(Kumar *et al.*, 2008; Lenoir *et al.*, 2011;**

Choupani *et al.*, 2014 ; Cheurfa and Allem., 2016; Sánchez-Marzo *et al.*, 2019). These phenolics are one of the most significant compounds which confer bioactive potential to plants (**Moshari - Nasirkandi *et al.*, 2020)**. The hydro-alcoholic extract of *Aloysia triphylla* has an antioxidant potential (**Cheurfa and Allem, 2016; Hosseinzadeh and Ebrahimzadeh, 2020**), antibacterial activity (**Bazzaz *et al.*, 2018; Jalal *et al.*, 2019**). **Funes *et al.* (2010)** had shown that verbascoside which is the major polyphenol in this plant, disrupts the structure of the phospholipid membrane of bacteria. For this reason, recent research has focused on improving the methods of their extraction, in order to optimize their recovery quantitatively and qualitatively **(Leyva-Jiménez *et al.*, 2020b)**.

Various classes of phytochemicals have been described in different parts of *Aloysia triphylla*, including terpenoids and phenolics, which are the most relevant groups of bioactive compounds **(Quirantes-Pine *et al.*, 2009)**. Fatty alcohols and ketones have also been identified **(Argyropoulou *et al.*, 2007; Parodi *et al.*, 2013)**.

I.2.1. Terpenes

Terpenes are one of the most diverse classes of phytochemicals. Terpenes, especially those found in the essential oils, are considered as suitable penetration enhancers in pharmaceutical industries since they can improve the permeability coefficient of poorly-absorbed drugs **(Williams and Barry, 2004)**. They are also used as flavor and fragrance in food industries due to their pleasant organoleptic properties **(Merfort, 2002)**. A wide variety of terpenes and terpenoids have been detected in *Aloysia triphylla*.

I.2.1.1. Monoterpenes and monoterpenoids

The essential oil of *Aloysia triphylla* contains 79.5-83.3% of monoterpenoids, including limonene (17.7%), geranial (10.1%) and neral (9.8%), was the major components of the essential oil **(Hudaib *et al.*, 2013**). Some monoterpene alcohols such as α- and γ-terpineol and monoterpene ketones such as myrcenone were also identified in the essential oil. Monoterpenes have an important role in the antibacterial activity of this plant. Indeed, low amounts of these compounds induced less potent antibacterial activity **(Parodi *et al.*, 2013)**. Iridoids, a subgroup of monoterpenoids, were identified also in their glycosylated form in the aqueous and methanolic extract of *Aloysia triphylla* aerial parts **(Quirantes-Pine *et al.*, 2009**).

I.2.1.2. Sesquiterpenes

According to **Argyropoulou *et al.* (2007),** sesquiterpenes of *Aloysia triphylla* cultivated in Greece, constitute only 14.1-15.1% of its essential oil. However, **Bahramsoltani *et al.* (2018)** and **Meshkatalsadat *et al.* (2011**) had identified 59.54% of monoterpene derivatives (including 15.70% monoterpenes and 43.84% oxygenated monoterpenes) and 26.93% sesquiterpenoids in the essential oil of the plant grown in Iran. It is worth mentioning that, unlike monoterpenes, the presence of sesquiterpene compounds is not limited to the essential oil as they can be isolated from different extracts of the plant. A pressurized leaf extract obtained by supercritical CO_2, contains 26% sesquiterpenes, these molecules contain β-caryophyllene (6.5%) and germacrene D (2.4%) (**Parodi *et al.*, 2013**). The ethanolic extract of the plant also contained some sesquiterpenes such as turpinionoside D, dihydrovomifoliol-O-β-D-glucopyranoside and Eudesm-4(15)-ene-1β,6α-diol (**Zhang *et al.*, 2015a, Zhang *et al.*, 2015b**).

I.2.2. Phenylethanoids and phenylpropanoids

Phenylpropanoids are phenolic compounds, a class of plant secondary metabolites that are products of the shikimic acid pathway (**Ferrer *et al.*, 2008**). The most important phenylethanoid isolated from *Aloysia triphylla* is verbascoside (acetoside) and its derivatives, **Quirantes-Pine *et al.* (2009)** reported that β-hydroxyverbascoside and β-hydroxyisoverbascoside could not be distinguished due to their similar fragmentation pattern in MS-MS spectrometry. Phenylpropanoids have a strong antioxidant capacity (**Vertuani *et al.*, 2011; Martino *et al.*, 2016**), and the development of extracts enriched with these compounds could lead to greater antioxidant effects and improved functional ingredients to prevent chronic diseases. So, the related biological effects are mainly attributed to verbascoside (**Sánchez-Marzo *et al.*, 2019**).

These phenolic compounds were summarized in the **figure 1**

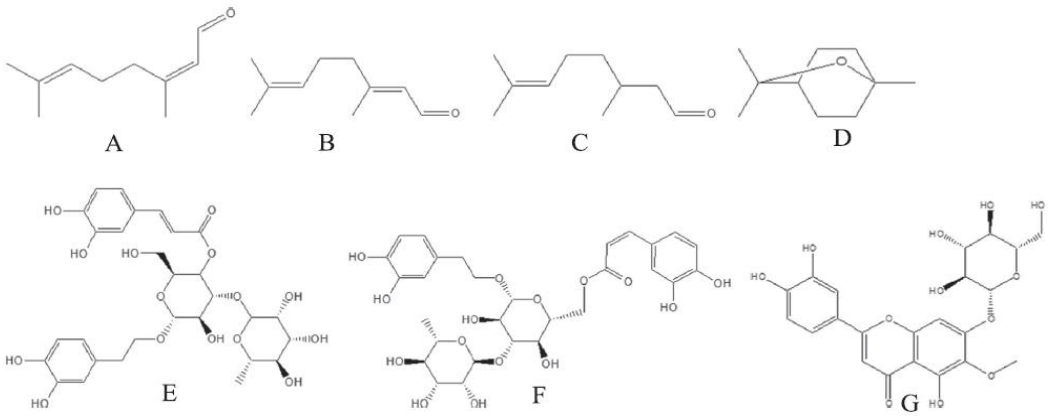

Figure 1: Chemical structure of the most popular components of *Aloysia triphylla*. A: neral, B: geranial, C: citronellal, D: 1,8-cineole, E: verbascoside, F: isoverbascoside, G: nepitrin (**Bahramsoltani *et al,* 2018**).

I.2.3. Miscellaneous compounds

In addition to the above-mentioned phytochemicals, some other chemical structures (**Table I**) such as fatty alcohols, ketones, lignans, and triterpenes are also isolated from the essential oil, aqueous extract, and alcoholic extracts of *Aloysia triphylla* aerial parts (**Bahramsoltani *et al.,* 2018**).

Table I: *Aloysia triphylla* phenolic compounds and their biological activities.

Therapeutic activity	Fraction	Compound	Reference
Antimicrobial activity (*Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa*)	Ethanolic extract/ Essential oil	Verbascoside	Mirzaie *et al.*, 2016; Oukerrou *et al.*, 2017
Larvicidal effects (*Culex quinquefasciatus*)	Essential oil	Neral and geranial	Benelli *et al.*,2017
Anesthesic and neuropsychological	Essential oil/ Aqueous extract	/	Parodi *et al.*, 2013 ; Abuhamdah *et al.*,

Effects/ Coordinator for anxiety			2015 ; Ragone *et al.*, 2010
Anticonvulsant activity	Ethanolic extract	/	Rashidian *et al.*, 2016
Gastroprotective effects	Aqueous / Ethanolic extracts	Vitexin and isovitexin, identified (flavonoids)	Mashayekhi-sardoo *et al.*, 2020
Antioxidant effect	Ethanolic/ Aqueous extract	Verbascoside (caffeoyl phenylethanoid glycoside	Cheurfa and Allem, 2016; Hosseinzadeh and Ebrahimzadeh, 2019; Di Giancamillo *et al.*, 2015
Antinociceptive and anti-inflammatory effects	Essential oil/ Organic extract	Citral/ verbascoside	Ponce-Monter *et al.*,2010
Antigenotoxic effect (ultraviolet radiation)	Essential oil	/	Quintero Ruiz *et al.*, 2017
Antihyperalgesic activity	Hydroalcoholic extract	verbascoside	Isacchi *et al.*, 2011
Metabolic effects	Organic exytact	Polyphenols	Herranz-Lopez *et al.*, 2015
Cardiovascular effects	Aqueous extracts	Verbascoside	Bahramsoltani *et al.*, 2018
Anticancer effects	Ethanolic extract	/	Mirzaie *et al.*, 2016.

I.3. Biological activities of *Aloysia triphylla*

I.3.1. Antioxidant activity

Several studies have shown that *Aloysia triphylla* has a strong antioxidant activity (**Sánchez-Marzo *et al.*, 2019**). After oral administration of this plant extract to rats, evaluation of malondialdehyde (MDA) generation, plasma iron reduction capacity (FRAP) and superoxide dismutase (SOD) activity in plasma samples proved the strong antioxidant activity of the plant (**Funes *et al.*, 2010**). **Quirantes-Pine *et al.* (2013**) demonstrated that administration of this extract to rats induced an increase in the activities of antioxidant enzymes, such as glutathione peroxidase (Gpx), catalase and glutathione reductase (Gred), reduced myeloperoxidase (MPO) activity and protection of blood cells against oxidative damage. According to **Portmann *et al.* (2012)**, *Aloysia triphylla* aqueous extract protects against lipid peroxidation and protein carbonylation. *Aloysia triphylla* essential oil exhibits a high antigenotoxic effect against ultraviolet radiation-induced DNA damage (**Quintero Ruiz *et al.*, 2017**).

Oral administration of verbascoside (maximum dose 3 mg/day) as a dietary supplement to hares indicated a protective effect on eye tissue and fluids (**Mosca *et al.*, 2014**). A study on suckling lambs showed that the consumption of verbascoside as a feed supplement has an effect on productive performance, plasma oxidative status and some blood metabolites. A remarkable decrease in reactive oxygen metabolites ($P < 0.01$), as well as a significant increase in serum levels of vitamin A ($p < 0.05$) and vitamin E ($P < 0.01$) was observed in rabbits treated with *Aloysia triphylla* extract (**Casamassima *et al.*, 2013**).

According to **Valentao *et al.* (2002**), the antioxidant effect of *Aloysia triphylla* extract can attenuate or stop the activity of superoxide, hydroxyl and hipochloric radicals. Furthermore, the antioxidant capacity values of this plant are comparable to those of a commercial green tea antioxidant drink (**Abderrahim *et al.*, 2011).**

I.3.2. Antibacterial activity

In recent years, there has been an increasing interest in using herbal products to overcome bacterial resistance **(Bazzaz *et al.*, 2018)**. Indeed, bacterial resistance is one of the greatest challenges of the twenty-first century **(Rasko and Sperandio, 2010)**. Infectious diseases caused by resistant bacterial strains have many negative effects, as their treatment requires higher doses of antibiotics, additional treatments, and prolonged hospitalizations results in higher mortality rates **(Khameneh *et al.*, 2016)**. Studies conducted on the antibacterial activity

of *Aloysia triphylla* had shown satisfactory results, according to **Oukerrou *et al.* (2017)**, the essential oil of this plant had an antibacterial function against *Escherchia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* with the MICs range from 2.84 to 8.37 mg/mL. The ethanolic extract of the aerial parts of *Aloysia triphylla* showed an important inhibitory effect on the growth of gram-negative bacteria *Pseudomonas aeroginosa* and gram-positive bacteria *Bacillus subtilis*, indicating the presence of strong antibacterial compounds in this plant **(Mirzaie *et al.*, 2016)**.

A study conducted by **Bazzaz *et al.* (2018)** on pure verbascoside and on *Aloysia triphylla* extract had shown that these extracts could synergistically potentiate the antibacterial action of Gentamicin against some clinical isolates of *Staphylococcus aureus* and *Escherichia coli.* According to this study, the antibacterial activity is due to the verbascoside, which is one of the most present secondary metabolites in the *Aloysia triphylla* leaves. Verbascoside belongs to the phenylpropanoid glycoside family with demonstrated anti-inflammatory, antioxidant, antitumor and antimicrobial properties (**Pardo *et al.*, 1993; Alipieva *et al.*, 2014**). That said, in comparison with *Aloysia triphylla* extract, the antibacterial activity of verbascoside pur was more pronounced. These results are consistent with previous works that showed that verbascoside has antibacterial activities (**Alipieva *et al.*, 2014)**, which support some ethnopharmacological uses of this plant (**Rocha *et al.*, 2019**).

1.3.3. Antiacetylcholinesterase activity

Aloysia triphylla essential oil has been previously shown to possess a range of useful neuropharmacological properties, including *in vitro* neuroprotective potential. **Abuhamdah *et al.* (2015)** studies showed, for the first time, that *Aloysia triphylla* essential oil provides complete and partial protection vs H_2O_2- and β-amyloid-induced neurotoxicity, respectively, both relevant to oxidative stress-induced damage in major neurodegenerative disease. In addition to the neuroprotective activity, the essential oil of this plant possesses an inhibiting activity of the acetylcholinesterase (AChE). Indeed, *Aloysia triphylla* essential oil has been described as a potential new candidate for treating Alzheimer's disease (AD). According to **Abuhamdah *et al,* (2018)**, three compounds of the essential oil namely, geranyl acetate, caryophyllene oxide, and β-/(Z)-α-bisabolene were found to have AChE inhibitory activity, a dose-response relationship showed an AChE inhibition with an IC_{50} of 5.3 µM for caryophyllene oxide and 244.5 µM for geranyl acetate. He had also shown that the methanolic extract of *Aloysia triphylla* exhibited an AchE activity of 68.03 ± 0.08 % at a concentration of

100 µg/mL. Therefore, this plant is among the plants most likely to reduce the risk of neurodegenerative diseases, including AD and should be considered as a candidate treatment for this disease.

I.4. Ethnobotanical study of *Aloysia triphylla*

I.4.1. General information

The medicinal species *Aloysia triphylla* belongs to the *Verbenaceae* family (**Boulos, 2002**). The genus Lippia has a rich genetic diversity, which allows it to synthesize various essential oil constituents in plants grown in different parts of the world (**Santos-Gomes *et al.*, 2005**). There are more than 200 species, of which *Aloysia triphylla* is the most important species (**Oladzad *et al.*, 2012**). This plant is classified as an astringent and aromatic herb. Its economic interest is mainly related to the commercial extraction of the essential oil from its leaves. This essential oil has a high market value due to its use by various industries, especially the pharmaceutical and perfumery ones (**Duarte *et al.*, 2007**). It is one of the well-known medicinal plants with several therapeutic activities (**Bahramsoltani *et al.*, 2018**).

I.4.2. International vernacular names

Aloysia triphylla has the following names: *Verbena citronella, verbena triphylla,* Arabic tea, Louise herbs, *Lippia citrodora, Lippia triphylla; Aloysia citriodora*. In South America, botanists gave it the name of the genus *Lippi*, in honour of Auguste LIPPI (1678-1704), a seventeenth-century French botanist, who left his name engraved to mark the plant. The term citriodora means "lemon scent" (**Armada and Barra., 1992**). In 1785, Palau a Spanish botanist named it "Aloysia" in honour of Maria Luisa, princess of Parma and wife of Charles IV of Spain, owner of the Real Jardin garden in Madrid where the plant was imported (**Ghédira and Goetz, 2017**). "citrodora" was given for the citron scent the plant gives off, with the Spanish name "Yerba de la Princesa". The common name of the Fragrant Verbena comes from the Latin "verbena", which meant “leafy branch”.

Aloysia triphylla has several names depending on the language of each country **(Jimenez-Ferrer *et al.*, 2017**):

Germany : Citronenkraut, Zitronenkraut, Zitronenverbene.

France : Herbe-Louise, Lemon Verbena, Verveine du Peru.

Great Britain: Lemon Verbena, Herb Louisa, Lemon Beebrush, scented Verbena.

Italy: Cedrina, Cedronella, Erba-Luigia, Verbena odorosa.

Spain: Cedron del peru, Cidren, Hierba Luisa, Hierba cidrera.

Nederland : Citroenkruid, Citroenverbena, Lemonverbena.

Latin-America : "cedrón", "cidron", "citró", "hierba Luisa", "Maria Luisa"

Brazil : "limonete", "erva-luísa" "falsa-erva-cidreira, salva-limão", "Lúcia-lima" or "cidrão"

I.4.3. Botanical description

Aloysia triphylla is a branched shrub with angular, fluted stems bearing rough, rapidly twisted, 3-cut leaves, when crumpled; they give off a characteristic lemon smell. The spiky flowers have 4 petals fused at the base into a tube and spread in 4 bicolored lobes: white on the outer side and purplish blue on the inner side" (**Ghédira and Goetz, 2017**). The shrub as showed in **figure 2**, reaches a height of 1.5-3 meters (**De Figueiredo *et al.*, 2004**). Stems are angular, fluted with straight and ramified branches (**Cheurfa and Allem, 2016**). The leaves are pale green, elongated, 3 to 7 centimeters long and 1 to 2 centimeters wide, whorled in three or four on the stems, with very short petioles, rough to the touch. There are usually 2 cutting periods: (1) May-June (when 50% of the plants have flowered), and (2) Late July-August. A third harvest can take place 1 to 2 months after the second harvest (**Elattir *et al.*, 2003**).

Figure 2: (A) *Aloysia triphylla* fresh leaves, **(B)** *Aloysia triphylla* dried leaves, **(C)** *Aloysia triphylla* flowers

I.4.4. Taxonomies and systematics

The botanical classification of *Aloysia triphylla* is summarized in **Table II** according to **Ghédira and Goetz, 2017**.

Table II: Botanical classification of *Aloysia triphylla*

Règne	*Plantae.*
Super-Division	*Embryophyta*
Division	*Tracheophyta*
Classe	*Magnoliopsida*
Superordre	*Asteranae*
Ordre	*Lamiales*
Famille	*Verbenaceae*
Genre	*Aloysia Juss*
Espèce	*Aloysia triphylla*

I.4.5. Habitat and geographical distribution

Aloysia triphylla is native to Chile and Peru (**Ghédira and Goetz, 2017**), and was introduced to Europe at the end of the 17th century by several botanists. It was then cultivated in temperate climates along the Mediterranean: southern Europe and northern Africa (**Naser Aldeen *et al.*, 2015**). Due to the pleasant lemony fragrance and its application in food industries and cosmetics, as well as its use as a home remedy for several health problems, the plant is currently available in other parts of the world, as well (**Bahramsoltani *et al.*, 2018**).

I.4.6. Traditional use

In South America, this plant is widely used for medicinal and aromatic purposes thanks to the irresistible lemony smell of its leaves. According to the literature, reports on the traditional use of this species date back to the 17th century, proving its ethnopharmacological importance as a

medicinal plant (**Elechosa *et al.,* 2017**). This aromatic plant is most commonly used in the preparation of infusions. *Aloysia triphylla* is considered worldwide as a citral-bearing plant specie. It is cultivated in several countries due to its high economic value (**Parodi *et al.,* 2013**).

In Brazil, *Aloysia triphylla* is cultivated in gardens and vegetable gardens, for medicinal purposes and as a seasoning in cooking and salads (**Rezaeifar *et al.,* 2020)**. The plant is traditionally used as essential oil for its bactericidal effect and to treat skin disorders (**Duarte *et al.,* 2007**). In addition, its infusion is consumed for many purposes such as fever, flu, nervous problems, acne, and as an insecticide, bactericide, tonic, antispasmodic, carminative and stimulant (**Ritter *et al.,* 2002; Santos-Gomes and Fernandes-Ferreira, 2005; Bahramsoltani *et al.,* 2018**).

In Morocco, this species has been cultivated for more than a century and has been used in folk medicine as herbal tea preparations, for its antispasmodic, digestive, stomach, sedative and antipyretic properties (**Jalal *et al.,* 2019**). According to **Miara *et al.* (2019),** *Aloysia triphylla* is one of the most used medicinal plants in traditional phytotherapy by the local population of some Algerian cities, in addition to other plants such as, *Allium cepa* L; , *Anthemis arvensis* L. *Asparagus horridus* L.; *Atriplex hortensis* L.; *Beta vulgaris* L.; *Bunium bulbocastanum* L.; Citrullus colocynthis (L.); and *Genista quadriflora* Munby. These plants are used for the treatment of skin problems; constipation; wounds; digestive problems; obesity; breast cancer, thanks to their biological activities. *Aloysia triphylla* is usually consumed as an infusion for its stimulating, relaxing properties and exhilarating smell, its leaves have a pleasant lemon smell when crushed (**Rocha *et al.,* 2019**).

I.4.7. Other uses of *Aloysia triphylla*

The *Verbenaceae* family is one of the most important families in the plant kingdom, which has several economic values due to its numerous uses in the food industries (**Pérez Zamora *et al.,* 2018; Moshari-Nasirkandi *et al.,* 2020**). In North Africa (Algeria, Tunisia), *Aloysia triphylla* is used as a main ingredient to produce infusions and soft drinks (**Gil *et al.,*2007**) as well as in the preparation of jams and refreshing sorbets. Therefore, *Aloysia triphylla* products can be considered as food products (**Funes *et al.,* 2010**). Indeed, species of *Verbenaceae* family are used as; food supplements (**Mari *et al.,* 2012**), sweetener (**Combrinck *et al.,* 2007**), food flavouring (**de Almeida *et al.,* 2018**) for flavouring fish, poultry, salads, jams, puddings, soft drinks (**Funes *et al.,* 2010**), edible coating for food storage and preservation (**Montanari *et al.,***

2011; Rahmanzadeh Ishkeh *et al.*, 2019), spice **(Sarrazin *et al.*, 2015**) and they are also used in food seasoning (**Marongiu *et al.*, 2010**). Concerning *Aloysia triphylla*, its aromas are used in perfumes and potpourri to scent homes (**Brant *et al.*, 2010**).

I.4.8. Safety and toxicity

Few studies have been carried out on the toxicology of *Aloysia triphylla*. **Etemad *et al.* (2016)**, showed that verbascoside has no negative impact on the embryo development at a dose of 1 g/kg/day in mice, another studies, showed that the median lethal dose (LD_{50}) of the aqueous extract and verbascoside was greater than 5 g/kg in the acute assessments (**Etemad *et al.*, 2015, Etemad *et al.*, 2016**). Clinical trials have concluded that the administration of *Aloysia triphylla* for a period of 21 to 28 days did not lead to any adverse effects (**Barhamsoltani *et al.*, 2018**). In the United States, *Aloysia triphylla* is a plant generally considered safe (**Jimenez-Ferrer *et al.*, 2017**), but the lack of studies regarding the use of this plant makes it difficult to decide on its administration, especially for the elderly, children, pregnant women and nursing mothers. Therefore, the long-term use of the plant, especially in chronic conditions, needs to be scientifically investigated. It is important to mention that this plant possesses phytochemicals that may be substrates for drug metabolizing enzymes. Thus, its concomitant use with conventional drugs by patients with underlying disorders risks plant-drug interactions (**Bahramsoltani *et al.*, 2018**).

According to the same authors, *Aloysia triphylla* is a valuable medicinal plant with several pharmacological activities demonstrated in experimental studies. However, future clinical trials are needed to clinically prove the safety and efficiency of this medicinal plant in humans.

Part II: Preparation of plant samples and extraction method

1. Generalities

Several bioactive compounds of medicinal plant like phenolic compounds are used as nutraceuticals. They are defined as compounds with health benefits for the treatment or prevention of certain diseases **(Bravo and Mateos, 2008)**. The initial stage in studying medicinal plants is the preparation of samples to preserve the biomolecules in the plants priorto extraction. Plants samples such as leaves, barks, roots, fruits and flowers can be extracted from fresh or dried plants material. Other pre-preparations such as grinding and drying also influence the preservation of phytochemicals in the final extracts **(Azwanida, 2015)**.

Due to the structural diversity and complexity of phenolic compound in plants, extraction is a very important step in their separation and characterization **(Bujor, 2018)**. The most common liquid/liquid and solid/liquid extractions are frequently employed to separate phenolic compounds. The phenolic nature of polyphenols makes them relatively hydrophilic. Thus, free phenolic compounds, including aglycones, glycosides, and oligomers, are extracted using water, polar organic solvents such as ethyl acetate, methanol, ethanol, chloroform, dietyl ether, acetonitrile and acetone, or their mixture with water **(Ignat *et al.*, 2011)**.

Several methods are used for the extraction of bioactive compounds. Nowadays, the overall environmental impact of an industrial extraction is taken into consideration. Indeed, the concept of green extraction is introduced to protect both the environment and consumers, while increasing the competition of industries to be more environmentally friendly, economical (less energy and solvent consumption), and innovative (**Chemat *et al.*, 2013; Bujor, 2018**). In line with this green extraction approach, other innovative and unconventional methods for phenolic compounds extraction are used. These methods are microwave extraction (**Mandal *et al.*, 2007**), ultrasound-assisted extraction (**Ghitescu *et al.*, 2015**), techniques based on the use of compressed fluids, such as subcritical water extraction (SWE) (**Dai and Mumper, 2010**), supercritical fluid extraction (SFE) (**Herrero *et al.*, 2010**) and pressurized fluid extraction (PFE) or accelerated solvent extraction (ASE) (**Kaufmann and Christen, 2002**) (**Bujor, 2018**).

II.2. Extraction methods

Extraction is the separation of medicinally active portions of plant using selective solvents through standard procedures **(Handa *et al.*, 2008)**. Its purpose is to separate the soluble plant

metabolites, leaving behind the insoluble cellular marc (residue). The initial crude extracts contain complex mixture of many plant metabolites, such as alkaloids, glycosides, phenolics, terpenoids and flavonoids. Some of the initially obtained extracts may be ready for use as medicinal agents in the form of fluid extracts but others need further processing. The different extraction methods commonly used are:

II.2.1. Maceration, infusion, percolation and decoction

Maceration involved soaking plant materials (coarse or powdered) in a stoppered container with a solvent and allowed to stand at room temperature for a period of minimum 3 days with frequent agitation **(Handa *et al.*, 2008)**. The processed intended to soften and break the plant's cell wall to release the soluble phytochemicals. After 3 days, the mixture is strained by filtration. For this conventional method, the choice of solvents will determine the type of compound extracted from the samples. Infusion and decoction uses the same principle as maceration; both are soaked in boiled or cold water. However, the maceration period for infusion is shorter and the sample is boiled in specified volume of water for a defined time **(Handa *et al.*, 2008)**. Decoction is only suitable for extracting heat-stable compounds, hard plants materials (e.g. roots and barks) and usually resulted in more oil-soluble compounds compared to maceration and infusion. However, organic waste come into an issue as large volume of solvents is used and proper management of the waste is needed **(Azwanida, 2015)**.

II.2.2. Soxhlet extraction or hot continuous extraction

In this method, finely ground sample is placed in a porous bag or "thimble" made from a strong filter paper or cellulose, which is place, is in thimble chamber of the Soxhlet apparatus. Extraction solvents is heated in the bottom flask, vaporizes into the sample thimble, condenses in the condenser and drip back. When the liquid contents reach the siphon arm, the siphon empties back into the lower vial and the process continues **(Azwanida, 2015)**. This method requires a smaller quantity of solvent compared to maceration **(Handa *et al.*, 2008)**. However, the Soxhlet extraction comes with disadvantage such as exposure to hazardous and flammable liquid organic solvents, with potential toxic emissions during extraction. Solvents used in the extraction system need to be of high-purity that might add to cost. This procedure is considered not environmental friendly and may contribute to pollution problem **(Naudé *et al.*, 1998)**.

II.2.3. Ultrasound-assisted extraction (UAE) or sonication extraction

Ultrasound-assisted extraction involves the use of ultrasound ranging from 20 kHz to 2000 kHz **(Handa *et al.*, 2008)**. The mechanic effect of acoustic cavitation from the ultrasound increases the surface contact between solvents and samples and permeability of cell walls. Physical and chemical properties of the materials subjected to ultrasound are altered and disrupt the plant cell wall; facilitating release of compounds and enhancing mass transport of the solvents into the plant cells **(Dhanani *et al.*, 2017)**. The procedure is simple and relatively low cost technology that can be used in both small and large scale of phytochemical extraction **(Azwanida, 2015)**. The benefits of UAE are mainly due to the reduction in extraction time and solvent consumption. However, use of ultrasound energy more than 20 kHz may have an effect on the active phytochemicals through the formation of free radicals **(Kaufmann and Christen, 2002)**.

II.2.4. Accelerated solvent extraction (ASE)

Accelerated solvent extraction is an efficient form of liquid solvent extraction compared to maceration and soxhlet extraction as the method uses minimal amount of solvent. Sample is packed with inert material such as sand in the stainless steel extraction cell. Packed ASE cell includes layers of sand-sample mixture in between cellulose filter paper and sand layers. This automated extraction technology is able to control temperature and pressure for each individual samples and requires less than an hour for extraction **(Azwanida, 2015)**.

II.2.5. Supercritical fluid extraction (SFE)

Supercritical fluid, or also called as dense-gas, is a substance that shares the physical properties of both gas and liquid at its critical point. Factors such as temperature and pressure are the determinants that push a substance into its critical region. Supercritical fluid (SF) behaves more like a gas but have the solvating characteristic of a liquid. An example of SF is CO_2 that become SF at above 31.1°C and 7380 kPa. The interest in supercritical CO2 (SC-CO_2) extraction is due to the fact that CO_2 is an excellent solvent for non-polar analytes, is readily available at low cost, and has low toxicity. Although supercritical CO_2 has low solubility for polar compounds, modifications such as adding small amounts of ethanol and methanol allow it to extract polar compounds. The strength of the supercritical solvents can be easily modified by changing the temperature and pressure, thus reducing the extraction time **(Patil *et al.*, 2013)**.

II.2.6. Microwave assisted extraction (MAE)

Microwave assisted extraction is the simplest and the most economical technique for extraction of organic compounds including polyphenols, from plant materials and foods (**Routray and Orsat, 2012**). This technique has reduced extraction time and solvent volume compared to conventional methods such as maceration and Soxhlet extraction. As an alternative to conventional heating techniques and as an environmentally friendly process, microwave irradiation is a fast, efficient, selective, low solvent consumption and energy efficient volumetric heating. The direct heating of the molecules is achieved by the interaction between the microwave energy and the molecular dipole moments of the starting materials. **(Dahmoune *et al.*, 2015; Nayak *et al.*, 2015)**.

II.3. Microwave assisted extraction

II.3.1. History

Microwave technology was originally developed in the 1930s for military radio applications. During the Second World War, scientists realized that the microwaves used in telecommunication systems could have other uses. From then on, the initial technology developed into a modern and sophisticated household appliance: the microwave oven (**Curet, 2014**) (**Figure 3**). The use of microwave radiation to heat treat products began in the 1950s-1960s with the first technological developments from the work of Percy Spencer and Raytheon (**Edgar & Osepchuk, 2001**). The main applications in the food industry are mainly for drying and tempering processes. There are also more specific applications related to chemical industry and plastics industry (vulcanization) (**Curet, 2014**).

The use of microwave energy as a heating source was analyzed in the laboratory in the early 1970s **(Abu Samra *et al.*, 1975)**, and the first studies on the use of this type of wave in the field of natural product extractions date from the middle of the 1980s **(Ganzler and Salgo, 1987)**. Dielectric microwave heating, as an eco-friendly process, offers an efficient alternative energy source for many chemical processes and reactions **(Mallakpour and Zadehnazari., 2012)**.

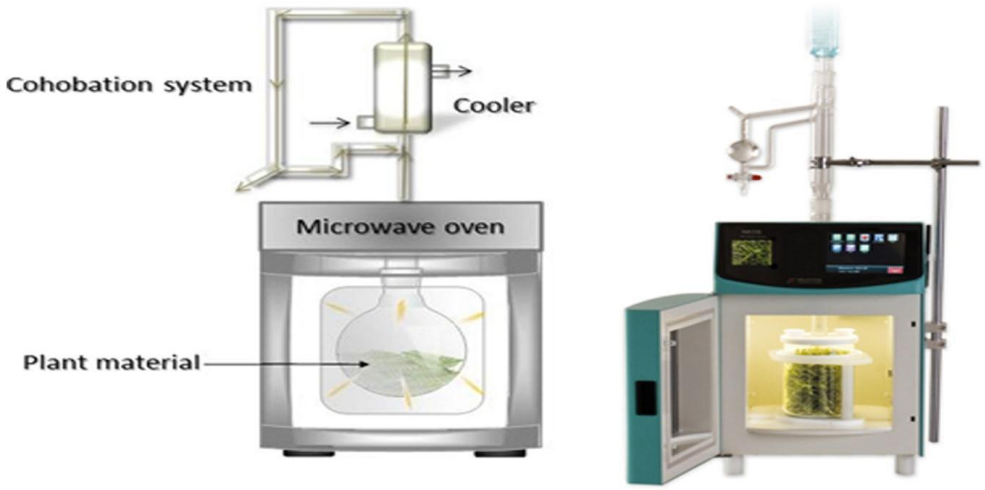

Figure 3: Microwave-assisted extraction system: an open (or focused) system

III.3.2. Principle

As shown in the **figure 4** and according to the official nomenclature, microwave radiation is characterized by frequencies ranging from 300 MHz to 300 GHz. They are therefore designated as decimetric to millimetric waves (wavelengths from 1mm to 1m), and located between infrared and radio frequency radiation (**Camel, 2001**).This range is the most used as a frequency for communications, especially radar, cellular phones, television and satellite applications **(Jang *et al.*, 2009; Menéndez *et al.*, 2010; Zhang *et al.*, 2011)**.

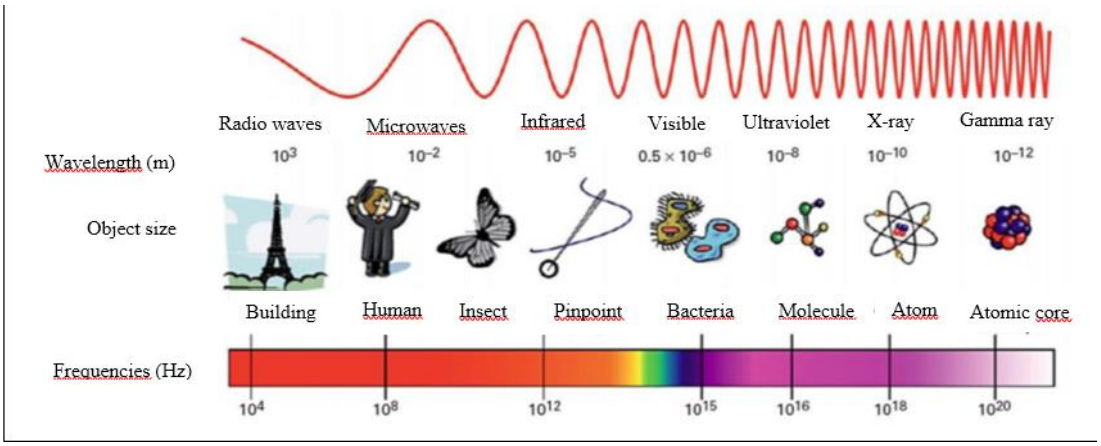

Figure 4: Positioning of microwaves in the frequency spectrum (**Curet., 2019**)

More specifically, the microwave domain can be divided into three sub-bands described by frequency decades:

- From 0.3 to 3 GHz: UHF band (Ultra High Frequency - decimeter waves)

- From 3 to 30 GHz: SHF band (Super High Frequency - centimetric waves)

- From 30 to 300 GHz: EHF band (Extra High Frequency - millimeter waves)

They are also called centimetric waves because the associated wavelengths range from millimeters to meters. On the electromagnetic spectrum, they are located between high frequencies and infrared (**Figure 5**) (**Curet, 2008**).

For applications dedicated to microwave heating, the most commonly used frequencies are 2.45 GHz (domestic microwaves and industrial applications, vacuum wavelength of about 12 cm) and 915 MHz (mainly industrial applications, vacuum wavelengths of about 33 cm) (**Curet, 2019)**.

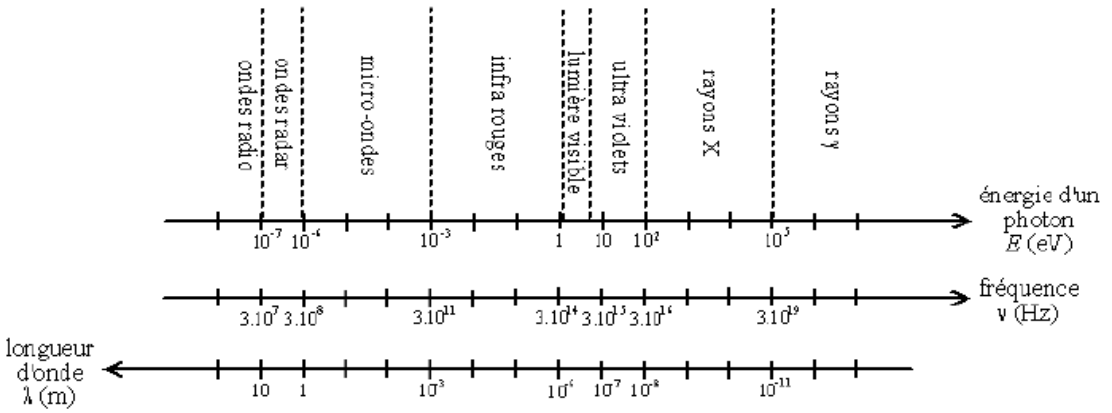

Figure 5: Spectrum of electromagnetic waves (**Curet, 2008**)

II.3.3. Principle of microwave heating

Microwave assisted extraction uses the energy of microwaves to cause molecular movement and rotation of liquids with a permanent dipole, leading to rapid heating of the solvent and the sample and increase the mass transfer rate of solutes from the sample matrix to the solvent (**Feng *et al.*, 2012).** In contrast to conventional heating where the heat penetrates slowly from

the outside to the inside of an object, in MAE the heating appears right in the core of the body that is being heated, and the heat spreads from the inside to the outside of that body (**Nitthiyah *et al.*, 2017**).

Microwaves are electromagnetic waves; they have both an electric field and a magnetic field perpendicular to each other (**Figure 6**). In free space, the maximum speed C_0 at which the wave can propagate corresponds to the speed of light, with 3×10^8 m/s. The electric field acts on the heating via two most important mechanisms, which are dipolar rotation and ionic conduction. Other factors also exist including dielectric properties, dielectric polarization (**Curet, 2008**).

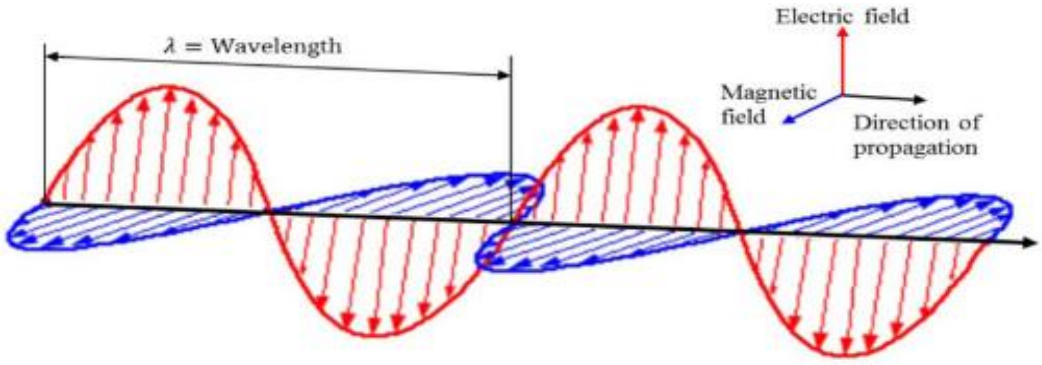

Figure 6: Component of an electromagnetic wave **(Tang *et al.*, 2015)**

II.3.3.1. Dipolar rotation

This mechanism depends on the presence of polar molecules in the material, in the case of food products; water is usually the major polar component. For the water molecule, the differences in electronegativities of the individual atoms lead to the existence of a permanent electric dipole (**Meda *et al.*, 2017**). When an electromagnetic field is applied, the water molecules tend to realign themselves along the direction of the electric field. Due to the high frequency electric field, this realignment occurs a million times per second and causes internal friction of the molecules leading to volume heating of the product **(Chandrasekaran *et al.*, 2013)**.

II.3.3.2. Ionic conduction

The **Figure 7** represents the two main mechanisms related to microwave heating: Dipolar rotation and ionic conduction. Heat is generated by the migration of ions in an oscillatory way under the action of a high frequency electric field (**Datta and Davidson, 2000**). These ionic

movements cause collisions between the ions and other molecules, resulting in the conversion of the kinetic energy of the ions into thermal energy **(Salazar- González *et al.*, 2012)**. According to **Kubo *et al*, (2019)**, the mechanisms responsible for the temperature rise differ depending on the microwave frequency used, thus around 300 MHz, ionic conduction phenomena in food matrices are mostly responsible for the temperature rise compared to dipole rotation. In this case, the degradation of the energy of the electromagnetic wave into heat is due to the electrical conductivity of the ions present in the matrices. On the other hand, at the frequency of 2450 MHz, the mechanisms related to the dipolar rotation of the molecules are in the majority (**Tang *et al.*, 2015**).

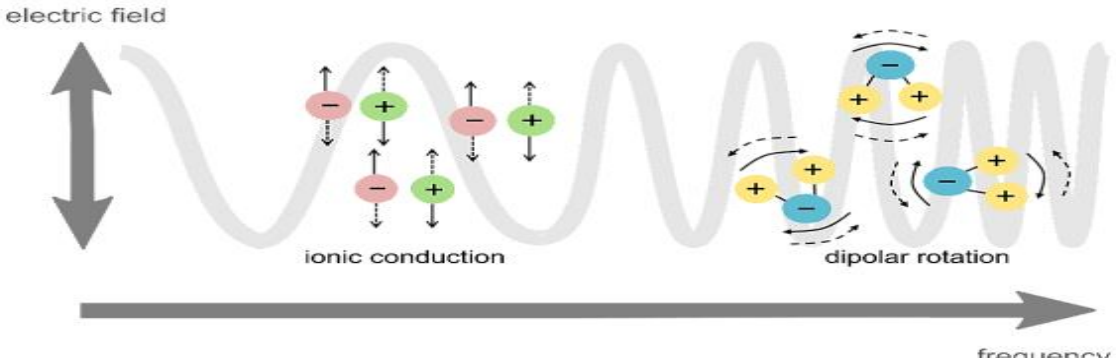

Figure 7: Schematic representation of the two main mechanisms related to microwave heating in the UHF bands: Dipolar rotation and ionic conduction **(Tang *et al.*, 2015)**.

II.3.3.3. Dielectric properties

Under the microwave action, only certain insulating materials or poor conductors of electricity are able to heat up. These materials are called dielectrics (**Figure 8**) as opposed to conductive materials which contain free charges. A perfect dielectric does not contain any free electrical charges and therefore has zero electrical conductivity. Macroscopically, dielectrics appear as entities that are globally neutral from an electrical point of view but with a dissymmetric distribution of their partial ionic charges (**figure 8**). That is, one part of the molecule is positively charged, while the other part is negatively charged. These molecules form electric dipoles (**Curet, 2008**).

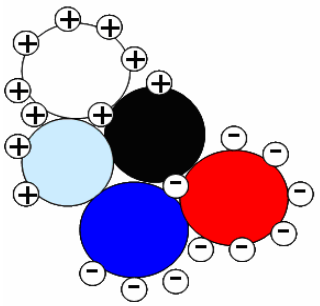

Figure 8: Simplified diagram of a dielectric **(Curet, 2008)**

In the absence of an electric field, the orientation of the dipoles is random, under the application of an electric field, the dipoles orient themselves in the direction of the field. Thus, in an alternating electric field, the orientation of the dipoles changes with each alternation of the electric field direction. For a non-perfect dielectric placed in a high frequency microwave field, the free charges of the molecule will migrate to the surface exposed to the microwave field. Similarly, the displacement of the bound electric charges in the direction of the field gives rise to polarization phenomena of the molecule (**Saltiel and Datta, 1999**). The combination of these two phenomena, conduction and polarization, induces a heating of the material (**Curet, 2008**). The frequency and intensity of the emitted radiation play an important role in the heat generation. The losses by conduction are important at low frequency. As the frequency increases, the time required for the transport of the charges towards the field decreases, and the losses by polarization then become preponderant.

During microwave heating, the polarization phenomena by dipolar alignment are important. In a variable electric field, intermolecular frictions accompany the rotation of the molecules on themselves (due to the successive polarization), which induces the production of heat (**figure 7**). At the frequency of 2.45 GHz, the oscillation of the dipoles in the direction of the electric field occurs at a rate of 4.9×10^9 times per second (**Gunasekaran, 2002**). Thus, the higher the frequency of the electric field, the more intense the friction and the greater the heat release (**Anizon *et al.*, 2003**).

The interactions of electromagnetic waves with a matrix are characterized by the intrinsic dielectric properties of the treated product (**Curet, 2019**). These are defined by a complex permittivity consisting of a real part (Ɛ'), which is the dielectric constant or relative permittivity.

It represents the ability of molecules to be polarized by an electric field and thereby to store electric energy. This part is called the real part of the dielectric properties, and the dielectric loss factor (Ɛ'') which is the amount of energy lost in the material to be dissipated into heat when it contacts the microwaves. Dielectric loss can be determined as the ability to convert electrical energy into heat and it is caused by free electron conductivity. It is considered the imaginary part of the dielectric properties, and as lower as its value, the material has less ability to absorb microwaves (**Zhao *et al.*, 2010; Motasemi *et al.*, 2014**). Dielectric properties are defined according to the following relationship:

$$\text{Ɛ} = \text{Ɛ}' - j\text{Ɛ}'' \quad (1)$$

Where $j = \sqrt{-1}$

The permittivity of vacuum, $Ɛ_0$= 8.85.10-12 F.m-1 allows the normalization of the permittivity of a material or medium by defining a relative permittivity Ɛr ($F.m^{-1}$), according to the following equation:

$$\text{Ɛ}r = \text{Ɛ}_0\,(\text{Ɛ}r' - j\text{Ɛ}r'') \quad (2)$$

This parameter is frequency dependent and reflects the ease with which a molecule can interact with electric waves **(Sahin and Sumnu, 2006)**. The dielectric constant reflects the ability of a molecule to orient itself in the direction of the electric field (polarization phenomenon) (**İçier and Baysal, 2004**), while the dielectric loss factor characterizes the degradation of the energy contained in the electromagnetic wave into heat (**Zhu *et al.*, 2012**). Products with a loss factor greater than 1 heat up easily under the influence of microwave radiation. Among these compounds with high dielectric losses, we find water in its liquid state, products naturally rich in water and polar solvents such as alcohols (**Curet, 2008**).

The **Table III** represents solvents with their corresponding dielectric losses, dielectric constants and loss tangents.

Table III: Solvents with their corresponding dielectric losses, dielectric constants, and loss tangents **(Nour *et al.*, 2021)**

Solvent	Dielectric loss	Dielectric constant	Loss tangent
Chloroform	0.437	4.8	0.091
Dimethyl sulfoxide	37.125	45.0	0.825
Dimethylformamide	6.079	37.7	0.161
Ethanol	22.866	24.3	0.941
Ethylene glycol	49.950	37.0	1.350
Hexane	0.038	1.9	0.020
Toluene	0.096	2.4	0.040
Water	12.3	80.4	9.889

The intermolecular friction phenomena due to the rotation of the electric dipoles are manifested by a slight delay in the orientation of the polarized materials after the application of the electric field. This phase shift is represented by the loss angle δ, which relates the loss factor to the real permittivity. The ratio of the relative dielectric loss factor to the relative dielectric constant relative defines the tangent to the loss angle or the dissipation factor (**Curet, 2008; Chandrasekaran *et al.*, 2013**). It is expressed as:

$$\tan \delta = \frac{\varepsilon''r}{\varepsilon'r} \qquad (3)$$

tan δ measures the ability of the matrix to absorb microwave energy and convert it into heat (**Kostas *et al.*, 2017**).

The materials can be classified according to the dielectric loss tangent present as conductive materials, non-conductors and dielectric materials. (i) Dielectric materials cannot penetrate by microwaves. They have tan δ < 0, for example the metal; (ii) Non-conductor materials are transparent to microwaves and have low or null dielectric loss, they have a level of tan δ from 0.1 to 0.5, some examples are teflon, glass, quartz and air. Non-conductors materials are the main materials used to manufacture vessels or reactors to heat with microwave; (iii) Dielectric materials: these absorb microwaves, having high levels of tan δ > 0.5, they are ideal materials

to heat by microwave, for example water (**Chandrasekaran *et al.*, 2013; Motasemi *et al.*, 2014**).

The power penetration depth (Dp) is the distance at which the power density drops to 1/e of its value at the surface and is expressed as follows (**Metaxas and Meredith, 1983**):

$$Dp = \frac{C}{\sqrt{2\pi f}\left[k\prime\left\{\sqrt{1+(\frac{K''}{K'})^2-1}\right\}\right]^{1/2}}$$

Where C is the velocity of light given as $C = (\mu_0 \varepsilon_0)^{-\frac{1}{2}}$

μ_0 is the permeability of free space ($\mu_0 = 4\pi$ x 10-7 H/m), Ɛ_0 = 8.85.10-12 F.m^{-1}

k ' and k " are relative dielectric constant and relative dielectric loss respectively, which are given as k ' =Ɛ '/Ɛ_0 and k " =Ɛ "/Ɛ_0.

II.4. Principle of microwave assisted extraction of plant matrix

Heating of the plant matrix in a microwave is due to the presence of moisture in a plant cell. In MAE, the objective of heating in the case of dried plant material is to heat this amount of moisture present in the cells. Heating this moisture causes evaporation and creates enormous pressure on the cell wall. The cell wall weakens from the inside due to this pressure and breaks down. In this way, exudation of potential constituents from the ruptured cell occurs, which improves the extraction yield of phytoconstituents (**Mandal *et al.*, 2007**). The extraction yield of phytoconstituents from plant matrices can also, be improved by impregnating the plant matrix in a solvent, or by increasing the extraction temperature, which promotes faster penetration of the solvent into the cell wall of the plant matrix (**Azmir *et al.*, 2013; Veggi *et al.*, 2013)**.

Three types of samples were studied using scanning electron micrographs to track the impact of different extraction methods on a plant matrix: an untreated sample, a sample from a reflux extraction, and a sample extracted by microwave. Complete cell wall disruption is only observed in the microwave extracted test sample. In the conventional reflux heat extraction method, extraction occurs through the action of penetration and solubilization to move plant components out of the plant cell (**Liu, *et al.*, 2018**). In MAE, the *in situ* water in the plant cells is heated under the microwave flow, which causes the expansion of the plant cells and the rupture of the cell wall, inducing the release of essential components (metabolites/secondary

constituents) from the plant matrix (**Bagade and Patil, 2021**). The **figure 9** represents the schematic of cell rupture in the case of MAE.

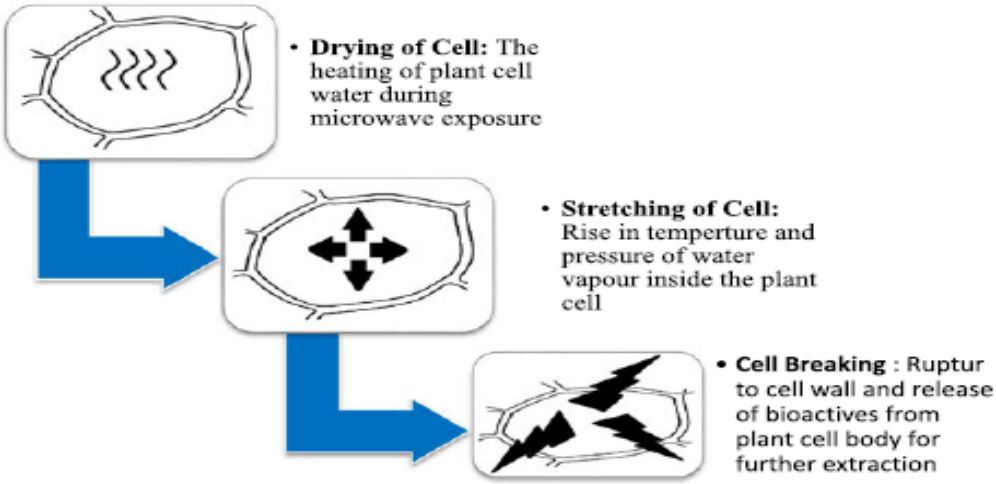

Figure 9: The effect of microwave flux on the plant cell during MAE (**Bagade and Patil, 2019)**.

Figure 10 shows that microwave-assisted extraction can damage and destroyed tissues and/or cells (even organelles) after microwave assisted extraction (**Zhang *et al.*, 2011**).

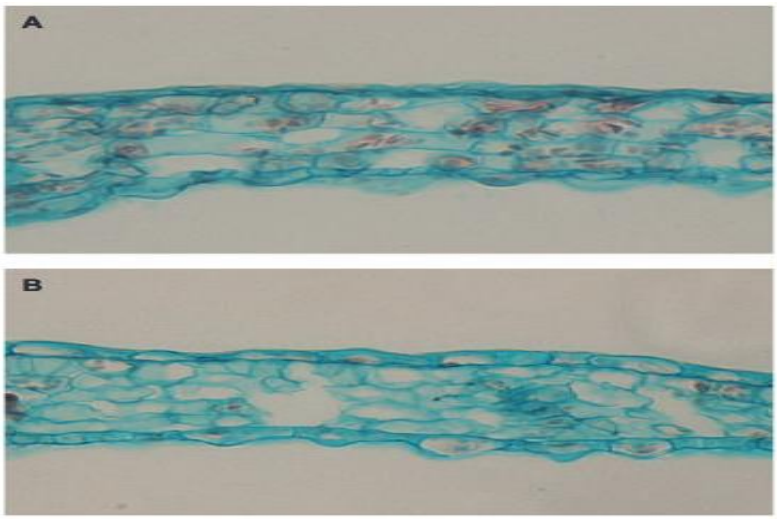

Figure 10: Light micrographs of Epimedium leaf samples: (A) untreated leaf sample, (B) leaf sample after microwave irradiation

In microwave assisted extraction, the process acceleration and high extraction yield may be the result of a synergistic combination of two transport phenomena: heat and mass gradients working in the same direction **(Bousbia *et al.*, 2009)**. On the other hand, in conventional extractions the mass transfer occurs from inside to the outside, although the heat transfer occurs from the outside to the inside of the substrate (**Figure 11**). In addition, although in conventional extraction the heat is transferred from the heating medium to the interior of the sample, in MAE the heat is dissipated volumetrically inside the irradiated medium **(Veggie *et al.*, 2013.**

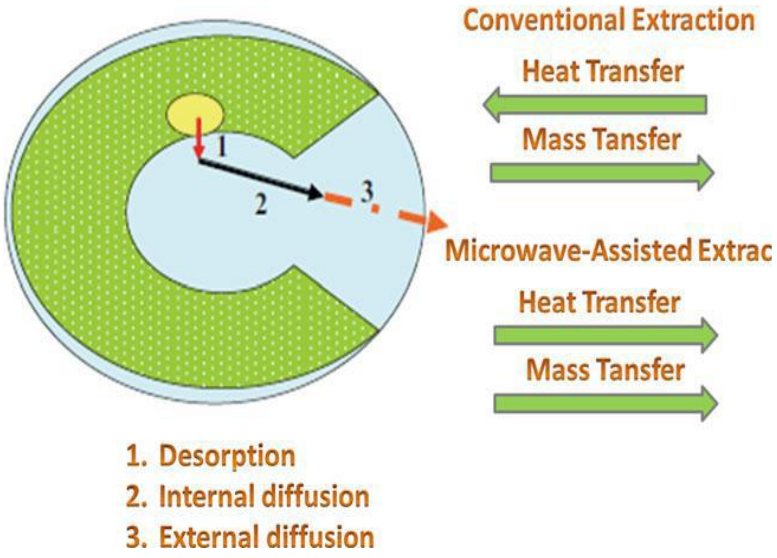

Figure 11: Basic heat and mass transfer mechanisms in microwave and conventional extraction of natural products (**Périno-Issartier *et al.*, 2011**).

II.5. Factors affecting MAE of plant matrix

The parameters that influence the extraction technique are represented in the **figure 12**. Namely, choice of solvent composition, solvent to feed ratio, power applied and temperature, extraction time, size and moisture of the plant material. The selection of parameters and their values depends on solubility, volatility, and stability of target compounds. It will also depend on the interaction of other compounds present in the plant material (**Desai *et al.*, 2010**).

II.5.1. Solvent properties

In microwave-assisted extraction, the choice of solvent is the key to successful extraction. A proper solvent choice will provide a more efficient extraction process. In the case of plant tissue

extraction, the compounds of interest are present in the cells and in different parts of the raw material. To extract these compounds, the solvent must reach them and dissolve them. The solvent usually attacks the cell wall of the raw materials and penetrates it to reach the compounds, but it can also dissolve various other impurities **(Rostagno and Prado, 2013)**. Solvent selection depends on the solubility of the compounds of interest, solvent penetration and its interaction with the sample matrix, its dielectric constant **(Chen, *et al.*, 2008**) and the mass transfer kinetics of the process **(Spigno and Faveri., 2009**). In addition, the larger the dipole moment of the solvent, the faster the solvent heats up under microwave irradiation. The solvent should preferably have a high selectivity toward the solutes of interest excluding undesired matrix components. Another important aspect is that the optimal extraction solvents cannot be selected directly from those used in conventional extractions. It depends on the capacity of the solvent to absorb the microwave energy and consequently heat up (**Chan *et al.*, 2011; Routray and Orsat., 2012)**. The capacity of the solvent to absorb microwave energy is high when the solvent presents high dielectric constant and dielectric loss (**Spigno and Faveri., 2009; Rostagno and Prado, 2013**). Solvents that are transparent to microwaves do not heat when submitted to them. Hexane is an example of microwave-transparent solvent whereas ethanol is an excellent microwave- absorbing solvent (**Mandal *et al.*, 2007**). Both polar and non-polar solvents can be used in MAE, and solvents such as ethanol, methanol, and water are sufficiently polar to be heated by microwave energy (**Brachet *et al.*, 2002**). In this context, the properties of the solvent can be modified when combining different solvents, which allow varying the its selectivity for different compounds (**Brachet *et al.*, 2002**). Studies have reported that ethanol or water can be added into poor microwave absorbers, such as hexane, to improve the extraction efficiency. One of the most used solvent mixtures is hexane-acetone, and only a small amount of water (about 10%) must be added in non-polar solvents such as hexane, xylene, or toluene to improve the heating rate (**Eskilsson, 2000**).

II.5.2. Extraction time

Irradiation time is another important factor affecting MAE. One of the advantages of this method over conventional ones is that the extraction time is very short (**Mandal *et al.*, 2007**). Increasing the extraction time increases the extraction yield, as it affects the dielectric property of the solvent used. However, increasing the extraction time also increases the risk of degradation of thermolabile components. Therefore, a balance between extraction yield and component stability must be achieved to ensure better extraction (**Delazar *et al.*, 2012; Alara**

***et al.,* 2019**). Indeed, solvents such as ethanol, water, and methanol can heat up rapidly upon prolonged exposure, which can lead to degradation of thermolabile compounds in extracts (**Chan *et al.,* 2011; Shams *et al.,* 2015**).

II.5.3. Microwave power

Microwave power and temperature are interrelated because high microwave power can bring up the temperature of the system and result in the increase of the extraction yield until it becomes insignificant or declines (**Hu *et al.,* 2008; Xiao *et al.,* 2008**). It is known that the temperature is controlled by incident microwave power that controls the amount of energy provided to the matrix, which is converted to heat energy in the dielectric material. It affects interactions and equilibrium rate and controls partition of analytes between sample and solvent (**Ma *et al.,* 2009a**). At high temperatures the solvent power increases because of a drop in viscosity and surface tension, facilitating the solvent to solubilize solutes, and improving matrix wetting and penetration (**Mandal *et al.,* 2007; Li *et al.,* 2010**). Generally, it has been observed that there is an increase in the yield of the extracted compound when there is an increase in the microwave power (**Hu *et al.,* 2008**). However, according to **Routray and Orsat, (2012)**, the efficiency increases with the increase in temperature until an optimum temperature is reached and then starts decreasing with the further increase in temperature. This happens because the selection of ideal extraction temperature is directly linked with the stability and, therefore, with the yield of the target compound (**Ma *et al.,* 2009a**).

II.5.4. Solvent-to-solid ratio

The solvent-to-solid ratio is an important parameter to be optimized. The solvent volume must be sufficient to guarantee that the entire sample is immersed in the solvent throughout the entire irradiation process, especially when using a matrix that will swell during the extraction (**Mandal *et al.,* 2007; Dahmoune *et al.,* 2013**). In many applications, a ratio 10:1 (mL/mg) to 20:1 (mL/mg) was found to be optimal **(Talebi *et al.,* 2004**). In addition, the solvent volume is an important factor to be considered because too much of the extracting solvent means more energy and time is required to condense the extraction solution in the later step and purification process. On the other hand, MAE may give lower recoveries because of no uniform distribution and exposure to microwaves (**Ibrahim *et al.,* 2018; Nour *et al.,* 2021**).

II.5.5. Particle size

The particle size of the matrix subjected to microwave extraction generally has an effect on the extraction yield. Like any other extraction technique, the smaller the particle size (the larger the surface area), the better the extraction by MAE due to better penetration of the microwave energy (**Delazar *et al.*, 2012; Routray and Orsat, 2012**). In the preparation step, the sample is ground and homogenized to increase the contact surface between the matrix and the solvent **(Eskilsson and Björklund, 2000)**. On the other hand, very fine particles can pose some technical problems. Therefore, centrifugation or filtration is applied to overcome this discomfort **(Mandal *et al.*, 2007; Chupin *et al.*, 2015)**. In many cases, extraction recovery is enhanced by the moisture in the matrix, which acts as a solvent. The matrix moisture is heated, evaporated, and generates internal pressure in the cell, which ruptures the cell to release solutes, thus improving extraction yield **(Wang *et al.*, 2006**).

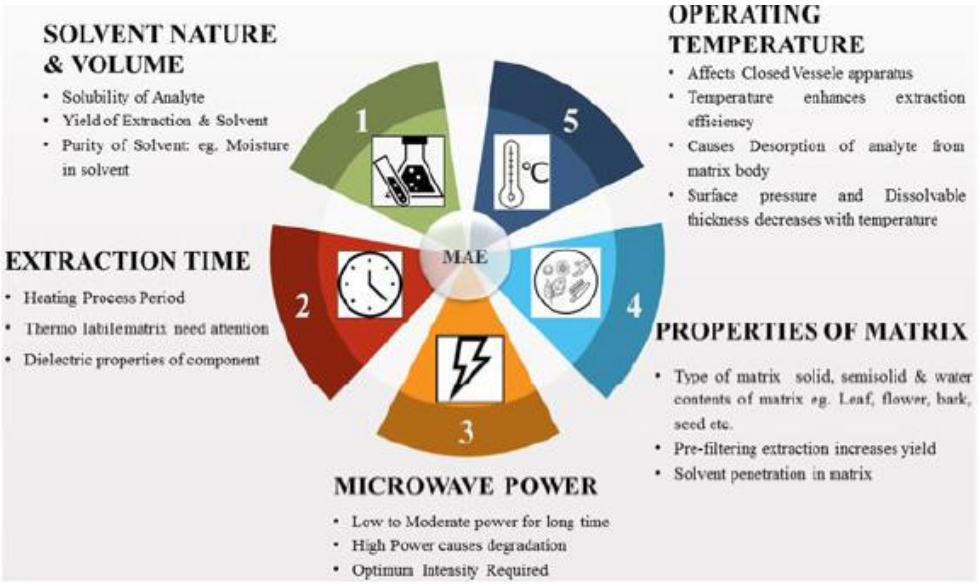

Figure 12: Factors affecting microwave assisted extraction of herbal matrices **(Bagade and Patil, 2019)**

II.6. Optimization of microwave assisted extraction by design of experiments

Optimizing refers to improving the performance of a system, a process, or a product in order to obtain the maximum benefit from it. The term optimization has been commonly used in analytical chemistry as a means of discovering conditions at which to apply a procedure that produces the best possible response **(Arau´ jo and Brereton, 1996)**.

Conventional methods in the optimization process require the determination of a dependent variable for each combination of independent variables, varying only one at a time while holding all others constant (**Singh *et al.*, 2013)**. This optimization technique is called "one-variable-at-a-time". Its main drawback is that it does not take into account the influence of interactions between independent variables (**Sahu *et al.*, 2009**). Therefore, this technique does not depict the full effects of the parameter on the response (**Lundstedt *et al.*, 1998**). Another disadvantage of one-factor optimization is the increase in the number of experiments needed to conduct the research, which leads to increased time and expense as well as increased consumption of reagents and materials **(Bezerraa *et al.*, 2008; Tak *et al.*, 2015)**. To address this problem, optimization of analytical procedures was performed using multivariate statistical techniques. The response surface methodology (RSM) is among the most relevant techniques used in analytical optimization (**Bezerraa *et al.*, 2008; Delazar *et al.*, 2012)**.

II.6.1. Response surface methodology (RSM)

Several studies have performed the RSM method to optimize microwave assisted extraction **(Dahmoune *et al.*, 2015; Radojković *et al.*, 2018, Djaoud *et al.*, 2020; Guemghar *et al.*, 2020; Wani and Uppaluri, 2022)**. Box and Wilson introduced this method in 1951. It is a combination of statistical and mathematical techniques. The RSM is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, which must describe the behavior of a data set with the objective of making statistical previsions. It presents the method that could be applied to determine the optimal extraction conditions using the effects of some process variables and their interactions to identify the relationship between the response function and these variables (**Bezerraa *et al.*, 2008)**. In addition, this method is easy to estimate and apply, even when the process is not well known (**Delazar *et al.*, 2012)**. By establishing a model equation, RSM allows the interaction between multiple parameters to be evaluated using quantitative data. Before applying the RSM methodology, it is first necessary to choose an experimental design that will define which experiments should be carried out in the experimental region being studied. There are some experimental matrices for this purpose. To approximate a response function to experimental data that cannot be described by linear functions, experimental designs for quadratic response surfaces should be used (**Bezerraa *et al.*, 2008)**.

II.6.2. Theory and steps for RSM application

The steps for applying RSM as an optimization technique are; (1) the selection of independent variables of major effects on the system through screening studies and the delimitation of the experimental region, according to the objective of the study and the experience of the researcher; (2) the choice of the experimental design and carrying out the experiments according to the selected experimental matrix; (3) the mathematic–statistical treatment of the obtained experimental data through the fit of a polynomial function; (4) the evaluation of the model's fitness and (5) obtaining the optimum values for each studied variable **(Bezerra *et al.*, 2008; Wang *et al.*, 2012)**.

II.6.2.1. Screening of variables

Many variables can affect the response of the system under study, and it is impossible to control the small contributions of each of them. Therefore, it is necessary to select the variables with major effects. Screening designs must be performed to determine which of the different experimental variables and their interactions have the most significant effects **(Lundstedt *et al.*, 1998)**.

II.6.2.2. Choice of the experimental design

The simplest model that can be used in the RSM is based on a linear function. It is applied in the case where the responses do not present curvatures. For its application, the equation that represents this model is:

$$Y = \beta_{\circ} \sum_{i=1}^{k} \beta_i x_i + \varepsilon \qquad (1)$$

Where k is the number of variables, $\beta_{\circ}$ is the constant term, βi represents the coefficients of the linear parameters, *xi* represents the variables, and ε is the residual associated to the experiments.

Two-level factorial designs are used in the estimation of first-order effects, but they fail when additional effects, such as second-order effects, are significant. So, a central point in two-level factorial designs can be used for evaluating curvature. The next level of the polynomial model should contain additional terms, which describe the interaction between the different experimental variables. This way, a model for a second-order interaction is presented as follow:

$$Y = \beta_{\circ} + \sum_{=1}^{k} \beta_i x_i + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

βij is the coefficients of the interaction parameters.

The polynomial function must contain quadratic terms in order to determine a critical point (maximum, minimum or saddle), according to the equation presented below:

$$Y = \beta_{\circ} + \sum_{=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j \quad (3)$$

Where Y represents the independent responses; β_0, β_i, β_{ii}, and β_{ij} represent the regression coefficient of the process variables for the intercept, linear, quadratic, and cross product terms, respectively (**Prakash Maran *et al.*, 2017**). In **Figure 13**, the condition at which the optimization can occur was explained, optimization can be confirmed when second order model can be optioned from statistical outcomes and which coincide with the optimum value **(Bhattacharya, 2021)**.

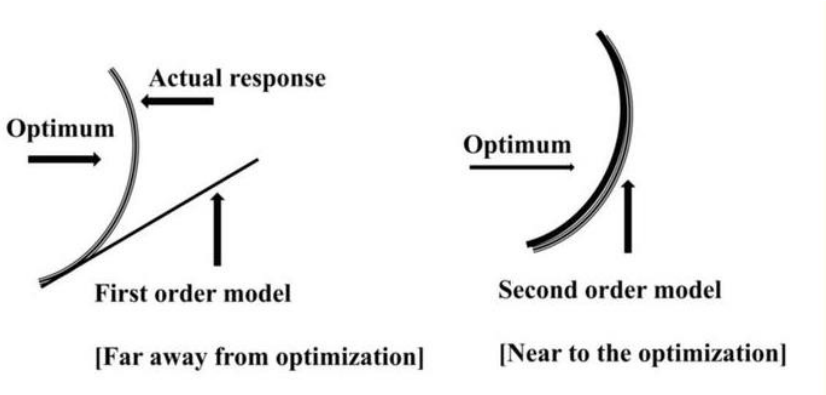

Figure 13 : Optimization conditions **(Bhattacharya, 2021)**

For the reliability test, analysis of variance (ANOVA) should be performed to assess the fit of the model through the R-squared response and the F-test for lack of fit (**Said and Amin, 2015**).

To study the parameters of equation (3), all the variables studied must be carried out at least at three factor levels. Thus, there are symmetric response surface designs. The best-known second-order symmetric designs are the three-level factorial design, the Box-Behnken design, the central composite design and the Doehlert design. These symmetric designs differ from each other in the selection of experimental points, the number of levels for the variables, and the number of series and blocks **(Bezerraa *et al.*, 2008)**.

II.6.2.2.1. Full three-level factorial designs

The three-level full factorial design is an experimental matrix that has limited application in RSM when the number of factors is greater than 2. This is because the number of experiments required for this design (calculated by the expression N=3k, where N is the number of experiments and k is the number of factors) is very large, making it inefficient in modeling quadratic functions. Since a full three-level factorial design for more than two variables requires more sets of experiments than are practically possible, designs with a smaller number of experimental points, such as the Box-Behnken, central composite, and Doehlert designs, are more often used, and for two variables, its efficiency is comparable to that of the central composite design (**Morris, 2000**).

II.6.2.2.2. Box–Behnken designs

This design suggests how to select the points of the three-level factorial design that allows the efficient estimation of the first and second order coefficients of the mathematical model. These designs are, in this way, more efficient and economical than their corresponding 3k designs, mainly for a large number of variables (**Bezerraa *et al.*, 2008**).

In this designs, the experimental points are located on a hypersphere equidistant from the central point, it requires an experiment number according to $N= 2k (k-1) + Cp$, where k is the number of factors and *Cp* is the number of the central points. All factor levels have to be adjusted only at three levels (−1, 0, +1) with equally spaced intervals between these levels (**Ferreira *et al.*, 2006; Bezerraa *et al.*, 2008**).

II.6.2.2.3. Doehlert design

It is developed by Doehlert, the design is a practical and economical method. It describes a circular domain for two variables, spherical for three variables, and hyperspherical for more than three variables. This design accents the uniformity of the studied variables in the experimental domain. It presents some advantages, such as requiring few experimental points for its application and high efficiency. This design requires an experiment number according to $N= k2 + k + Cp$, where k is the factor number and (*Cp*) is the replicate number of the central point. In this plan, each variable is studied at a different number of levels, a particularly important characteristic when some variables are subject to restrictions such as cost or when it is interesting to study a variable at a major or minor number of levels (**Bezerraa *et al.*, 2008**).

II.6.2.2.4. Central Composite Design (CCD)

Many applications of the central composite design in the optimization of analytical procedures can be found in literature (**Shahid *et al.*, 2017; Barbosa-Pereira *et al.*, 2018; Guemghar *et al.*, 2020; Hayder *et al.*, 2021; Unlu, 2021**). Based on the outcomes and empirical models from various experimental design, the CCD gives us a direction to logically think and exercised multivariable analysis **(Esbensen *et al.*, 2002)**.The optimization performed using CCD can screen a wide range of parameters as well as the role of each factor **(Şahin, *et al.*, 2011)**. CCD is also capable of evaluating a single variable or the cumulative effect of variables on the response with a considerable reduction in experimental runs **(Sun *et al.*, 2010)**.

Three design points are prerequisite to establish second-order polynomial equation in CCD model (**Chu and Fan., 1998**). The variables are coded, and the codes are represented by -1, 0, and +1 **(Said and Amin, 2015)**. When two levels of fractional factorial design need to be established, 2^k should have possible +1 and -1 levels of factors. In similar patterns, 2 k needs to be calculated, which can be otherwise called star points, and α forms the center to generate quadratic terms **(Bhattacharya, 2021)**.

$$\boldsymbol{N = K^2 + 2K + n}$$

Where N is the actual number of experiments, n is a number of repetition and k is the number of different factors which were incorporated within the study.

To determine the local axial point, it is necessary to identify the alpha value in the CCD model. This alpha value can be calculated using the following equation:

$\alpha = (2^K)^{\mathbf{0.25}}$

If α value comes equals 1, the position of axial points stands within the factorial region. This is otherwise called a face-centered design, with three levels of factors that need to be kept in the design matrix. To calculate and analyze experimental results from response surface methodology, a polynomial equation needs to be implemented to study the correlation between dependent and independent variables **(Bhattacharya, 2021)**.

$$Y = \beta_\circ + \sum_{=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 \sum_{1 \le i \le j}^{k} \beta_{ij}\, x_i x_j \qquad (4)$$

Figure 14 shows representations of central composite designs for two and three variable optimizations, respectively (**Bezerra *et al.*, 2008**).

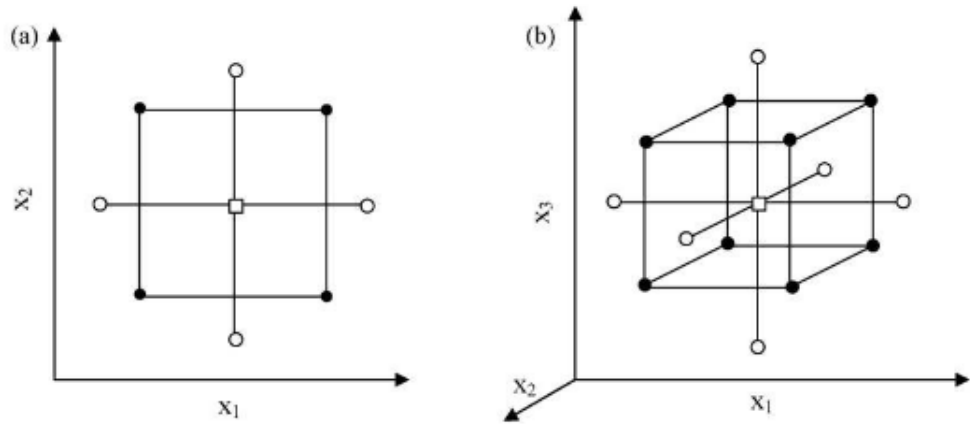

Figure 14: Central Composit Design for the optimization of: a (two variables), and b (three variables).

II.6.3. Statistical treatment of data

After acquiring data related to each experimental point of a chosen design, it is necessary to fit a mathematical equation to describe the behavior of the response according to the levels of values studied, therefore, in matrix notation, equations (1) and (3) can be represented as:

$$y_m X_i = X_m X_n b_n X_1 + e_m X_1 \qquad (5)$$

Where y is the response vector, X is the matrix of the chosen experimental design, b is the vector constituted by the parameters of the model, e is the residual, m and n represent the numbers of lines and columns from the matrices, respectively. Equation (5) is solved by using a statistical approach called the method of least square (MLS) (**Bas and Boyaci, 2007**). MLS is a multiple regression technique used to fit a mathematical model to a set of experimental data generating the lowest residual possible. After mathematical transformations of equation (5), a vector b containing the parameters can be obtained by the following equation:

$$b_{n\cdot 1} = (X^T_{n\cdot m} X_{m\cdot n})^{-1} (X^T_{n\cdot m} y_{m\cdot i}) \qquad (6)$$

Equation (6) is used in the construction of the response surface that describes the behavior of the response in the experimental domain (**Bezerra *et al.*, 2008**).

II.6.3.1. Evaluation of the fitted model

The mathematical model constructed after fitting the function to the data may in some cases not satisfactorily describe the experimental domain studied. The most reliable way to assess the

quality of the fitted model is to apply analysis of variance (ANOVA). The main purpose of ANOVA is to compare the variation due to the treatment (change in the combination of variable levels) with the variation due to the random errors inherent in the generated response measures. From this comparison, it is possible to assess the significance of the regression used to predict the responses by considering the sources of experimental variance. In ANOVA, the evaluation of data set variation is made by studying its dispersion. The evaluation of the deviation (di) that each observation (yi) or its replicates (yij) present in relation to the media ($\bar{y}$), or, more precisely, the square of this deviation, is

$$d_i^2 = (y_{ij} - \bar{y})$$

The sum of the square of all observational deviations from the mean is called the total sum of the square (SS_{tot}). It is composed of the sum of the square due to the fitted mathematical model (due to the regression, SSreg), and the sum of the square due to the model-generated residuals (SSres), according to this equation:

$SS_{tot} = SS_{reg} + SS_{res}$

It is possible to estimate the pure error associated with the repetitions of the central point, so the sum of the squares of the residuals can be divided into two parts: the sum of the square due to the pure error (SS_{pe}) and the sum of the square due to the absence of adjustment (SS_{lof}):

$SS_{res} = SS_{pe} + SS_{lof}$

The sum of the square for each source of variation (total, regression, residual, lack of fit and pure error) is represented by the "media of the squares" (MS). The significance of regression can be evaluated by the ratio between the media of the square of regression (MS_{reg}) and the media of the square of residuals (MS_{res}), by comparing these variation sources using the Fisher distribution (F test), taking into account its respective degrees of freedom associated to regression (v_{reg}) and to residual (v_{res}) variances:

$MS_{reg}/MS_{res} \approx F_{vreg,\ vres}$

A statistically significant value for this ratio must be higher than the F value. This condition is an indication that the mathematical model is well fitted to the experimental data.

Lack of fit test is another way to evaluate the model. It expresses the variation of the data around the fitted model. A model will be well fitted to the experimental data if it presents a significant regression and a non-significant lack of fit. In other words, the major part of variation

observation must be described by the equation of regression, and the remainder of the variation will certainly be due to the residuals. Most variation related to residuals is due to pure error and not to the lack of fit, which is directly related to the model quality (**Bezerra *et al.*, 2008**).

II.6.3.2. Determination of the optimal conditions

The surfaces generated by linear models can be used to indicate the direction in which the original design should be moved to achieve the optimal conditions. if the experimental region cannot be displaced due to physical or instrumental reasons, the search must find the best operating condition within the experimental condition being studied by visual inspection **(Bezerra *et al.*, 2008)**.

II.6.3.3. Application to the microwave assisted extraction

A Central Composite Design (CCD) was used throughout this work, to analyze total phenolic compounds (TPC), in order to study the influence of the operating parameters required during extractions. Four independent factors were evaluated: ethanol concentration (%), microwave power (second), extraction time and liquid-to-solid ratio, the linear effect, mutual interaction and quadratic effect are also evaluated.

II.7. Comparison between microwave extraction and conventional methods

Conventional heating relies on the process of conduction and convection to transfer heat from an external source (steam, hot water). This process requires relatively long processing times (**Vadivambal and Jayas, 2010**). As shown in the **figure 15**, during conventional heating, the heat is diffused from the external surface to the interior of the matrix. Conversely, microwave heating has the potential to generate heat throughout the product due to volume heat generation, resulting in a significant reduction in heating time. The heat is generated within the volume and the temperature gradient can be reversed compared to the conventional heating process. The problem of product surface damage due to overheating by the conventional process is thus avoided (**Curet, 2019**).

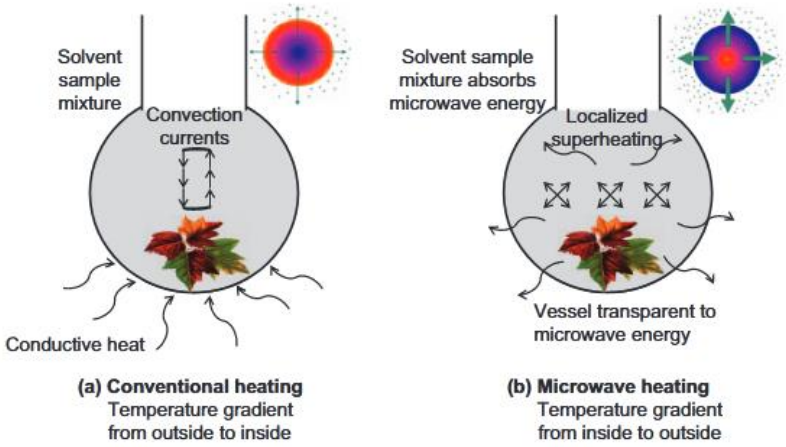

Figure 15: Difference between conventional and microwave heating **(Rostagno and Prado, 2013)**

Microwave heating is a promising technology, because it offers several advantages over conventional methods. It is known for its low energy and water consumption, higher yield, better accuracy, adaptation to thermolabile chemical components and a cleaner working environment (**Curet, 2019**). The MAE method is used in different types of samples, including geological, environmental and biological matrices. In recent times, the MAE method is generally used to obtain bioactive compounds from plant samples, which has significantly improved the total interest in the development and research fields. Microwave heating better preserves thermolabile compounds and maintains the antioxidant activity of the plant matrix **(Delazar *et al.*, 2012; Guo *et al.*, 2017; Pérez-Grijalva *et al.*, 2018**). This method allows to faster the recovery of solutes from plant samples with more efficient extraction (appreciable extraction efficiency) compared to traditional techniques (**Routray and Orsat, 2012; Flórez *et al.*, 2015)**. It employs reduced extraction time and requires minimal solvent consumption. It is considered as an effective green technology for the extraction of bioactive compounds from plant samples (**Auta *et al.*, 2018**).In the many published papers comparing MAE with other advanced and conventional extraction methods, MAE has been accepted as a potential and powerful alternative for the extraction of organic compounds from plant materials (**Li *et al.*, 2012a**).

There have been numerous reviews and research on the advances of different extraction techniques by comparing their results. In the extraction of bioactive compounds from plants,

MAE was reported to be more efficient compared to conventional techniques such as Soxhlet and advanced methods of extraction including ultrasound-assisted extraction (UAE), pressurized liquid extractions (PLE), and supercritical fluid extraction (SFE), which have emerged as energy-saving technologies, and have been adopted over decades in laboratories and industry (**Chemat and Cravotto, 2013)**.

Practical part

Plant material and phytochemical analysis

I. Plant material

Aloysia triphylla leaves were collected from June to September, in the region of Bejaia (Algeria), precisely in the region of Toudja (36° 45 '31 "North and 4° 53' 36" East). As shown in the figure **figure 16**, fresh *Aloysia triphylla* are leaves oven-dried at 40 °C until the stabilization of the weight and then ground into powder with an electrical grinder (IKA model A11Basic, Germany). The powder was passed through 125, 250 and 500 µm sieves, then stored in the dark at 4 °C in airtight jars until use.

Figure 16: Preparation of *Aloysia triphylla* leaf powders for experimental work

II. Chemical reagents

The chemicals used in this work, are mentioned in the **table I** (Annex).

III. Phytochemical screening

III.1. Moisture content determination:

The moisture content of the sample was determined by the evaporative drying method (**Boulekbache *et al.*, 2013**) with some modifications. 5 g *Aloysia triphylla* samples were dried at 105 ± 2 °C for 3 hours, the drying operation is repeated until the weight is stabilized. The experiments were performed in triplicate.

The calculation of the moisture content is as follows:

M.C (%) = (W0/ Wi) × 100

M.C: Moisture content

Wi: Initial leaf weight

W0: Weight of leaves after drying

III.2. Extraction yield

The extraction yield of phenolics from Lemon verbena powder is calculated as follows:

$$\text{Yield } (\%) = \frac{\text{Mass of extract}}{\text{Mass of dried material}} \times 100$$

Mass of dried material = 1g.

This parameter is usually used as an indicator of the effects of the extraction conditions **(Dhanani *et al.*, 2017)**.

IV. Approximative composition: Determination of ash content:

This was done using the method of **A.O.A.C (1990).** The total ash content of a substance is the percentage of inorganic residue remaining after the organic matter has been ignited (**Agoreyo *et al.*, 2012**). 2 g of the *Aloysia triphylla* samples was placed in a crucible and ignited in a muffle furnace at 600 ^{o}C for 6 hours. Thereafter cooled in a desiccator and weighed at room temperature to get the weight of the ash. The experiments were performed in triplicate.

The ash content was determined in percentage (%) according to the following formula:

$$\textbf{Ash } (\%) = \frac{\mathbf{m1 - m2}}{\mathbf{mi}}\mathbf{x100}$$

Where: m1: mass (g) of crucible with ashes;

m2 : mass (g) of empty crucible;

mi : initial mass (g) of sample.

V. Extraction of total phenolic compounds (TPC)

The phenolic compounds were extracted using two methods. In order to standardize the extraction conditions, the same amounts of *Aloysia triphylla* powder, the same extraction solvent and the same ratios were used.

V.1. Microwave assisted extraction (MAE)

The extraction of polyphenols was carried out using a domestic microwave oven with dimensions of 28.1 cm × 48.3 cm × 38.7 cm and a frequency of 2450 MHz (2450 MHz, Maxipower Model MAXMO23S, China). The microwave operates at powers ranging from 100W to 1000W. Precautions have been taken to prevent the loss of vapors formed during the

extraction process. One gram of the powdered *Aloysia triphylla* leaves was placed in a 100 mL volumetric flask containing the extraction solvents. The contents were subjected to microwave oven irradiation. Depending on the test, different particle sizes, solvents, irradiation times, microwave power and solvent-to-solid ratio were used. After each extraction, the volumetric flask was allowed to cool at room temperature. The obtained extract was filtered through Whatman No. 1 paper; the filtrates were stuck in shaded bottles and stored at 4 °C until further use.

V.1.1. Optimization of MAE

The principle of this method is to vary the value of the studied variable, while leaving constant the variables which are not. Each test was performed three times. To start the experiments, several particle sizes were tested, namely 500 µm, 250 µm, 125 µm, in order to select the size of the particles allowing the best yield of the TPCs. Constant parameters were set at 500W, 120 s, 20:1 mL/g and 50% ethanol, for microwave power, irradiation time, solvent-to-solid ratio, solvent type and concentration, respectively. The particle size with 125 µm of diameter was chosen to define the adequate solvent for the extraction. To do this, different mixtures of solvent/water, namely ethanol, methanol and acetone (1:1 v/v; organic solvent: water) were used, and ethanol was chosen and studied to define the best concentration (20-80%). By testing the influence of the two factors (type of solvent and its concentration), the constant values of microwave power, irradiation time, solvent-to-solid ratio and particle size were 500W, 120 s, 20:1 mL/g and 125 µm, respectively.

After fixing the concentration of the solvent (60% ethanol), the best ratio was selected by varying the latter from 15:1 to 45:1 mL/g and the constant values of the other factors were (60% ethanol, 500 W, 120 s and 125 µm). Thus, the adequate ratio was set at 25:1 mL/g. To define the optimal power for the extraction, the other parameters were set as follows: 125 µm, 60% ethanol, 120 s and 25:1 mL/g, whereas the irradiation power varied between 200 W and 700 W, the optimum power was set at 300 W. For the irradiation time, six times were studied, namely (90, 120, 150, 180, 210, 240 seconds), fixing the other factors (125 µm, ethanol 60 %, 25:1 mL/g, 300 W), the adequate irradiation time was set at 180 s.

Based on the obtained single-factor experimental results, the main influencing factors were selected. Subsequently, an RSM based on a CCD (Central Composite Design) was carried out to optimize the MAE process. Regression analysis of the data to fit a second-order polynomial

equation (quadratic model) was performed according to the following general equation (Equation 1) which was then used to predict the optimal conditions for extracting polyphenols:

$$Y = ß_{\circ} + \sum_{i=1}^{k} ß_i x_j + \sum_{i=1}^{k} ß_{ii} x^2 \sum_{i>j}^{k} ß_{ij} x_i x_j + E \quad (1)$$

Where $ß_{\circ}$ is the constant coefficient of the model, $ß_i$, $ß_{ii}$ and $ß_{ij}$ are the coefficients of linear, quadratic and interactive terms, respectively, X_i and X_j represent the coded independent variables. The factors corresponding to each independent variable were X_1-solvent concentration (%, v/v), X_2-irradiation time (s), X_3-microwave power (W) and X_4-solvent-to-solide ratio (mL/ mg).

The TPC yield was the dependent response variable (Y). Analysis of variance was performed for the response variable using the full model, where the *P* values that are partitioned into linear and interaction factors indicate whether the terms are significant or not. To check the adequacy of the models, additional extraction experiments were carried out under the optimal conditions provided with the RSM. The experimental data obtained were compared with the values predicted by the regression model. The polyphenols content of the extract optimized by MAE was then compared to an extra reference obtained by conventional extraction.

V.2. Conventional extraction (CE)

The conventional extraction was achieved by adopting the protocol of **Cheurfa and Allem (2016)**. One gram of powder was mixed with 40 mL of ethanol (40%: v/v), the mixture was shaken well and left to macerate for 3 days at room temperature, then the filtrate was stored at 4°C until its analysis.

VI. Spectrophotometric determination

VI.1. Total phenolic content

The Folin-Ciocalteu test was widely used in clinical and nutritional studies to measure the TPCs in plant-derived foods and biological samples. This method was originally designed to analyze proteins, but it was later adopted by **Singleton *et al.* (1999),** in order to analyze the phenolic components in wine, after which it became a routine test for the antioxidant evaluation of food and plant extracts (**Singleton *et al.*, 1999**). This assay is based on the quantification of the total concentration of hydroxyl groups present in the extract. 1.25 mL of diluted Folin Ciolcateu reagent (diluted ten times 1/10) was added to 200 µL of the extracts. The mixture was incubated

for 2 min at room temperature, and then 0.8 mL of saturated sodium carbonate solution (7.5%) was added. The mixture was incubated for 15 min at 50 °C and then cooled in ice water bath. The specific absorbance at 765 nm was immediately measured by a spectrophotometer (UV-mini 1240, Shimadzu, Japan). The absorbance of the extract was compared to a standard Gallic acid curve (**Fifure 1, annexe 2**) to estimate the concentration of total polyphenols in the sample. The TPC was expressed as mg Gallic acid equivalents (GAE) per gram dry weight (DW).

VI.2. Flavonoids content

The flavonoid content was determined according to the method of **Çam and Hisil (2010**). 192 µL of ethanol (40%), 30 µL of sample (triplicate extracts) or catechin (duplicate), 9 µL of $NaNO_2$ (5%) and 9% solution of $AlCl_3$ (10%) were very well mixed. After 6 minutes of incubation, 60 µL of NaOH solution (1M) was added. Absorbance was read at 510 nm, in microplate reader ((Synergy HT, Bio Tek (Winooski, USA)); with Gen5TM2.0 data analysis software spectrophotometer. The results were expressed in terms of mg catechin equivalent (CE) per gramme of dry weight (CE/g DW).

VI.3. Condensed tannin content

Condensed tannins were determined by the method of **Vermerris and Nicholson. (2007)**, which consisted of mixing 250 µL of extract with 2.5 mL of an acidic solution (Fe2 (SO4)3 + n-butanol/HCl). After incubating the mixture at 95°C for 50 minutes, the absorbance was read at 550 nm against a blank. The concentration of condensed tannins was calculated according to the following equation:

[Tanins] = (A× DF× MW) /Ɛ×L;

DF: Dilution factor;

MW: Molecular weight of cyanidin (287 g/mol);

Ɛ: Molecular extinction coefficient (34700L/ mol/cm) ;

L: 1 cm.

Condensed tannins are expressed as mg cyanidin eq / g dry weight.

VI.4. HPLC-DAD-ESI-MS/MS

Leaves and flowers Phenolic compounds were analyzed with HPLC-MS/MS, on a Waters Alliance HPLC system (Waters®, 2695 separation module, Ireland) equipped with a quaternary

pump, solvent degasser, auto sampler and column oven, coupled to a Photodiode Array (PDA) Detector Waters 2996 (Waters, Ireland). Chromatographic separation of compounds was carried out on a Lichrocart RP-18 column (250 x 4 mm, particle size 5 µm, Merck) and a Manu-cart® RP-18 pre-column in a thermostated oven at 35°C. The mobile phase consisted of water: formic acid (99.5%: 0.5%) as eluent A and acetonitrile: formic acid (99.5%: 0.5%) as eluent B, at a flow rate of 0.30 mL min-1. The system was run with the following gradient program: 0-10 min at 95% eluent A, 10-30 min from 95 to 82% eluent A, 30-44 min from 82 to 64% eluent A, 44-64 min at 64% eluent A, 64-90 min from 64 to 10% eluent A, and finally returning to the initial conditions for 20 minutes. The injection volume was 20 µL. PDA was used to scan wavelength absorption from 210 to 600 nm. Tandem mass spectrometry (MS/MS) detection was performed on a Micromass® Quattro Micro triple quadrupole (Waters®, Ireland) using an electrospray ionization (ESI) source operating at 120°C, capillary voltage of 2.5 kV, cone voltage of 30 V and collision energy of 20 eV. The compounds were ionized in both positive and negative modes and spectra were recorded in the range m/z 60-1100. High purity nitrogen (N_2) was used both as drying gas and as a nebulizing gas. Ultra-high purity Argon (Ar) was used as collision gas.

VII. Evaluation of biological activities of extracts

VII.1. Evaluation of antioxidant activities

Oxidation processes are complex, and a single method cannot reflect the antioxidant profile of a sample. For this reason, this work focuses on the study of several antioxidant activities, namely total antioxidant activity, ferric ion reduction, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging, ABTS $^{+}$ radical cation scavenging, ORAC assay and HOSC assay. The principle of these methods is color change followed by absorbance readings at specific wavelengths.

VII.1.1. Evaluation of antioxidant activity by DPPH

DPPH$^{\bullet}$ is a π-radical, present in its monomer form in a solid state, as well as in solution (**Williams, 1966**). The DPPH radical is soluble in different organic solvents, but not in water. It usually dissolves in methanol, ethanol, or their aqueous mixtures. In this final case, the water content should not exceed 60% to make the radical more readily soluble (**Staško *et al.*, 2007**). In solution, DPPH is a stable radical, it is purple in appearance and absorbs at 515 nm in methanol (**Krishnaiah *et al.*, 2011**). The DPPH test is based on donating electrons from the

antioxidants in order to neutralize the DPPH radical. In this test, antioxidants reduce purple 1-diphenyl-picrylhydrazyl to a yellow compound, 1-diphenyl-picrylhydrazine, whose color intensity is inversely proportional to the capacity of the antioxidants present in the reaction medium (**Sanchez-Moreno, 2002; Mishra *et al.*, 2012**). Antioxidant activity by the DPPH neutralization method is often reported as IC_{50}, which is defined as the efficient concentration of the antioxidant necessary to reduce the initial DPPH concentration by 50% (**Foti *et al.*, 2015**). The application of this test allows the comprehension of the various chemical phenomena and has obvious advantages, like low cost, ease of performing experiments, reproducibility, applicability at room temperature, as well as automation possibilities **(Munteanu and Apetrei, 2021)**. It is comparable to other methods like ABTS, superoxide anion reduction and lipid peroxidation inhibition (**Villaño *et al.*, 2007**).

The ability of the extracts to remove the DPPH radical was determined according to the procedure of **Brand-Williams *et al.* (1995)** with some modifications. 50 µL of various concentrations of the hydroalcoholic extracts were added to 2 mL of 0.004 % DPPH methanolic solution. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 minutes. The absorbance was measured at 517 nm, and the activity was expressed using the following equation:

% DPPH inhibition = {(AB-AA) / AB} x 100

AB: Absorbance of DPPH solution;

AA: Absorbance in presence of extract or standard;

Results are expressed as % DPPH inhibition and IC_{50}. Where the IC_{50} of DPPH scavenging activity is the concentration of sample or standard that inhibit 50% of DPPH radicals, it was obtained by linear regression analysis of dose-response curve plotting between the % of inhibition and concentration.

VII.1.2. Iron Reduction (FRAP)

The indirect assay based on single electron transfer (SET) methodology FRAP test (**Bridi *et al.*, 2021**) is simple, fast, cost-effective and does not require specialized equipment. It is used globally on a large scale, providing results for a variety of purposes including the estimation of the antioxidant content in foods and their contribution to the supply of antioxidants, FRAP is used too, to investigate the effect of storage, growth, draught, solar radiation, processing, genetic modification of dietary agents, and to compare the relative content of antioxidants in

foods, medicines, traditional medicines, herbs, spices, teas and wines for product differentiation, quality, control and development. This test may be also used to detect water contamination and to study the effect of radiations, pollution. The high sensitivity and accuracy of the test allows for sample differentiation. It is used to assess the absorption and systemic distribution of antioxidants "bioavailability" after ingestion of food, beverages, drugs or supplements (**Munteanu and Apetrei, 2021**).

The FRAP assay measures the reduction of ferric iron (Fe III) and 2,3,5-triphenyl-1,3,4-triaza-azoniacyclopenta-1,4-diene chloride to blue ferrous (Fe II) complex by antioxidants (**Antolovich *et al.*, 2002; Haida and Hakiman., 2019**).

The ability of the extracts to reduce ferric iron was evaluated according to the method of **(Yildirim *et al.*, 2001**). 1 mL of extract is mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide ($K_3Fe(CN)_6$; 1%), the mixture was incubated at 50 °C for 30 minutes. After incubation, 2.5 mL trichloroacetic acid (TCA, 10%) was added and the mixture was centrifuged at 1650 g for 10 min. 2.5 mL of the supernatant was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm. For reducing power, the IC50 is the extract concentration where the absorbance is 0.5, and is calculated from the graph of absorbance at 700 nm against the extract concentration (**Rezig *et al.*, 2019**). The lowest IC_{50} means that the sample had the highest antioxidant capacity (**Fidrianny *et al.*, 2015**).

VII.1.3. ABTS+• radical cation

This assay was performed using the methodology described by **Re *et al.* (1999)**. It can be evaluated over a wide pH range, which is useful to study the effect of pH on antioxidant mechanisms for food components. Furthermore, the ABTS radical is soluble in water and organic solvents, enabling the determination of antioxidant capacity of both lipophilic and hydrophilic compounds (**Munteanu and Apetrei, 2021**). This tests allow the determination of a large variety of antioxidant substances, since ABTS•+ radical reacts rapidly with both synthetic and natural ones (i.e., phenols, amino acids, peptides, vitamin E and vitamin C) in food components (**Walker *et al.*, 2009**). Furthermore, this assay is cheap and operationally simple. DPPH method and ABTS radical scavenging method is one of the most extensively used antioxidant assays for plant samples **(Krishnaiah *et al.*, 2011)**.

ABTS, was dissolved in distilled water at a concentration of 7.4 mM and mixed with 2.45 mM potassium persulfate ($K_2S_2O_8$). The reaction mixture was allowed to stand at room temperature in the dark for 12-16 hours before use. The $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.020 at 734 nm. 1 mL of diluted ABTS solution was mixed with 10 µL of plant extracts and the absorbance was measured at 734 nm exactly 6 min after mixing. The standard (Gallic acid) was prepared in 40% ethanol and tested under the same conditions. Appropriate solvent blanks were made in each assay. The percentage inhibition was calculated and plotted against the concentration of Gallic acid. The results were expressed in terms of the equivalent antioxidant capacity of Gallic acid (mM Gallic acid equivalent per milligram of dry extract: Mm GAeq/mg dE).

VII.1.4. Evaluation of total antioxidant activity by phosphomolybdate

This assay is based on the reduction of Mo (VI) to Mo (V) by the sample and subsequent formation of a green phosphate/Mo (V) complex at acidic pH (**Prieto *et al.,* 1999**). An aliquot of 0.1 mL of sample at different concentrations was mixed with 1 mL of reagent sulphuric acid (H_2SO_4, 0.6 M), sodium phosphate (Na_3PO_4, 28 mM) and ammonium molybdate ($(NH_4)_6$ Mo_7O_{24}, H2O, 4 mM). The sealed tubes are incubated at 95°C for 90 minutes. After cooling at room temperature, absorbance was measured at 695 nm against a blank (which contain solvent with reagent) and incubated under the same conditions as the rest of samples. All assays were carried out in triplicate.

VII.1.5. ORAC assay

Oxygen Radical Absorbance Capacity Assay (ORAC) is a method that was first developed by **Cao *et al.* (1993).** It is a direct assay method based on hydrogen atom transfer (HAT) (**Bridi *et al.,* 2021**); the ORAC test measures the splitting ability of the radical chain reaction by antioxidants through monitoring the inhibition of the oxidation of the peroxyl radical. Peroxyl radicals are characterized as free radicals that predominate in lipid oxidation in biological systems and also in foodstuffs, under physiological conditions. As a result, ORAC values are appreciated by certain researchers as biologically relevant, a benchmark for antioxidant efficiency (**Munteanu and Apetrei., 2021**).

VII.1.6. HOSC assay

The hydroxyl radical is one of the most reactive free radicals in a biological system (**Liang and Kitts., 2014**). Hydroxyl radicals evaluates the hydroxyl radical scavenging capacity of a sample

using fluorescein as a probe and a classic Fenton reaction with Fe (III) and H_2O_2 as a source of hydroxyl radicals (**Bordalo *et al.*, 2021**).

The test was performed according to the **Moore *et al.* (2006)** method on black 96-well plates, using FL 800 microplate fluorescence reader. 170 µL of fluorescein (9.96×10^{-8} M) used as probe (classic Fenton reaction), 30 µL of blank, extract or standard, 40 µL of H_2O_2 (0.2 M) and 60 µL of $FeCl_3$ (3.42 mM) which were used as hydroxyl radical generating system, were mixed. Trolox was used for the calibration curve and the results are expressed as micromoles of Trolox equivalent antioxidant capacity (TEAC) per gram of extract (DW).

VII.2. Evaluation of antibacterial activity

VII.2.1. Microorganisms and culture conditions

The antimicrobial activity of the *Aloysia triphylla* leaves extracts (MAE and conventional) were evaluated against the following strains provided by the Algerian Pasteur Institute:

– Gram-positive bacterial strains: *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633.

– Gram-negative: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853

All bacteria were grown in Tryptone Soya Agar (TSA) and then incubated at 37 ºC for 24 hours. All experiments were conducted twice independently.

VII.2.2. Disc diffusion assay

The antimicrobial activity of the extracts was evaluated by the disk diffusion method (**Wikler, 2006**). Each bacterial suspension with a concentration of 10^6 CFU/mL (at 625 nm) was grown on Petri dishes and the inoculum was prepared by dilution in Mueller-Hinton broth. Paper discs (6 mm) were soaked with 20 µL of 100 mg/mL extract prepared in DMSO and placed on the inoculated agar. The Petri dishes were incubated at 37 °C for 24 h. The DMSO-impregnated discs were used as a negative control. Antibacterial activity was determined by measuring the zones of inhibition (ZI) in millimeters (mm) against the microorganisms. An extract is active when its ZI around the disc is greater than 6 mm (**Daas Amiour *et al.*, 2014**). The experiments were performed in duplicate.

VII.2.3. Minimum inhibitory concentration (MIC) and minimum bactericid concentration (MBC) assay

The MIC was determined in 96-well microplates device by the method of successive dilutions **(Wikler, 2006)**. The tested dilutions of extracts were ranging from 25 to 0.05 mg/mL. In each well, 100 µL of an appropriate dilution of extracts or 100 uL of antibiotics were added to the wells containing 100 µL of 24 hours' inoculum at 10^7 CFU/mL. The microplates were incubated at 37 °C for 24 h and the tests were performed in triplicate. The growth of bacteria in treatment wells was compared to positives controls (100 µL of MH media + 100 µL inoculum and negative control (200 µL of MH media). The absorbance was measured at 620 nm using an absorbance microplate reader (Spectrophotometer UV-Visible SRECTROSCAN50). The MBC was determined from the MIC values. The MIC was the lowest concentration of the extract at which growth of bacteria was not observed and the MBC is the concentration required to kill them.

VII.3. Anti-acetylcholinesterase activity

VII.3.1. Determination of anti- acetylcholinesterase activity

Acetylcholinesterase enzymatic activity was measured by the Ellman test (**Ferreira *et al.*, 2006**). 400 µL of 50 mM Tris buffer (pH 8), 50 µL of plant extract in ethanol with different concentrations and 25 µL of an enzyme solution containing 0.26 UI/mL were incubated during 15 min. Subsequently, 75 µL of a solution of acetylthiocholine iodide (AChI) 15 mM and 475 µL of 3 mM 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) were added. Absorbance of the mixture was measured at 412 nm when the reaction reached the equilibrium. A control mixture was prepared, using ethanol 20% instead of extract and was considered 100% activity. Inhibition, in %, was calculated in the following way:

$$I\ (\%) = 100 - (V_{extract} / V_{control}) \times 100$$

where I was the percent inhibition of acetylcholinesterase, V_{sample} is the initial velocity of the extract containing reaction and $V_{control}$ is the initial velocity of the control reaction. A blank with Tris buffer instead of enzyme solution was used. Extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting the inhibition percentage against extract solution.

VII.3.2. Protocol for the determination of antiacetylcholinesterase activity

The experiments were conducted in triplicates as shown in the **table IV**.

Table IV: Treatment protocol for the *in vitro* determination of anti-acetyl cholinesterase activities of *Aloysia triphylla* leaves conventional and microwave extracts

Treatment Group	Treatment of extracts
Blank	None + buffer + crude enzyme extract (AChE) +ATCI+DTNB
Experimental group 1	0.1 mg/mL of plant extract +crude enzyme extract(AChE) + buffer + ATCI + DTNB
Experimental group 2	0.2 mg/mL of plant extract +crude enzyme extract(AChE) + buffer + ATCI + DTNB
Experimental group 3	0.35 mg/mL of plant extract +crude enzyme extract (AChE) + buffer + ATCI + DTNB
Experimental group 4	0.5 mg/mL of plant extract +crude enzyme extract (AChE) + buffer + ATCI + DTNB
Experimental group 5	0.6 mg/mL of plant extract +crude enzyme extract (AChE) + buffer + ATCI + DTNB
Experimental group 6	0.75 mg/mL of plant extract +crude enzyme extract (AChE) + buffer + ATCI + DTNB

ACTI: Acetylthiocholine iodide (substract) / DTNB: 5-5 dithiobis-2-nitrobenzoic acid

VII.3.3. Acetylcholinesterase sensitivity to different concentrations of the extract

For sensitivity tests, different concentrations of the aqueous and organic extracts were tested to determine the extract concentration that inhibited 50% of the enzyme activity. The value of median inhibition concentration (IC_{50}) for the inhibitors was calculated based on log (inhibitor concentration) *versus* probit (percentage of inhibition) linear regression. Concentration response curve of change in absorbance against concentration was plotted to show the sensitivity of the enzyme to the extracts (**Anne *et al.*, 2016**).

VIII. Statistical analysis

Concerning the optimization of microwave assisted extraction, the experiments were performed in triplicate, and all the data have been reported as means ± SD. The influence of each factor

on the TPC yield for the MAE was statistically evaluated by the analysis of variance (ANOVA) and the Tukey's post hoc test with a 95% confidence level. To construct the CCD approach, JMP software (version 10.0, SAS, USA) was used. The Tukey's post hoc test, $p < 0.05$ and $p < 0.01$ were taken as significant and highly significant level, respectively.

Concerning conventional extraction of phenolic compounds, antioxidant, antibacterial and acetylcholinesterase activity, the results were submitted to one-way analysis of variance (ANOVA), considering as critical level $p < 0.05$ to evaluate significant difference between the experimental groups, followed by Tukey's Test, using GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, USA) software. Graphs also were designed using the GraphPad Prism 5.0 software.

The efficiency of the two methods used for leaf extracts, namely the non-conventional method (MAE) and the conventional method (CE), were compared based on the recovery rate of phenolic compounds, the quality of recovered phenolic compounds and the antioxidant, antibacterial and anti-acetylcholinesterase activities.

Results and discussion

I. Phytochemical sceening

I.1. Moisture content of *Aloysia triphylla* leaves

The leaves of *Aloysia triphylla* have high moisture content (MC) which was 72 ± 1.38 % (**Figure 17**), demonstrating that this plant is rich in water. A moisture content of 75.76 % was recorded for bergamot mint (*Mentha piperita*) (Lamiaceae) **(Grzeszczuk and Jadczak., 2009)**, 75 % for *Cordia verbenacea DC*. leaves **(Goneli *et al.*, 2014)**, it was 81.5 ± 0.71 % for the leaves of *Lippia multiflora* (*Verbenaceae*) **(Kane *et al.*, 2010)**, and 94.4 ± 0.04 % for *Vitex Pinnata* Linn leaves (Lamiaceae) **(Thenmozhi and Subasini, 2016)**. This high moisture content implies that the plant can retain enough moisture to support microbial growth, which would make the plant susceptible to microbial degradation during storage. For effective storage, drying may be required by applying heat or drying over a longer period of time to reduce moisture content, and by storing under a specific humidity or conditioning/treating with an appropriate drying agent (**Omoregie *et al.*, 2015**).

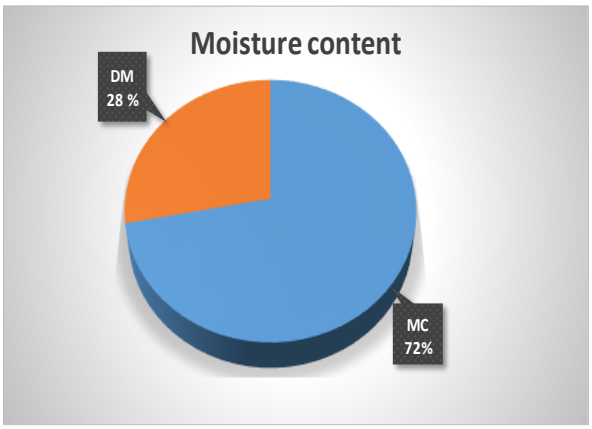

Figure 17 : Moisture content of *Aloysia triphylla* leaves

I.2. Leaf extraction yield

Selection of an extraction method would mainly depend on the advantages and disadvantages of the processes such as extraction yield, complexity, production cost, environmental friendliness and safety (**Li *et al.*, 2010**). MAE yielded a dry extract that was not statistically different from conventional extraction ($P<0.05$), it was 27.3±0.015 % and 26.6±0.007% for

MAE and conventional extracts, respectively. However, MAE should be preferred, given that it is faster and more environmentally friendly (**Dahmoune *et al.*, 2015**).

The yields of dry extracts vary from one plant to another of the same family because the yield depends on several parameters namely the methods and conditions of solid-liquid extraction of polyphenols: temperature, extraction solvent, particle size and diffusion coefficient of the solvent (**Wang *et al.*, 2006; Penchev, 2010; Michel, 2011**). **Choupani *et al.* (2014)** performed extraction with ethanol by maceration of *Aloysia triphylla* powder at room temperature. The extraction rate was 9.53±0.5 % of powder, which is much lower than the extraction rate obtained in this work.

According to **Chen *et al.* (2016)**, swelling of herb tissue by maceration enhances the extraction yield of a chemical marker. It is possibly due to the fact that, there are so many water soluble substances. When water is present in the maceration solvent, it dissolves and removes the water soluble substances, and therefore increases the availability of chemical marker to be extracted by the mixture of ethanol and water.

II. Approximative composition: Determination of ash content

The total ash test provides informations on the mineral content, as minerals are not transformed into volatiles at high temperatures, unlike organic matter. The total ash content was evaluated at 2.83±0.35 %. It represents the mineral fraction of *Aloysia triphylla* leaves. It is much lower than that found by **Pereira *et al.* (2015)** which was 8.5±0.3 %. This variation is probably due to several biotic and abiotic factors, namely soil composition, climatic factors and even geographical origin.

According to **Yeo *et al.* (2021),** *Aloysia triphylla* infusion contains several mineral elements such as: P (10.7 ppm), K (15977.0 ppm), Zn (991.2 ppm), Ca (192888.9 ppm), Fe (50062.5 ppm), I (9.18 ppm). The presence of some metal traces may have both curative and preventive roles in combating diseases (**Aliyu *et al.*, 2008**).

According to **Ajasa *et al.* (2004),** the growing interest in the use of herbal medicine is due to its minimal side effects, availability, and acceptability by the majority of the population in third world countries. Their consumption contributes to the intake of minerals (essential and non-essential) in infants and the elderly. Indeed, the human body requires a number of minerals in order to maintain good health. A number of minerals essential to human nutrition are accumulated in different parts of plants as it accumulates minerals essential for growth from

the environment. Macro- and micro-elements influence biochemical processes in the human body, and the study of elements in relation to native medicinal plants reveals that major elements and trace elements have important roles in combating a variety of human ailments and diseases (**Shirin *et al.*, 2010**).

III. Optimization of microwave extraction of phenolic compounds by experimental design

Determining the optimal conditions is crucial for the extraction of phenolic and antioxidant compounds from plant materials, and these extraction conditions are different from one plant matrix to another due to their phytochemical, pharmacognostic and anatomical diversity. The efficiency of microwave-assisted extraction of a compound depends on several parameters such as microwave power, irradiation time, solvent type and concentration, liquid-solid ratio; and their effects can be either independent or interactive **(Dahmoune *et al.*, 2015; Djaoud *et al.*, 2020; Guemhgar *et al.*, 2020)**. This study was conducted in several steps. The first step was the determination of the lower, middle and upper levels of the design variables employed in the response surface methodology (RSM). These levels of independent variables were determined based on the values obtained in the preliminary study.

Analysis of variance was performed for the response variable using *p*-values that are divided into linear and interaction factors. The *p*-value is used to check the significance of each coefficient and the interaction pattern between the variables. To check the adequacy (validation) of the models, additional extraction tests were performed under the optimal conditions predicted by the RSM mathematical models, and the experimental data obtained were compared with the values predicted by the regression model.

III.1. Single factor analysis method for MAE and effect of independent variables

Microwave extraction is influenced by several parameters such as: particle size, extraction time, solvent-to-solid ratio, microwave power and type of solvent. The preliminary study results of the microwave-assisted extraction of TPC are shown in **Table V**.

III.1.1. Effect of particle size

Particle size has a considerable effect on polyphenols extraction. This extraction rate increases with decreasing particle size (**Pinelo *et al.*, 2007; Chupin *et al.*, 2015**). In this study, the particle

size that resulted in a maximum extraction efficiency of 64.84±1.82 mg GAE/g DW was 125 µm, compared to particle size 250 µm and 500 µm which gave 56±1.55 mg GAE/g DW and 55.33±1.73 mg GAE/g DW, respectively. These results corroborate with those of **Rajha *et al.* (2014)**, since the limiting step in extraction is often the diffusion of chemicals out of the plant matrix. Fine powder can improve the extraction, the larger surface area of a fine powder allows contact between the plant matrix and the solvent and improves mass transfer. In addition, the extraction rate, which becomes better in the case of smaller particles, could be attributed to the opening of a larger number of pores (closed in case of larger fractions), facilitating contact with the solvent (**Rajha *et al.*, 2014; Çavdar *et al.*, 2017**). Furthermore, the diffusion distance of the solute in the solid is reduced when the particle size is smaller. Thus, the solute reaches the surface in a shorter time (**Pinelo *et al.*, 2007**). Therefore, in this study, we have chosen dried powder with particle size of 125 µm in order to evaluate the effect of solvent extraction on TPC assay.

III.1.2. Effect of extraction solvent

The choice of solvent has a significant effect on the extraction efficiency of TPC because they differ in their dielectric properties (**Choupani *et al.*, 2014; Dahmoune *et al.*, 2015**). It depends on the solubility of the biomaterials of interest through the interaction between the solvent and the plant matrix, and finally on the microwave energy absorption properties of the solvent, which is determined by its dielectric constant (**Wang and Weller., 2006**). Ethanol, methanol and water have a high dielectric constant, which facilitates the heating process. Microwave heating induces cell wall rupture and is followed by the release of the molecules to be extracted into the solvent (**Eskilsson and Bjorklund, 2000**). In this study, several extraction solvents were used (methanol, ethanol, water and acetone). The best TPC yield is achieved by ethanol (54±1.18 mg GAE / g DW) and methanol (57.69 ± 1.3461 mg GAE/g DW), with statistically equal values, followed by acetone (52.76 ± 1.74 mg GAE/g DW) and finally distilled water (46.04 ± 1.21 mg GAE/g DW). The ethanol was chosen for the RSM assays, because it is the most widely used solvent, due to its high extraction efficiency, low cost and low toxicity. It could be used safely in the food, pharmaceutical and cosmetic industries (**Yuan *et al.*, 2019; Guemghar *et al.*, 2020; Neshat *et al.*, 2020**); while methanol is potentially dangerous to human health (**Wu *et al.*, 2012**). In the recent study (**Rashid *et al.*, 2022**), the ethanolic extract of fresh *Aloysia triphylla* revealed the highest Gallic acid equivalent amount with a value of

117.13 mg GAE/g of dry weight (DW), followed by ethyl acetate extract with 73.44 mg GAE/g DW, while water revealed the lowest phenolic amount with a value of 66.6 mg GAE /g DW.

III.1.3. Effect of ethanol concentration

According to **Chew *et al.* (2011)**, ethanol concentration has a significant effect on the phenolic content. A low concentration of hydro-ethanol solution may enter the cell while a high concentration of this solvent causes protein denaturation and slows the dissolution of polyphenols, thus decreasing the extraction rate. Solvents of different concentrations have different polarities, so it is necessary to find an appropriate concentration to obtain a better extraction rate (**Yang *et al.*, 2009**). The principle of heating using microwave is based on its direct impacts on polar materials (**Letellier and Budzinski., 1999**). Indeed, microwave electromagnetic energy is converted to heat by ion conduction and dipole rotation mechanisms (**Jain, 2009**). Therefore, heating is proportional to the dielectric constant of the solvent. Microwave heating promotes the migration of dissolved ions and penetration of solvents into the matrix. On the other hand, higher viscosity of the medium lowers this mechanism by affecting the rotation of molecules (**Kaufmann and Christen, 2002**). Adding to this, in non-polar solvents, heating is not efficient because the energy is transferred by dielectric absorption only (**Handa *et al.*, 2008**). That is why, microwave extraction can be considered as selective methods that favor polar molecules and solvents with high dielectric constants (**Azwanida, 2015**). Indeed, water with dielectric constant of 78.5 (20°C) guarantees immediate overheating, which allows a better absorption of microwave energy, that increases the temperature in the plant cells, and enhances the dissolution and extraction yield of polyphenols (**Guemghar *et al.*, 2020**). However, low concentration of ethanol can also induce a low extraction of TPC, due to the difference in dielectric properties of the solvent towards microwave heating **(Dahmoune *et al.*, 2015; Simić *et al.*, 2016)**. Therefore, it is necessary to find an appropriate concentration to obtain a better extraction rate. Indeed, 60% and 80% ethanol gave maximum TPC yield (**Figure 18.C**). The 60% ethanol concentration was set for future single-factor experiments. It should also be noted that for the statistical analysis presented in **Table V**, the concentration range that was chosen for the central composite design tests is between 40 and 80%.

Lower concentrations of ethanol than 60% induce a low extraction of TPCs, it is probably due to poor solubilization of phenolic compounds. Indeed, the variation in solubility of phenolic compounds in different solvents is due to their structural diversification (**Ashraf et *al.*, 2016**),

and the polarity of the solvent affects the solubility of phenolic compounds (**Nackz and Shahidi, 2004; Mu'azu *et al.*, 2017**).

III.1.4. Effect of irradiation time

The irradiation time has a significant effect on the extraction rate of phenolic compounds by the fact that the extraction duration is proportional to the extraction yield **(Dahmoune *et al.*, 2015; Djaoud *et al.*, 2020)**. Indeed, the results show that the extraction yield of phenolic compounds increases with the increase in irradiation time, reached its maximum after 180 s of irradiation (64.37±1.51 mg GAE/g DW). Beyond this value, the extraction yield decreases progressively as the irradiation time is prolonged. Thus, a prolonged exposure to microwave radiation leads to thermal degradation of the phenolic compounds (**Xiao *et al.*, 2008; Luo *et al.*, 2010; Dahmoune *et al.*, 2015**). Therefore, 180 s is the optimal point used for single-factor tests and the range between 150 - 210 s was used to perform the RSM tests.

III.1.5. Effect of microwave power

Microwave power effect on the polyphenols extraction yield was performed between 200 W and 700 W. Like other factors, microwave power has a significant impact on phenolic compound extraction. The TPC yield increase from 200 W (55.43±1.00 mg GAE/g DW) to reach a maximal level at 300 W (57.53±0.55 mg GAE/g DW). After that, it begins to decrease progressively with microwave power increase. The lowest extraction yield is observed at the highest power which was 700 W (50.23±1.15 mg GAE/g DW).

Microwave heating is caused by the ability of materials to absorb energy by dipolar and ionic mechanisms. It affects the interactions and distribution of the analytes between the sample and the solvent (**Hayat *et al.*, 2009; Lefsih *et al.*, 2017**). **Chandrasekaran *et al.* (2013)** showed that the presence of water in solvent (60 % ethanol), causes dielectric heating due to the dipolar nature of water, the permanently polarized dipole molecules try to realign themselves in the direction of electric field. High frequency of the electric field, induces a realignment of a billion (2.4×10^9) times per second and causes internal friction between the molecules, resulting in volumetric heating of the material.

Microwave power intensity controls the amount of energy supplied to the sample that is converted into thermal energy in the dielectric material to raise its temperature. It affects the interactions and distribution of analytes between sample and solvent **(Ma *et al.*, 2009; Hayat et al., 2009; Lefsih *et al.*, 2017). Yang and Zhai. (2010)** and **Li *et al.* (2012a),** proved that

increase of microwave power, increased the extraction efficiency of phenolic compounds. However, a high microwave power can increase the temperature of the processed product, which allows decrease in yield extraction due to thermic degradation of the compounds **(Xiao *et al.*, 2012; Guemghar *et al.*, 2020).** Effectively, results showed a decreasing in TPC yield extraction as the microwave power is higher. These results corroborate too with those of **Dahmoune *et al.* (2015).** He reported that extraction yield of phenolic compounds from *Myrtus communis* L. leaves decrease with increasing microwave power. Hence, 300 W is selected as optimal level used for single-factor tests, and 200 W to 600 W was the range used to perform CCD design.

III.1.6. Effect of solvent-to-solid ratio

The results presented in **Table V** shows that the solvent-solid ratio has a significant influence on the extraction yield of phenolic compounds. Accordingly, the extraction yield of TPCs increases as the solvent-solid ratio increases progressively from 15:1 (mL/g) (54.56±1.46 mg GAE/g DW) up to a maximum of 35: 1 (mL/g) (62.73±1.14 mg GAE/g DW)

Table V: Results of single-factor experiments for the MAE of TPC from *Aloysia triphylla* leaves.

<table>
<tr><th colspan="2">Particle size</th><th colspan="2">Solvent</th><th colspan="2">Ethanol concentration</th><th colspan="2">Irradiation time</th><th colspan="2">Microwave power</th><th colspan="2">Solvent solid ratioμm</th></tr>
<tr><th>μm</th><th>TPC yield</th><th>Type</th><th>TPC yield</th><th>(%v/v)</th><th>TPC yield</th><th>(S)</th><th>TPC yield</th><th>(W)</th><th>TPC yield</th><th>(mg/ml)</th><th>TPC yield</th></tr>
<tr><td>125</td><td>64.84±1.82^{a}</td><td>Methanol</td><td>57.69±1.34^{a}</td><td>20</td><td>48.74±1.14^{c}</td><td>90</td><td>54.4±1.25^{c}</td><td>200</td><td>55.43±1.00^{ab}</td><td>15</td><td>54.56±1.46^{c}</td></tr>
<tr><td>250</td><td>56±1.55^{b}</td><td>Ethanol</td><td>54±1.18^{ab}</td><td>40</td><td>50.31±1.02^{bc}</td><td>120</td><td>54.7±1.16^{c}</td><td>300</td><td>57.53±0.55^{a}</td><td>20</td><td>57.22±0.77^{b}</td></tr>
<tr><td>500</td><td>55.33±1.73^{b}</td><td>Acetone</td><td>52.76±1.74^{b}</td><td>50</td><td>51.52±1.64^{b}</td><td>150</td><td>59.13±1.59^{b}</td><td>400</td><td>53.78±1.09^{b}</td><td>25</td><td>61.95±0.96^{a}</td></tr>
<tr><td colspan="2" rowspan="5">125μm</td><td>Water</td><td>46.04±1.21^{c}</td><td>60</td><td>55.71±1.26^{a}</td><td>180</td><td>64.37±1.51^{a}</td><td>500</td><td>53.46±0.99^{b}</td><td>30</td><td>60.48±1.01^{a}</td></tr>
<tr><td colspan="2" rowspan="4">Ethanol</td><td>80</td><td>55.53±1.06^{a}</td><td>210</td><td>55.18±1.70^{c}</td><td>600</td><td>51.1±1.21^{c}</td><td>35</td><td>62.73±1.14^{a}</td></tr>
<tr><td colspan="2" rowspan="3">40-80</td><td>240</td><td>49.33±1.46^{d}</td><td>700</td><td>50.23±1.15^{c}</td><td>40</td><td>57.67±1.05^{b}</td></tr>
<tr><td colspan="2" rowspan="2">150-210</td><td colspan="2" rowspan="2">200-600</td><td>45</td><td>54.41±0.48^{c}</td></tr>
<tr><td colspan="2">20-40</td></tr>
</table>

Results are reported as means ± S.D. Same letters in the same column refer to means not statistically different according to ANOVA and Tukey's test. TPC: total phenols yield referred to dry weight (DW) of *Aloysia triphylla* leaves. GAE, Gallic Acid equivalents.

Thereafter, the extraction efficiency progressively decreases for the other ratios 40: 1 (mL/g) (57.67±1.05 mg GAE/g DW) and 45:1 (mL/g) (54.41±0.48 mg GAE/g DW), respectively. According to **Luo *et al.* (2010)**, this is due to the excessive swelling of the material by water (ethanol 60%) and absorbing the effective constituent. In the same perspective, this can be explained by the fact that a larger volume of solvent requires greater absorption of microwave energy, but this energy may not be sufficient to destroy the cell walls and release the target

components (**Song *et al.*, 2011; Dahmoune *et al.*, 2015**). Therefore, range of ratio 20-40 mL/g was used to realize CCD design.

III.2. Optimization of MAE by RSM

III.2.1. Statistical analysis

Central Composit Design (CCD) was used to optimize the extracting conditions of phenolic compounds from the *Aloysia triphylla* leaves. The levels of independante variable were chosen based on the values obtained in single-factor experiments. Four factors were studied: Ethanol concentration (X_1, %, V/V), irradiation time (X_2, s), microwave power (X_3, W) and liquid-to-solid ratio (X_4, mL/g). Thirty experiments were performed with three levels for each factor. The values of responses to different experimental combinations of coded variables are shown in **Table VI**. Response (Y) represents the phenolic compounds content (Y, mg GAE/ g DW). As shown, the TPC yield varied between 42.6 mg GAE/ g DW and 64.4 mg GAE/ g DW.

Table VI: CCD with the experimental and predicted values for the TPC yield using the MAE.

Run	X_1-Ethanol concentration %(v/v)	X_2-Irradiation time (min)	X_3-Microwave power (W)	X_4-Solvent to ratio (g/mL)	Recovery of TPC (mg GAE/g DW)	
					Experimental	Predicted
1	80 (+1)	150 (-1)	200 (-1)	40 (+1)	46.0 ± 0.90^{kl}	47.00
2	60 (0)	180 (0)	400 (0)	30 (0)	54.2 ± 0.20^{cdefghi}	53.21
3	80 (+1)	210 (+1)	600 (+1)	20 (-1)	54.8 ± 1.13^{cdefgh}	54.59
4	40 (-1)	210 (+1)	600 (+1)	40 (+1)	62.6 ± 1.31^{ab}	63.15
5	80 (+1)	210 (+1)	200 (-1)	20 (-1)	47.0 ± 1.41^{jkl}	47.59
6	60 (0)	180 (0)	400 (0)	20 (-1)	64.4 ± 2.44^{a}	64.41
7	40 (-1)	150 (-1)	200 (-1)	40 (+1)	57.2 ± 1.73^{bcdef}	56.45
8	80 (+1)	210 (+1)	600 (+1)	40 (+1)	58.8 ± 1.92^{abcde}	58.66
9	40 (-1)	210 (+1)	600 (+1)	20 (-1)	59.2 ± 2.26^{abcd}	59,03
10	40 (-1)	150 (-1)	200 (-1)	20 (-1)	59.8 ± 0.41^{abc}	60.78
11	80 (+1)	150 (-1)	200 (-1)	20 (-1)	52.9 ± 3.25^{efghij}	51.38
12	80 (+1)	180 (0)	400 (0)	30 (0)	48.2 ± 2.88^{ijkl}	49.02
13	60 (0)	210 (+1)	400 (0)	30 (0)	46.2 ± 3.12^{kl}	45.92
14	60 (0)	180 (0)	600 (+1)	30 (0)	53.8 ± 1.48^{cdefghi}	54.24
15	60 (0)	180 (0)	400 (0)	30 (0)	53.6 ± 2.68^{defghi}	53.21
16	80 (+1)	150 (-1)	600 (+1)	20 (-1)	51.6 ± 3.44^{fghijk}	52.44

17	60 (0)	180 (0)	400 (0)	30 (0)	53.8±0.50^{cdefghi}	53.21
18	80 (+1)	150 (-1)	600 (+1)	40 (+1)	50.8±3.11^{ghijk}	49.80
19	80 (+1)	210 (+1)	200 (-1)	40 (+1)	50.3±1.55^{hijk}	49.91
20	60 (0)	180 (0)	400 (0)	40 (+1)	63.8±1.70^{a}	64.27
21	40 (-1)	180 (0)	400 (0)	30 (0)	56.3±1.11^{cdefgh}	55.96
22	60 (0)	180 (0)	400 (0)	30 (0)	53.5±2.83^{defghi}	53.21
23	60 (0)	150 (-1)	400 (0)	30 (0)	42.7±2.12^{l}	43.46
24	60 (0)	180 (0)	400 (0)	30 (0)	53.0±0.28^{efghij}	53.21
25	40 (-1)	150 (-1)	600 (+1)	40 (+1)	54.2±0.11^{cdefghi}	54.45
26	60 (0)	180 (0)	200 (-1)	30 (0)	51.7±0.14^{fghijk}	51.74
27	40 (-1)	150 (-1)	600 (+1)	20 (-1)	57.6±1.40^{bcdef}	57.03
28	60 (0)	180 (0)	400 (0)	30 (0)	52.6±1.37^{fghij}	53.21
29	40 (-1)	210 (+1)	200 (-1)	40 (+1)	59.2±0.85^{abcd}	59.20
30	40 (-1)	210 (+1)	200 (-1)	20 (-1)	56.8±1.36^{bcdefg}	56.83

X_1: Ethanol concentration, X_2: Irradiation time, X_3: Microwave power, X_4: Solvent-to-solid ratio, GAE: gallic acid equivalent and DW: dry weight. a, b, c, d, e, f, g, h, i, j, k, l. Different letters, per column, indicate significant differences between mean values ($p < 0.05$). Equal letters indicate non-significant differences ($p > 0.05$).

III.2.2. Modeling and model fitting

In this study, the least square technique was used to calculate the regression coefficients of the intercept, linear, quadratic and interaction terms of the model (**Table VII**) as mentioned by **Zhang *et al.* (2013)**.

Table VII: Analysis of variance (ANOVA) for the fitted quadratic polynomial model for total phenolic compounds from *Aloysia triphylla* leaves by using microwave-assisted process.

***Parameter*[a]**	***Estimated Coefficients***	***Standard error***	***DF***	***Sum of squares***	***F-value***	***Prob > F***
Model	53.207895	0.273901	14	787.66551	72.3626	<0.0001*
Intercept						
B0	53.207895	0.273901		787.66551	72.3626	<0.0001*
Linear						
X_1	-3.472222	0.207833	1	217.01389	279.1179	<0.0001*
X_2	1.2277778	0.207833	1	27.13389	34.8989	<0.0001*
X_3	1.25	0.207833	1	28.12500	36.1737	<0.0001*

X_4	-0.066667	0.207833	1	0.080000	0.1029	0.7528
Quadratic						
X_1^2	-0.715789	0.547802	1	1.32746	1.7074	0.2110
X_2^2	-8.515789	0.547802	1	187.88928	241.6585	<0.0001*
X_3^2	-0.215789	0.547802	1	0.12065	0.1552	0.6992
X_4^2	11.13211	0.547802	1	321.19667	413.1152	<0.0001*
Interaction						
X_1/X_2	0.0375	0.22044	1	0.02250	0.0289	0.8672
X_1/X_3	1,2	0.22044	1	23.04000	29.6335	<0.0001*
X_1/X_4	-0.0125	0.22044	1	0.00250	0.0032	0.9555
X_2/X_3	1.4875	0,22044	1	35.40250	45.5338	<0.0001*
X_2/X_4	1.675	0.22044	1	44.89000	57.7364	<0.0001*
X_3/X_4	0.4375	0.22044	1	3.06250	3.9389	0.0658
Lack of fit			10	10.027485	3.0665	0.1140
Pure error			5	1.635000	3.0665	0.1140
Residual			15	11.66249		
R^2					0.98541	
R^2*Adjusted*					0.971792	
C.V. %	1.2992					
RMSE*	0.8818					
Corr Total			29	799.32800		

X_1, Ethanol concentration; X_2, Irradiation time; X_3, Microwave power; X_4, Liquid to solid ratio

The second-order polynomial model that predicts TPC content from Lemon verbena leaves is written as follows:

$$Y\ (TPC)= 53{,}21 - 3{,}47\ X_1 + 1{,}23\ X_2 + 1{,}25\ X_3 - 0{,}066\ X_4 - 0{,}71\ X_1^2 - 8{,}51\ X_2^2 - 0{,}21\ X_3^2 + 11{,}13\ X_4^2 + 0{,}04\ X_1X_2 + 1{,}2\ X_1X_3 - 0{,}01\ X_1X_4 + 1{,}49\ X_2X_3 + 1{,}67\ X_2X_4 + 0{,}44\ X_3X_4 \quad (4)$$

The linear parameters which are ethanol concentration (X_1), irradiation time (X_2), microwave power (X_3), quadratic effects of irradiation time (X_2^2) and liquid-to-solid ration (X_4^2) as well as interactions X_1X_3, X_2X_3 and X_2X_4 were highly significant (<0.0001*). The linear effect of

X_2 and X_3 are positive, meaning that increasing these factors could increase the extraction rate. While liquid-to-solid ratio factor (X_4), quadratic effects X_1^2, X_3^2 and interactions X_1X_4 and X_3X_4 were not significant (> 0.05). The *p*-value is used as an important way to check the significance of each coefficient, and can indicate the interaction pattern between the variables. The lower the *p*-value, the more significant of corresponding coefficient is (**Ji *et al.*, 2012**). F-value (72.36) and p-value (<0.0001*) show that the model is extremely significant **(Song *et al.*, 2011)**.

R^2 coefficient and the adjusted determination adjacent R^2 coefficient are 0.9854 and 0.972 respectively, they were closely related. It demonstrates the good fit of the model to the experimental results **(Karazhiyan *et al.*, 2011)**. R^2 which is the coefficient of determination is a measurement of the degree of fitness **(Nath and Chattopadhyay., 2007)**. High R^2 value indicates a high percentage of variability in responses that can be explained by these patterns **(Ji *et al.*, 2012; Djaoud *et al.*, 2020)**. Indeed, this coefficient of determination R^2 means that 98.54% of the variations in the sample were due to the independent variables, and 1.46 % of total variations could not be explained by this model **(Song *et al.*, 2011)**.

Low coefficient of variation (C.V. %) of 1.29% indicates a good model reproducibility. In addition, a non-significant lack of fit validates the model (0.1140 > 0.005) **(Simic´ *et al.*, 2016)**. Indeed, the lack of fit is a measure of the inability of a model to represent data in the experimental domain where points were not included in the regression **(Montgomery, 2001)**. Coefficient of variation (C.V.) indicates the relative dispersion of experimental points from the prediction of the model **(Nath and Chattopadhyay, 2007)**. It can also be defined as the standard deviation in percentage of the mean TPC, a low C.V. gives better reproducibility and a C.V. greater than 10 shows the amount of variation in the mean TPC value and does not allow for proper development of an adequate response model. **(Ji *et al.*, 2012)**.

The linear effect of the ethanol concentration is significant and negative which means that the increase in ethanol concentration (X_1), could decrease the extraction yield. The linear effects of irradiation time (X_2) and microwave power (X_3) are significant and positive, which means that by increasing them, it is possible to increase the extraction yield. These results corroborate with those of **Bai *et al.* (2010)** and **Liu *et al.* (2012**). The non-significant factors and interactions were removed from the second-order polynomial equation in order to obtain a simpler final model, which was determined as follows (Eq. (2)):

$Y(TPC)= 53,21 - 3,47\ X_1 + 1,23\ X_2 + 1,25\ X_3 – 8,51X_2^2 + 11,13\ X_4^2 + 1,2\ X_1X_3 + 1,49\ X_2X_3 + 1,67\ X_2X_4$

III.2.3. Response surface analysis

The effect of the independent variables and their cross-effect on the phenolic performance was observed by a three-dimensional response surface curve, which is shown in **Figure 18 (A-F)**. The response was plotted on the z-axis with respect to the two studied independent variables, while keeping the other two remaining independent variables at their zero levels (**Hayat *et al.*, 2009**).

Figure 18.A illustrates the interaction between ethanol concentration and extraction time on the yield of phenolic compounds from *Aloysia triphylla* leaves. This figure shows that increasing the extraction time from 150 s to 180 s induces an increase in extraction yield, to reach a maximum value of 56.3 mg GAE/ g DW with a 40 % ethanol concentration, while above 180 s there was a gradual decrease in response which reaches 46.2 mg GAE/ g DW at 210 s (60% ethanol). Indeed, long exposure to microwave radiation could induce the thermo-degradation of phenolic compounds **(Luo *et al.*, 2010; Dahmoune *et al.*, 2015)**. It is important to note that the extraction rate of TPC depends mainly on the extraction time as its linear and quadratic effects were very significant (<0.0001*) (**Table VI**). The parabolic trajectory of the response as a function of the extraction time for all the ethanol concentrations, microwave powers and ratios, shows its very significant effect (**Dahmoune *et al.*, 2015)**. TPC yield decreases with increasing ethanol concentration. These results corroborate with those of **Huang *et al.* (2009),** which showed that the increase in solvent concentration induces a change in polarity that would extract more impurities and induce a low TPC yield. On the other hand, the presence of an adequate amount of water in the solvent could swell the plant matrix and consequently increase the contact surface of this matrix with the solvent, leading to an increase in extraction yield **(Huang *et al.*, 2009)**. In addition, water allows overheating, which facilitates the absorption of microwave energy by the plant, inducing the destruction of plant cells and the release of phenolic compounds **(Proestos and Komaitis., 2008)**. This figure shows that the cross-effect of these two factors is not significant on TPC yield as shown in **Table VII**.

Figure 18.B illustrates the effect of microwave power and ethanol concentration on TPC extraction yield. The linear effect of the factors X_1 and X_3 as well as their cross-effect X_1X_3 is highly significant, which shows that power and ethanol concentration has a strong influence on the response. Indeed, the extraction rate, which was 53.8 mg GAE/ g DW at 40% ethanol and

400 W microwave power, decreases continuously with increasing ethanol concentration until it reaches 48.2 mg GAE/ g DW at 80 % at the same power. These results are consistent with those of **Luo *et al.* (2010)** and **Handa *et al.* (2008)**, who concluded that ethanol concentration has a strong effect on the dissolution of phenolic compounds, and that during microwave extraction, heat transmission is not efficient in apolar solvents, due to their low dielectric constant. Because of its high dielectric constant, water present in the solvent penetrates the plant cells and absorbs the microwave energy which induces a rapid overheating that destroys the plant cells, and facilitates desorption of chemicals from the matrix, allowing the release of active compounds and improving the extraction rate **(Wang and Weller., 2006)**.

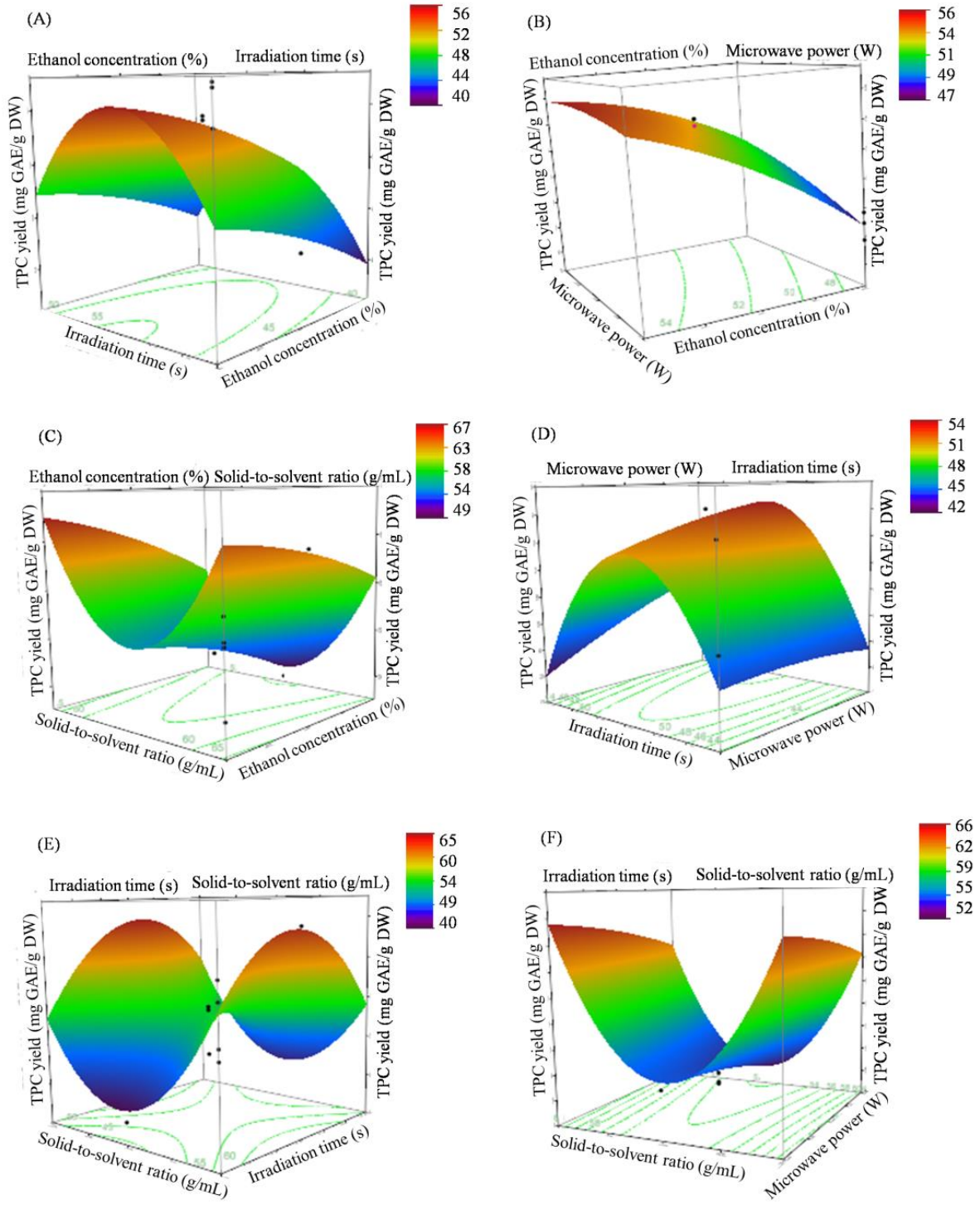

Figure18: Response surface plots for the effect of ethanol concentration and irradiation time (A); Ethanol concentration and microwave power (B); Ethanol concentration and solvent-to-solid ratio (C); Microwave power and irradiation time (D); Irradiation time and solvent-to-solid ratio (E) and Microwave power and solvent-to-solid ratio (F), on the TPC yield from *Aloysia triphylla* leaves obtained with MAE.

The effect of the interaction between the independent variables, ethanol concentration and solvent-to-solid ratio extraction is shown in **Figure 18.C**. As the ethanol concentration increases, the yield of TPC decreases slightly. The TPC yield decreases rapidly from 64.4 GAE/ g DW to 48.2 GAE/ g DW at a ratio of 30:1 (mL/g). Above this value, the response increases rapidly with increasing solvent-to-solid ratio to reach 63.8 GAE/ g DW at 40:1 (mL/g), which may be due to better solubility of phenolic compounds. Note that the interactive effect of ethanol concentration and solvent-to-solid ratio on TPC yield was insignificant.

Figure 18.D shows the interaction of the effect of extraction time (X_2) and microwave power (X_3) on the TPC extraction yield. The response increases from 52.9 mg GAE/g DW to 64.4 mg GAE/g DW when the irradiation time increases from 150 to 180 s and the microwave power increases from 200 W to 400 W, indicating good extraction conditions. The cross-effect of time and microwave power can be favorable to improving the extraction rate, by decreasing the viscosity of the solvent and the solubility of the phenolic compounds, thus accelerating the dissolution and release of TPC (**Dahmoune *et al.*, 2015**). Above these values, the TPC efficiency decreases rapidly as the irradiation time increases to the minimum value of 50.8 mg GAE/g DW (210 s, 200 W). Prolonged exposure to microwave radiation, leads to thermal degradation of total phenolic compounds (**Xiao *et al.*, 2008; Luo *et al.*, 2010**). It should be noted that the linear effects X_2, X_3 and the cross effect (X_2, X_3) are highly significant.

The linear effect of the extraction time (X_2), solvent- to-solid ratio (X_4) and their interactive effect X_2X_4 were highly significant (< 0.0001*). As shown in **Figure 18.E**, the TPC increased with increasing the extraction time from 150 to 180 s and the solvent-to-solid ratio from 30:1 to 40:1 mL/g to reach a maximum yield of 64.60 mg GAE/g DW. Beyond 180 s the response decreased rapidly to reach 42.7 mg GAE/g DW for a ratio of 40:1 mL/g.

The cross-effect of microwave power and solvent-to-solid ratio is shown in **Figure 18.F**. The extraction rate, which was maximal (64.40 mg GAE/g DW) at an extraction ratio of 20:1 mL/ g and a microwave power of 400 W, decreased significantly with increasing the ratio up to 30:1 mL/ g (53.8 mg GAE/g DW). Above this value, the response increased quickly with increasing the solvent-to-solid ratio to 40:1 mL/g at a microwave power of 400 W. The extraction rate decreased slightly with increasing the microwave power.

III.2.4. Optimal extraction conditions and model validation

The results obtained by the RSM as the predicted values of optimal conditions under microwave irradiation were: 40 % (V/V), 188 s, 600 W, 40:1 mL/g, for ethanol concentration, irradiation time, microwave power, and solvent-to solid ratio respectively **(figure 19)**. The predicted extraction rate under the above conditions was 67.87±1.61 mg GAE/g DW; this value is very significantly concordant with the extraction rate of phenolic compounds calculated experimentally under the same optimal conditions which was 67.86±0.92 mg GAE/g DW. These results allowed the validation of the regression model developed in the optimization of extraction conditions **(Zhang *et al.*, 2013)**. It is very important to note that the irradiation time was consistent with the single-factor tests because the level of phenolic compounds reached a maximum when this variable was at an average level (values coded 0). The other variables, ethanol concentration, microwave power, and solvent-to-solid ratio, showed a lower influence, which is inconsistent with the single-factor experiments.

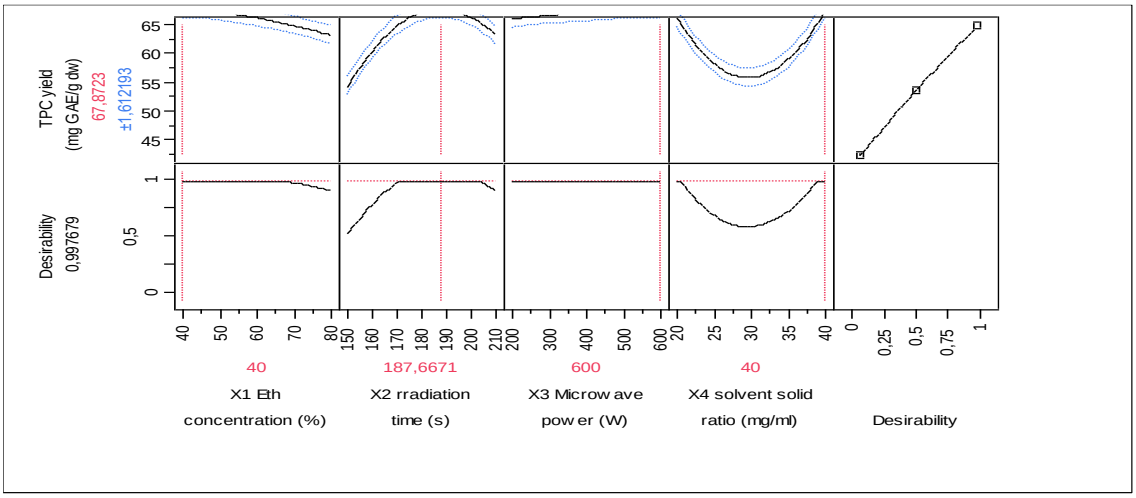

Figure 19: Optimal conditions for microwave extraction of phenol compounds from *Aloysia triphylla* leaves

The **figure 20** shows the relationship between the actual and predicted values of TPC yield of *Aloysia triphylla*. It also indicates that the developed model is adequate and the predicted results are in good agreement with the measured data.

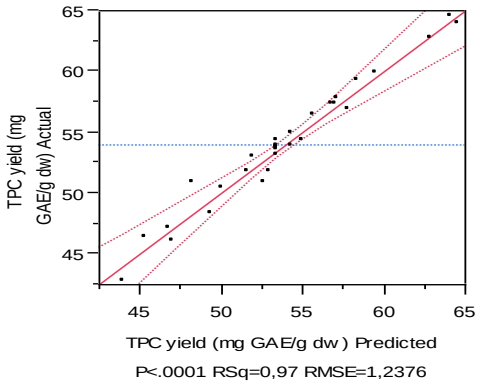

Figure 20: Comparison of the experimental and predicted TPC values of the optimized

IV. Spectrophotometric determination of optimized and conventional extracts

IV.1. Phenolic compound content

Under optimal conditions, MAE gave a TPC yield of 67.86±0.92$_a$ mg GAE / g DW) (188 s) (**Figure 19**). This value is significantly higher ($P < 0.001$) than the yield obtained with conventional extraction, which was 34.55±0.90$_b$ mg GAE / g DW (3 days).

To the best of our knowledge, there are more studies on the essential oils composition and their biological activities (**Bahramsoltani *et al.*, 2018; Djadouni, 2020; Pérez Zamora *et al.*, 2018; Bekara *et al.*, 2020; Mashayekhi-sardoo *et al.*, 2020; Sandner *et al.*, 2020**), than on the phenolic compounds of the studied plant. It should also be noted that the works carried out on its phenolic compounds were focused on their characterization (**Quirantes-Piné *et al.*, 2009; Quirantes-Piné *et al.*, 2010**) as well as on the biological activity of its different extracts or beverages (**Moshari-Nasirkandi *et al.*, 2020; Sandner *et al.*, 2020**). Indeed, few studies were conducted on TPC and TFC on the studied plant. Therefore, we tried to compare our results with those reported in previous works on this plant, but unfortunately the data were different. This could be due to uncontrolled external factors: agro-climatic conditions, soil composition, harvesting periods, and to controlled factors such as the extraction methods and conditions. **Zheng and Wang. (2001)** reported a content of 1.55±0.1 mg GAE/g of fresh weight from phosphate buffer extract. However, **Yoo *et al.* (2008)** found higher values of 770.7±2.2 mg GAE/100 g, and 431.60±1.42 mg catechin equivalents/100 g of fresh weight in the hydro-

methanolic extract. **Dadé *et al.* (2009)** reported 1.70±0.19 µmol of caffeic acid equivalent/mg dry matter and 0.50±0.04 µmol of rutin equivalent/mg dry matter from an infusion extract. Recently **Jalal *et al.* (2019)** obtained 0.86 mg of GAE/mg dry matter and 312.9 mg of rutin equivalent per 100 g of dry matter from an ethanolic extract.

Rafiee *et al.* (2011), had proved that ethanol was the most efficient extraction solvent, producing the highest extraction yield compared to other solvents and the highest phenolic concentration was obtained at 24 h (69.027 mg TAE/ g) with maceration extraction and at 15 min (88.298 mg TAE/ g) of exposition during MAE.. Therefore, MAE had a higher extraction yield compared with the maceration method (**Figure 21 (A)**).

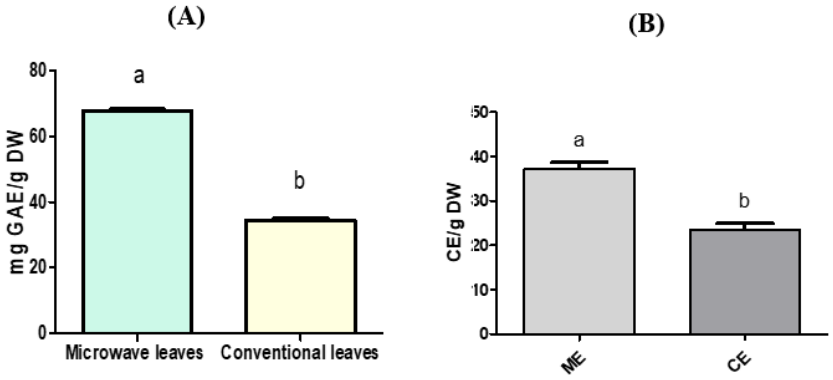

Figure 21: TPC **(A)** and flavonoids **(B)** content of microwave extract **(ME),** and conventional extract **(CE)**

IV.2. Flavonoids content

Flavonoids are molecules with various biological activities, especially for their antioxidant properties (**Fuhrman *et al.*, 1995**). The action of flavonoids is not only limited to scavenging and inhibiting free radicals, but it also participates in neutralizing enzymes responsible for the production of free radicals such as xanthine oxidase which is a biological source of the superoxide radical (**Cotelle, 2001**).

The flavonoid obtained with MAE extract was 37.27±1.62 mg_a CE/g DW, which was statistically different ($P<0.001$), then the flavonoid content of the conventional extract which was 23.25±1.47$_b$ mg CE/g DW (**Figure 21 (B)**). This shows the better efficiency of MAE compared to conventional extraction, adding to time that is much reduced in MAE process. These results corroborate with those of**,** **Dahmoun *et al.* (2013)**, **Bouras *et al.* (2015)**. This

higher content of polyphenols and flavonoids obtained with microwave extraction compared to conventional extraction is due to the effect of microwave waves on the plant matrix.

Indeed, MAE guarantees a rapid transfer of energy from the solvent to the plant matrix, inducing rapid and homogeneous heating improving TPC recovery (**Lefsih *et al.*, 2017**). On the other hand, a long extraction time may result in the degradation or conversion of the analytes (**Hayat *et al.*, 2009; Guemghar *et al.*, 2020**). As the water in the plant matrix absorbs the microwave energy, the destruction of cells is promoted by internal overheating, which facilitates the disintegration of biomaterials, improving their recovery (**Eskilsson and Bjorklund, 2000; Kaufmann and Christen, 2002**).

IV.3. Condensed tannins content

The tanoids, which are phenolic compounds, are produced by plants in response to environmental stress caused by various environmental factors: nutrient deficiency, drought, overheating and light intensity (**Leinmüller *et al.*, 1991**). Their concentration varies considerably between different plant species and within the same species where it depends on the degree of maturity, age of leaves and season (**Skadhauge *et al.*, 2004**).

The determination of tannins was carried out by the n-Butanol / HCl method adapted by **Vermerris and Nicholson., (2007)**. Cyanidine was used as a standard reference, and tannin content was expressed as mg cyanidine equivalent /100 g dry weight. The tannin content of optimized extract of *Aloysia triphylla* was 1.826 ± 0.402 mg cyanidine / 100 g dry weight. The qualitative studies carried out by **Kalita *et al.* (2011)** on the aqueous extract of *Lantana Camara* L. leaves. (Verbenaceae) and by **Karmakar *et al.* (2011)** on the leaf extract of *Premna integrifolia* Linn (Verbenaceae) showed the presence of tannins. The tannin content of *Vitex mollis* fruits (Verbenaceae) evaluated by **Montiel-Herrera *et al.* (2004)**, was 0.127 ± 0.011 catechine Eq g / 100g of Ms.

V. Identification of phenolic compounds by LC-DAD-ESI-MS/MS

A more accurate molecular characterization was performed by using a High performance liquid chromatography, coupled with a diode array detector and electrospray ionization tandem mass spectrometry (HPLC -DAD-ESI-MS/MS) method, to detect each polyphenolic compound. The great difference between this technology based on MS and the colorimetric assays is related to its targeted nature, which allows the identification of polyphenols not based on their total content but on specific fragmentation reactions for each molecule (**Illiano *et al.*, 2022**).

Data of phenolic compounds identification by HPLC-DAD-ESI-MS/MS were recorded in the negative and positive ionization mode (retention time, ƛ max, deprotonated molecules m/z values), to obtain maximum information on the composition of the different extracts of *Aloysia triphylla*. The low-energy collision induced dissociation tandem mass spectrometric (CID) fragmentation pathways analysis, and tentative product ions identification are presented in **Tables VIII** and **IX**. The analysis of the data were performed using MassLynx Software.

The chromatograms obtained for the different extracts are complex, but the HPLC method used allowed a good separation of the compounds present in the extracts and there was no variation observed in the duplicate analysis. Ionization conditions in the mass spectrometer were optimized in order to detect the m/z corresponding to the precursor ion. When the ionization conditions are adequate, this m/z value corresponds to the most intense peak in the mass spectra (base peak). However, often this situation is difficult to occur for all the compounds detected and some fragmentation at the ion source is responsible for a decrease in the signal corresponding to the precursor ion, making more difficult the study of the MS spectra. Analyses were performed in SCAN mode and fragmentation experiments (MS/MS) were also conducted.

Phenolics were monitored by a diode array detector set at 4 wavelengths (280 nm, 320 nm, 360 nm and 520 nm).The chromatograms of the conventional and microwave extracts of *Aloysia triphylla* leaves at these different wavelengths, show very similar peaks, as shown in the figures **4,5,6,7 (annexe 2).** However, the chromatographic profile of microwave and conventional extracts are represented by a common chromatogramme.

The comparison of the UV spectra of compounds detected showed that they could be arranged into several families presenting a characteristic spectrum. Phenolic acids and flavonoids show characteristic UV-range absorbance patterns from 190 to 380 nm (**Ma *et al.*, 2014**). Hydroxycinnamic acids exhibit UV absorption maxima at 320 nm and flavonoids exhibit a typical UV spectrum between 300 and 380 nm range (**Kheyar *et al.*, 2018**). UV-range absorbance for flavones was between 310 and 350 nm, while for flavonols it is between 350 and 385 nm. Flavanones show a characteristic UV spectrum with in the 300-330 nm range in the main peak, and flavanols show maximum UV spectra at 280 nm (**Munekata *et al.*, 2016**).

V.1. Characterization with the negative ionization mode

Table VIII shows a list of 30 compounds tentatively identified through LC–DAD–ESI-MS/MS experiments in negative mode along with their retention time (t_R), UV absorption maxima,

precursor ion, the MS/MS product ions and the bibliographic references to support the identification. These compounds were numbered according to their elution order.

Table VIII: Characterization of phenolic compounds in the *Aloysia triphylla* conventional and MAE extracts by LC–DAD–ESI–MS/MS in negative ionization mode

Peak	t_R (min)	λ max (nm)	[M-H]- m/z	HPLC–DAD–ESI MS/MS *m/z* (% base peak)	Tentative identification	Compound Group	References
1	8.58	228	133	**115 (100)**, 71(61)	Malic acid ($C_9H_8O_4$)	Phenolic acids (Hydroxybutanedioic acids)	Al-Rawahi *et al.*, 2014
2	12.13	228	117	**73(100),** 99(25)	succinic acid ($C_4H_6O_4$)	Phenolic acids	Mass bank
3	20.96	230	391	**123 (100),** 167 (60), 229(28), 211(4), 149(7), 141(32)	Shanzhiside (C_{16} H_{24} O_{11})	Iridoid glycosides	Quirantes-Piné *et al.*, 2010
4	26.55	230	373	**123(100),** 149(95), 167(27), 193(4), 211(6)	Gardoside (C_{16} H_{22} O_{10})	Iridoids	Quirantes-Piné *et al.*, 2009
5	26.85	230	461	**161 (100),** 135 (5)	Verbasoside (C_{20} H_{30} O_{12})	Phenylethanoid compounds	Quirantes-Piné *et al.*, 2010; Abdel-Hady *et al.*, 2018
6	30.08	280	375	**151 (100),**213 (50),169 (40), 125 (35), 95 (30), 195 (15)	Loganic acid ($C_{16}H_{24}O_{10}$)	Iridoids	Cao *et al.*, 2011 Kucharska and Fecka, 2016
7	32.94	228, 330	179	**135 (100) [M-H COOH],** 79 (45), 163 (42), 117 (40), 101 (40), 108 (27)	cafeic acid ($C_9H_8O_4$)	Phenolic acids	Abdelaziz *et al.*, 2020
8	33.00	230, 330	487	**179 (100),** 161 (20), 135 (9),	Cistanoside F (C_{21} H_{28} O_{13})	Phenylethanoid compounds	Quirantes-Piné *et al.*, 2009; Quirantes-Piné *et al.*, 2010; Abdel-Hady *et al.*, 2018
9	33.26	230, 330	389	**371(100),**212 (6), 209 (5), 179 (1)	Theveside ($C_{16}H_{22}O_{11}$)	Iridoid glucosides	Quirantes-Piné *et al.*, 2010; Quirantes-Piné *et al.*,2009
10	40.18	280,329	387	**185 (100),** 101 (25), 207 (32), 163 (22), 341 (6)	12-hydroxyjasmonic acid glucoside (C_{18} H_{28} O_9)	fatty acid glucoside	Marzouk *et al.*, 2018 ;
11	43.20	230, 330	639	**621 (100),** 179 (77), 161 (75), 459(18), 529(1)	hydroxyverbascoside ($C_{29}H_{36}O_{16}$)	Phenylethanoid compounds	Quirantes-Piné *et al.*, 2009
12	43.99	230, 275, 346	637	**351 (100),** 285 (54), 237 (15), 113 (10), 425 (9), 132 (7), 509 (6), 251 (6)	Luteolin-7-diglucuronide ($C_{27}H_{26}O_{18}$)	Flavones	Quirantes-Piné *et al.*, 2009 ; Pereira *et al.*, 2018
13	44.39	329	637	**351(100),** 285(61),281(36),100(30), 175(18)	Eukovoside ($C_{31}H_{38}O_{16}$)	Phenylpropanoid compounds	Smiljković *et al.*, 2018
14	45.51	230, 267, 332	463	**301 (100),** 138 (67), 431 (54), 333(45),257(37), 89 (15)	Quercetin-3-O-glucoside ($C_{21}H_{19}O_{12}$)	Flavonols	Hao *et al.*, 2021
15	46.43	231, 267, 332	621	**351 (100),** 113 (44**)**, 193 (40)	Apigenin-7-diglucuronide ($C_{25}H_{27}O_{17}$)	Flavones	Quirantes-Piné *et al.*, 2009
16	47.61	230, 335	651	**351 (100), 113 (100),**167(34), 324(30), 358 (6)	Chrysoeriol-7-diglucuronide (C_{28} $H_{28}O_{18}$)	Flavones	Quirantes-Piné *et al.*, 2009
17	48.07	329	637	175(78), 324(36), 263(32), 461(20), 161(13) ,193(10)	Cistanoside C (C_{30} $H_{38}O_{15}$)	Phenylethanoid compounds	Han *et al.*, 2012 ; Ma *et al.*, 2014 Abdel-Hady *et al.*, 2018
18	48.1	231, 260, 332	447	**285 (100),** 285(20), 259(18)	Astragalin ($C_{21}H_{20}O_{11}$)	flavonols	Hao *et al.*, 2021

19	48.30	230, 330	623	**161 (100),** 461 (25)	Glycosylated verbascoside ($C_{29}H_{35}O_{15}$)	Phenylethanoids compounds	Quirantes-Piné *et al.*, 2009; Quirantes-Piné *et al.*, 2010
20	48.50	231, 338	477	**285 (100),** 315 (14)	Derhamnosylverbas-coside ($C_{23}H_{26}O_{11}$)	Phenylethanoids compounds	Abderrahim *et al.*, 2011
21	48.76	231, 280, 338	461	**285** (100), 193 (11), 113 (10), 118(63)	Scutellarin ($C_{21}H_{18}O_{12}$)	flavones	Hao *et al.*, 2021
22	51.37	*338,346*	445	**269(100),** 113(22), 104(38), 85(35)	Baicalin ($C_{21}H_{18}O_{11}$)	Flavones	Baali *et al.*, 2022
23	52.13	231, 332	635	**351 (90)**	Acacetin-7-diglucoronide ($C_{28}H_{28}O_{17}$)	Flavones	Quirantes-Piné *et al.*, 2010
24	59.70	341	315	**300(100),** 185(10), 181(10)	Nepetin ($C_{16}H_{12}O_7$)	Flavones	Kheyar-Kraouche *et al.*, 2018
25	59.78	229, 275, 338	285	**133 (90),**151(15)	Luteolin ($C_{15}H_9O_6$)	Flavones	Shi *et al.*, 2013 Aguado *et al.*, 2016
26	59.92	229, 275, 338	315	**300 (100),** 181(10),185(10)	isorhamnetin ($C_{16}H_{12}O_7$)	Flavonols	Aguado *et al.*, 2016
27	68.59	230, 267, 338	269	**151 (100),**117(75), 149(60), 225(35), 107 (55)	Apigenin ($C_{15}H_{10}O_5$)	Flavones	Hao *et al.*, 2021
28	71.76	275, 330	299	**284 (100)**	Diosmetin ($C_{16}H_{12}O_6$)	Flavones	Kheyar-Kraouche *et al.*, 2018
29	86.42	275, 330	283	**268 (100)**	genkwanin ($C_{16}H_{12}O_5$)	Flavones	Borrás-Linares *et al.*, 2014
30	97.05	329,334	339	121(100),179(83)/148(60), 267(60), 151 (37)/311(12)	Methylophiopogono-ne A ($C_{19}H_{16}O_6$)	Homoisoflavonoid	Lin *et al.*, 2010; Wang *et al.*, 2017

V.1.1. Phenolic acids

Most of the compounds found in this study have been previously described in *Aloysia triphylla* leaves, and which are well-known compounds of this plant. The phenolic acids identified are malic acid, succinic acid and caffeic acid, which exhibit ƛ max between 228 and 338 nm.

Compound 1 (t_R = 8.58 min), **2** (t_R = 12.13 min) with [M-H]- ions at m/z 133 and 117 were identified as malic (**Figure 22**) and succinic acids (**Figure 23**), respectively, according to their product ions (**Al-Rawahi *et al.*, 2014; Mass bank**). As a result of LC-MS/MS analysis for malic acid, the loss of water [M-H-H2O]- provided an ion at m/z 115 with the loss of CO_2, and an intense ion at m/z 71 (**Baskaran *et al.*, 2016; Yilmaz *et al.*, 2022)**. Succinic acid gave a molecular ion at m/z 117 and MS^2 analyses showed the presence of the two main fragment ions at m/z 73 and at m/z 99 corresponding to the loss of a carboxylic group and a water molecule, respectively (**Papetti *et al.*, 2017**). Succinic acid is previously observed in Verbenaceae plants like *Caryopteris odorata* leaves (**Joshi *et al.*, 2019**); *Clerodendrum bungei* (**Kasmaei *et al.*, 2022**), but it is being proposed for the first time in *Aloysia triphylla* leaves.

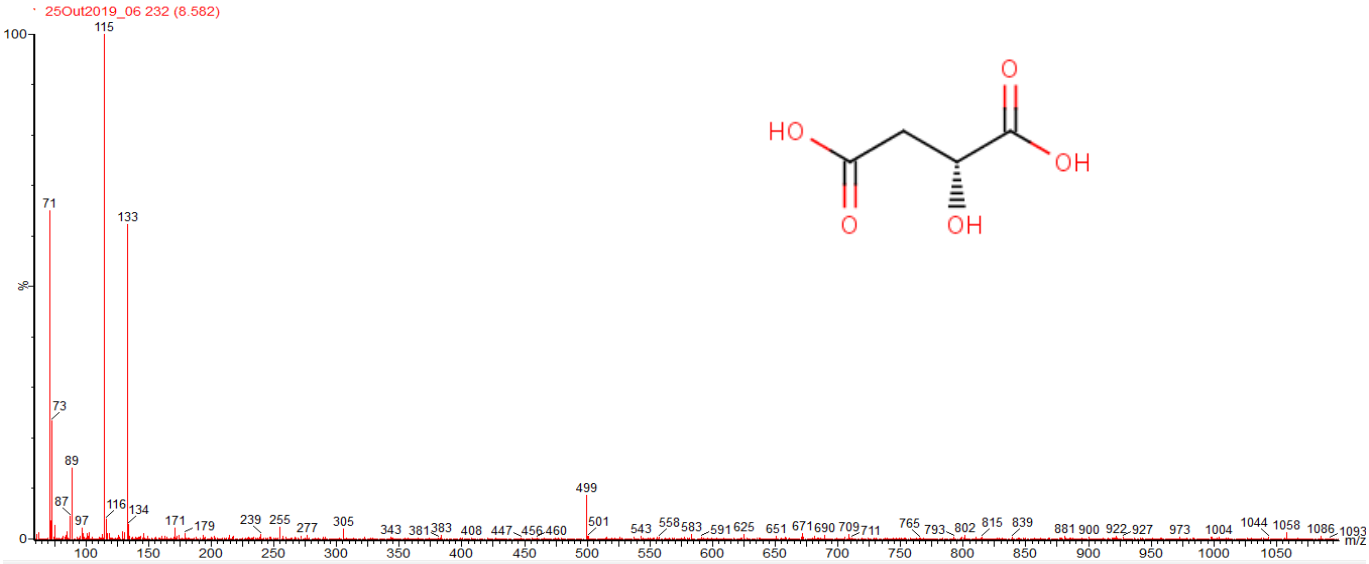

Figure 22: ESI-MS/MS spectra of malic acid

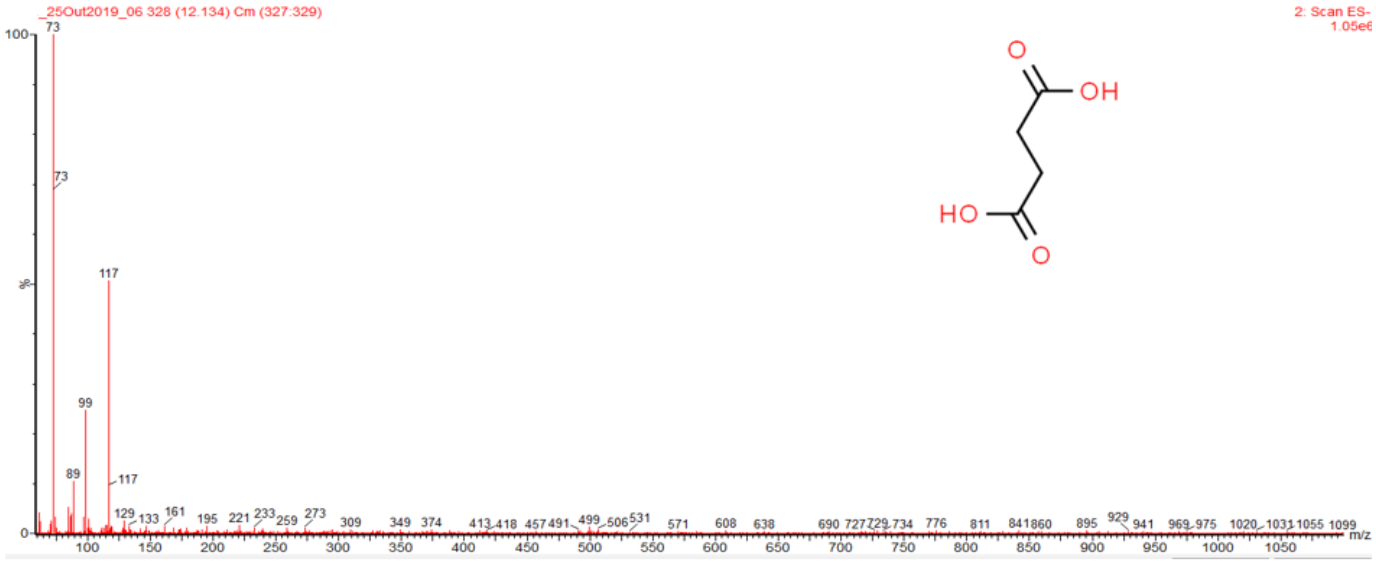

Figure 23: ESI-MS/MS spectra of succinic acid

Compound 7 with a precursor ion [M-H] - at m/z 179 has been tentatively assigned as caffeic acid **(Figure 24)**, in the MS/MS spectra, the product ion at m/z 135 was detected. The ion m/z 135 is the decarboxylated product from the caffeate ion [M-H-44]- which is the precursor ion of caffeic acid (**Tang and Sojinu, 2012; Kheyar-Kerouche *et al.*, 2018**). This compound is being proposed in areal part of *Aloysia triphylla* (**Pereira *et al.*, 2017; Hosseini *et al.*, 2021; Cheurfa *et al.*, 2022**). Caffeic acid and its phenethyl ester are naturally occurring hydroxycinnamic acids with an interesting array of biological activities; like antioxidant, anti-inflammatory, antimicrobial, antiviral, cytostatic. Their antioxidant, anti-inflammatory and anti-angiogenic properties contribute to an important anti-atherosclerotic effect, and protect tissues against ischemia/ reperfusion injuries and the cellular dysfunction caused by different physicochemical agents (**Silva and Lopes, 2020**).

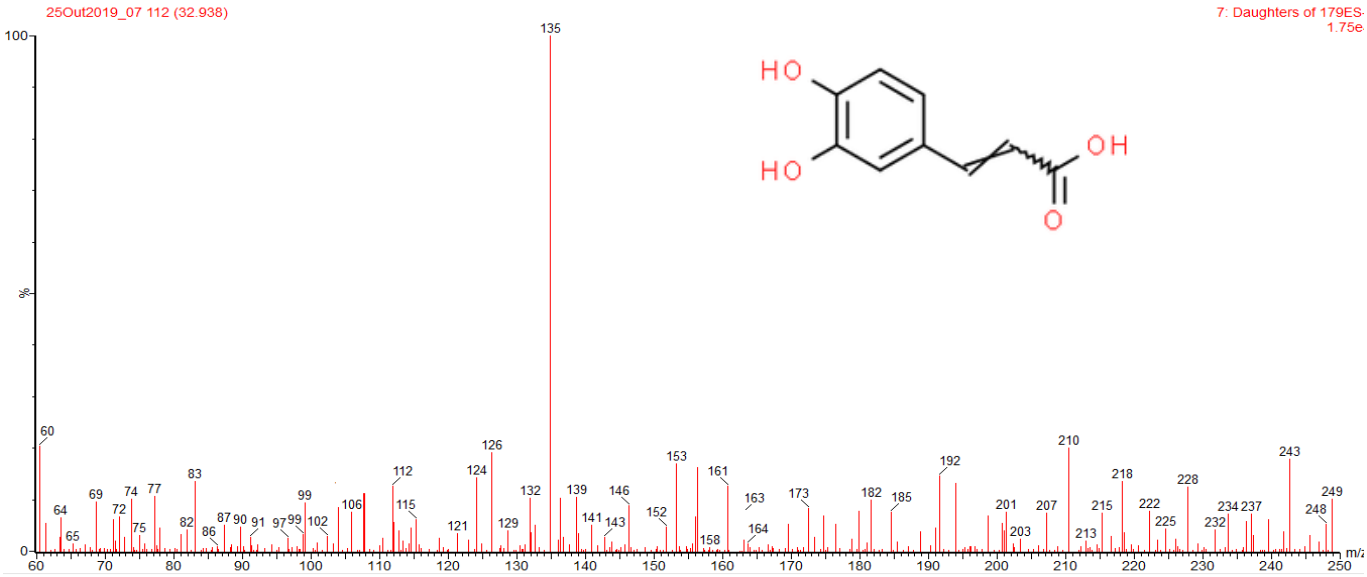

Figure 24: ESI-MS/MS spectra of caffeic acid

V.1.2. Iridoid compounds

Compound 3 with molecular ion peak at m/z 391 [M-H] was identified as shanzhiside (**Figure 26**), its MS/MS spectrum showed fragments at m/z 229, 211, 167, 123, 167, 149 and 141 corresponding to [M-H-glucose]- (229) and the successive losses of water (211), as well as subsequent decarboxylation (167) and dehydration (149) from fragment 211. Another fragment was found at m/z 141, corresponding to [M-glucose-88]-, which was obtained by the loss of the

3-oxopropanic acid molecule (**Quirantes-Pine´ *et al.*, 2010),** according to the fragmentation pathway shown in **Figure 25**.

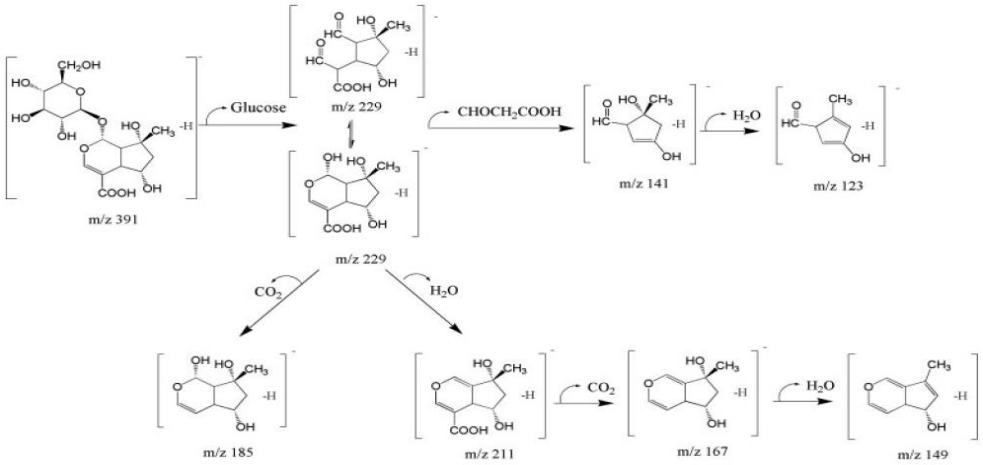

Figure25: Proposed fragmentation pathway for shanzhiside **(Quirantes-Pine´ *et al.*, 2010)**

This compound has been previously reported in other species from genus Lippia, like *Lantana camara* L. and *Lantana montevidensis* (**de Sousa *et al.*, 2018**), *Aloysia polystachya* (Griseb.), and in *Aloysia triphylla* (**Mohammad hosseini *et al.*, 2021**).

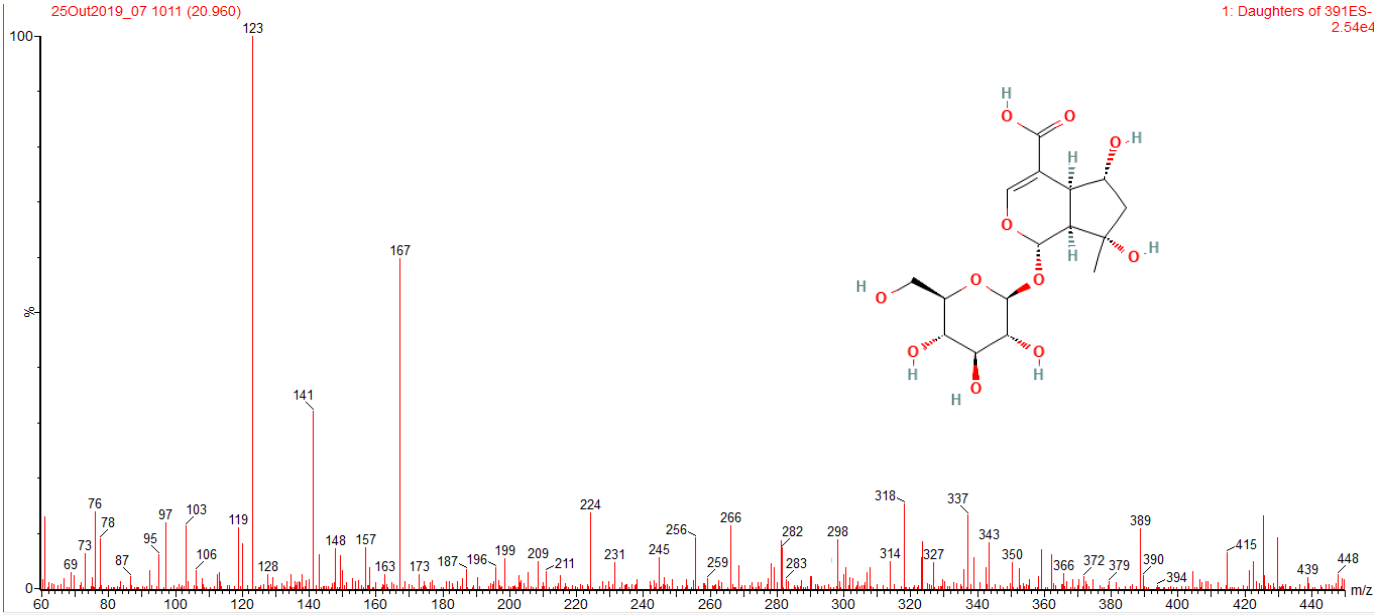

Figure 26: ESI-MS/MS spectra of Shanzhiside

Compound 4 was identified as gardoside taking into account the molecular formula provided for its accurate mass. This iridoid glycoside has been reported previously in other species from Verbenaceae family (*Lippia alba* (Mill.)) (**Filho *et al.*, 2007**). MS^2 spectrum of this compound showed fragments at m/z 211, 123, 193, 167 and 149, corresponding to [M-glucose]- (211) and the successive losses of water (193) and CO_2 (167), respectively, as well as the simultaneous elimination of water and CO_2 (149). The main fragment was found at m/z 123, corresponding to [M-glucose-88]-, which was obtained by the loss of the 3-oxopropanic acid molecule according to the fragmentation pathway shown in **Figure 27**.

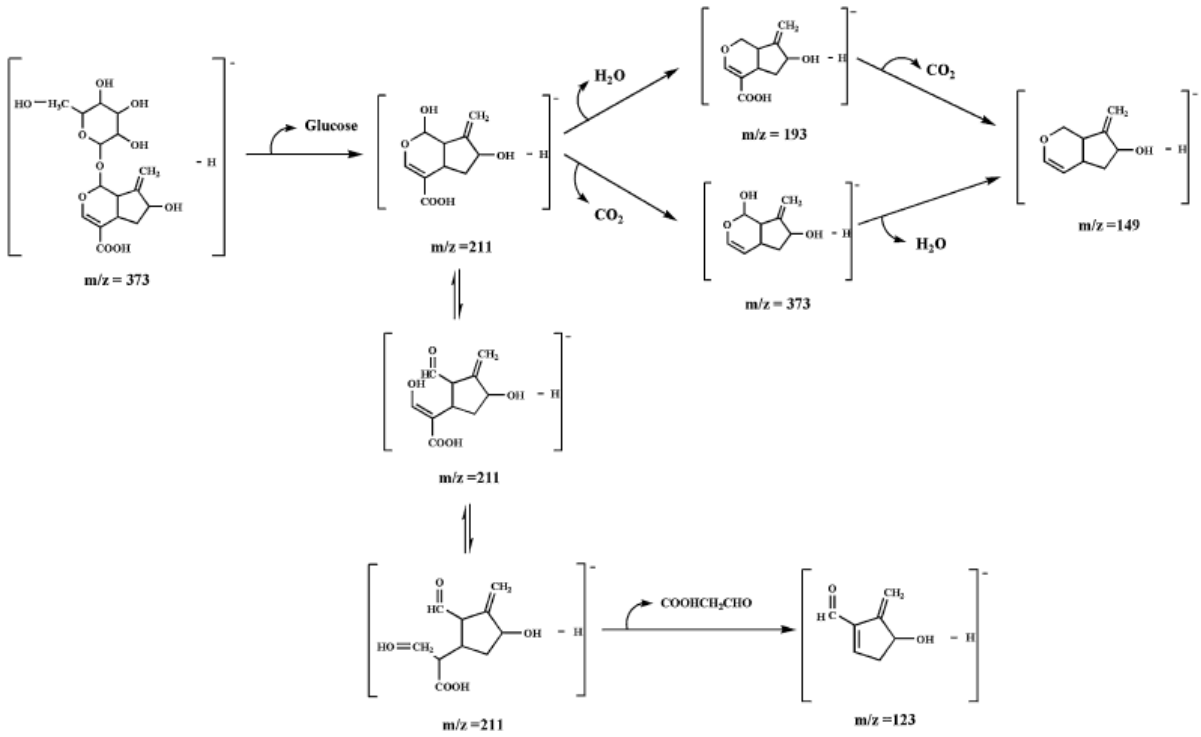

Figure 27: Proposed fragmentation pathway for gardoside (**Quirantes-Pine´ *et al.*, 2009**)

These fragments were also similar to previous data reported for other iridoid glycosides (**Quirantes-Pine´ *et al.*, 2009**). Gardoside (**Figure 28**) was previously reported in the genus Lippia like *Lippia alba* (**Ombito *et al.*, 2014**), and *Aloysia triphylla* (**Quirantes-Pine´ *et al.*, 2009; Sánchez-Marzo *et al.*, 2019).**

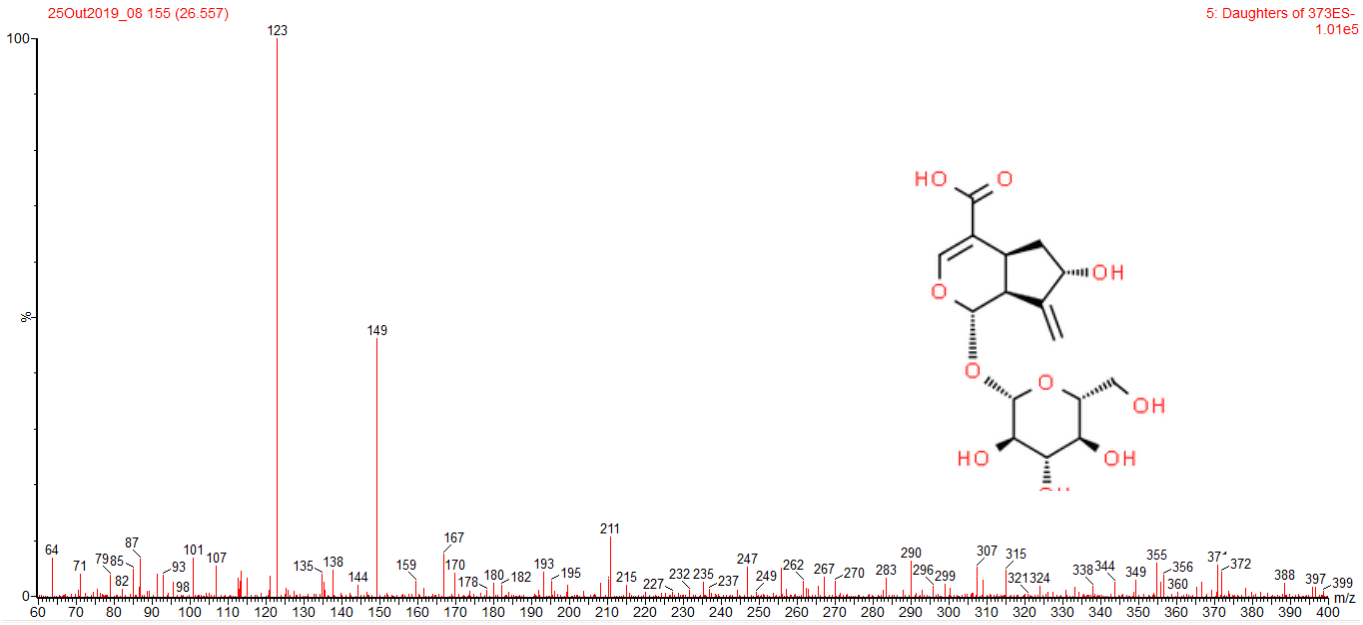

Figure 28: ESI-MS/MS spectra of gardoside

Compound 6 (m/z 375) was identified as loganic acid (**Figure 29**), it is an iridoid glycoside with a product ion at m/z 151 ([M-162-18-44-H]-), 213 ([M-162-H]-),169 ([M-162-44-H]-), 125 ([M-162-18-44-26-H]-), 95, 195 ([M-162-18-H]-) (**Kucharska and Fecka, 2016; Dzydzan *et al.*, 2020**). This compound had been previously reported in *Aloysia triphylla* (**Sánchez-Marzo *et al.*, 2019**) and in other plants like of *Cornus officinalis Sieb.* and *Lonicera caerulea* L. var. *kamtschatica* Sevast. (**Cao *et al.*, 2011; Kucharska and Fecka, 2016**). Loganic acid has strong free radical scavenging activity and remarkable cytoprotective effect against heavy metal mediated toxicity through the mechanism of inhibiting ROS generation. This compound showed remarkable antioxidant activity DPPH scavenging, superoxide radical scavenging and hydroxyl radical scavenging (**Abirami *et al.*, 2022**).

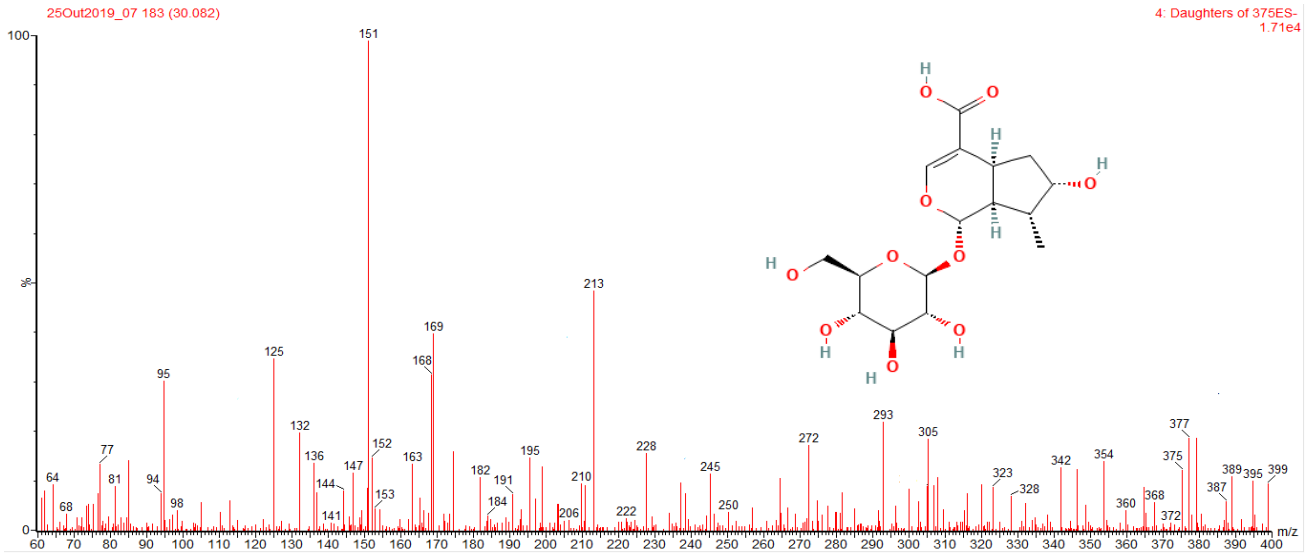

Figure 29: ESI-MS/MS spectra of loganic acid

Compound 9 was tentatively identified as theveside, another iridoid glycoside. This compound presented fragments at m/z 371 and 209 (**Figure 30**) which were consistent with the loss of water, and glucose moiety, respectively. The fragment at m/z 121 corresponded to the elimination of the 3-oxopropanic acid molecule from the fragment [M-glucose]- according to the fragmentation pathway previously described for gardoside. Both compounds showed an UV absorbance band at 230 nm which is characteristic of these iridoid glycosides (**Quirantes-Pine´ *et al.*, 2009; Abdel-Hady *et al.*, 2018**). Theveside is present in *Lippia alba* (**Hennebelle *et al.*, 2008**), and it is also present in *Aloysia triphylla* arial parts (**Quirantes-Pine´ *et al.*, 2009; Prado *et al.*, 2014**).

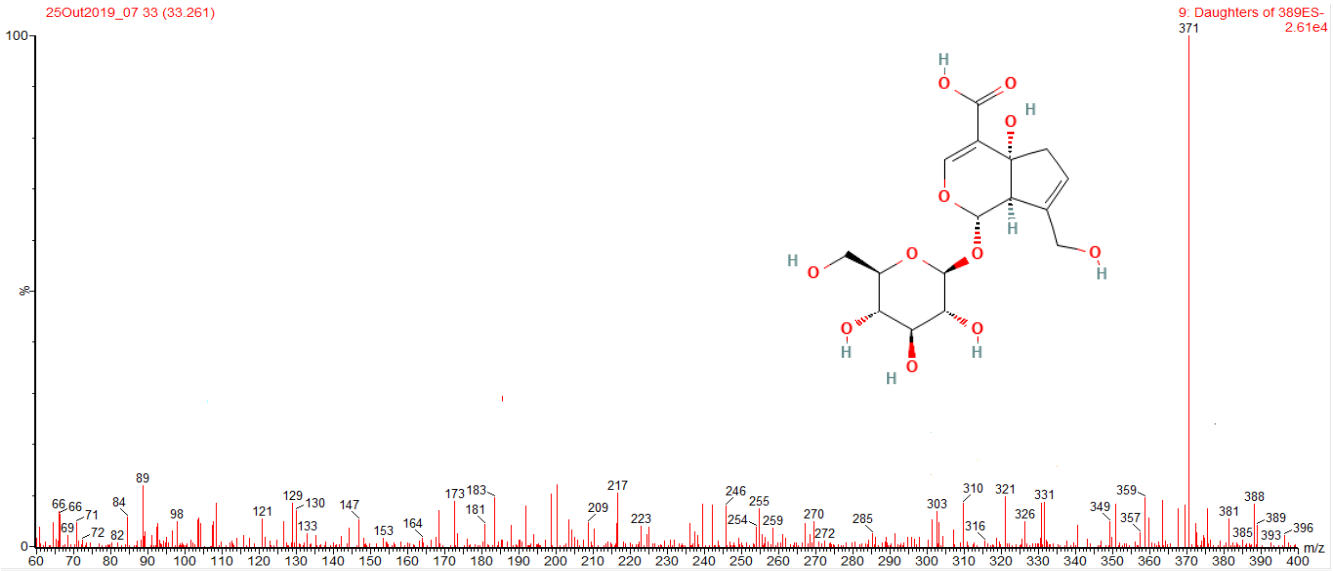

Figure 30: ESI-MS/MS spectra of theveside

V.1.3. Phenylethanoids compounds

TThe **compound 5** (t_R = 26.85 min) with [M-H]- ion at m/z 461 and a product ions at m/z 161/ 135 was identified as verbascoside (acteoside) (**Figure 31**), also known as decaffeoyl verbascoside. The ion at m/z 161 was consistent with deoxyhexose group and the fragment at m/z 135 represented the hydroxytyrosol moiety after loss of water (**Quirantes-Pine´ *et al.*, 2009**). This compound has been detected previously in Lippia genus (**Sánchez-Marzo *et al.*, 2019; Tammar *et al.*, 2021**). Verbascoside has been well reported in Verbenaceae family, such as *Verbena Carolina* (**Lara-Issasi *et al.*, 2019**); *Aloysia polystachya* (**Ortiz *et al.*, 2022**); *Lippia scaberrima* (**Maroyi *et al.*, 2019**); *Lippia multiflora* (**Masunda *et al.*, 2020**). Its presence was also reported in *Aloysia triphylla* (**Quirantes-Pine´ *et al.*, 2010; Pereira *et al.*, 2017; Sánchez-Marzo *et al.*, 2019**). It is the most abundant phenylpropanoid (97%) found in *Aloysia triphylla* leaves (**Razavi et al., 2017**).

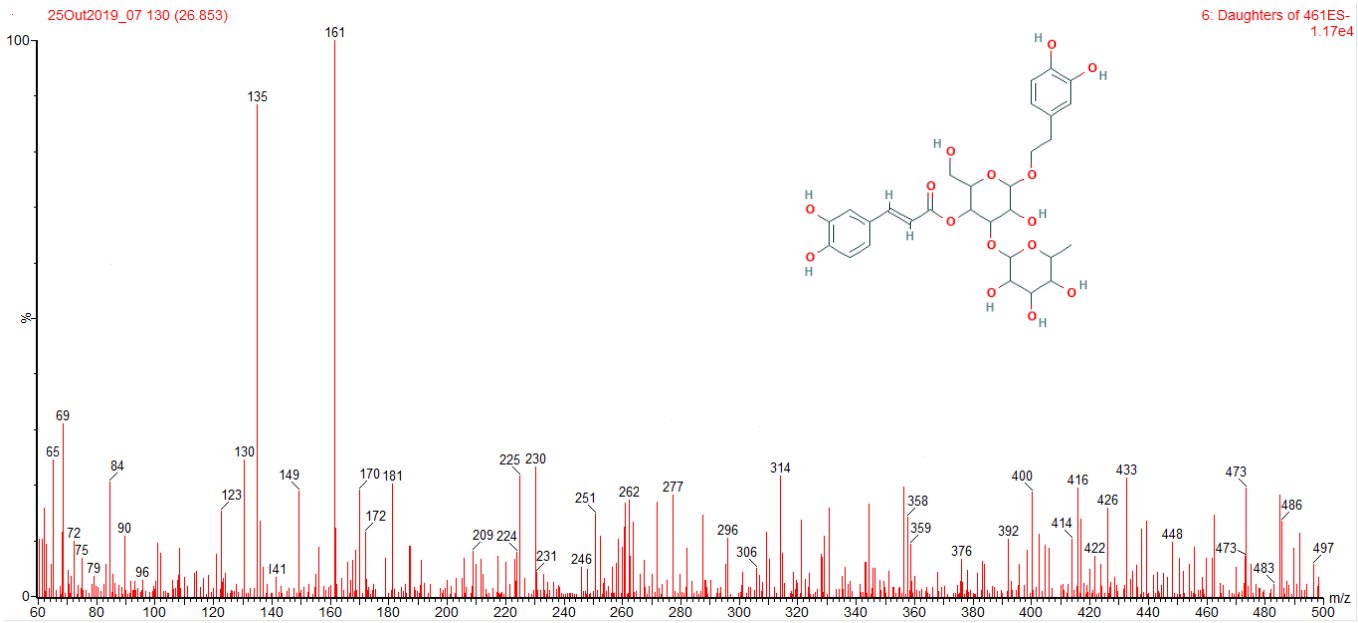

Figure 31: ESI-MS/MS spectra of verbascoside

Phenylethanoid-phenylpropanoid glycosides (PPGs) are widely distributed compounds in the plants kingdom. Most of them isolated from medicinal plants, are soluble in water and organic solvents. Attention has been growing about verbascoside, due to the high number of literatures that define its evident part in the prophylaxis and treatment of different disorders and diseases (**Khalaf *et al.*, 2021**). Indeed, verbascosides possesses different bioactivities including neuroprotection (**Wei *et al.*, 2019**), antidiabetic (**Boudjelal *et al.*, 2012**), anticancer (**Chen *et al.*, 2013**), anti-inflammatory, anti- androgen, antimicrobial (**Bazza *et al.*, 2018**) and antioxidant activities (**Rehecho *et al.*, 2011**). Verbascoside exhibited a higher capacity to scavenge free radicals within a hydrophobic environment than other antioxidants, such as hydroxytyrosol and caffeic acid. Furthermore, this phenylpropanoid was much stronger than these compounds and as potent as quercetin in inhibiting lipid peroxidation (**Funes *et al.*, 2009**). A fact that may related to its affinity for phospholipid membranes (**Funes *et al.*, 2010**; **Vertuani *et al.*, 2011**). Toxicity studies on verbascosides showed its high level of safety as oral LD_{50} less than 2000 mg/kg (**Etemad *et al.*, 2015**).

Cistanoside F **(Compound 8)** exhibited a pseudo-molecular ion [M-H]- at m/z 487, as shown in the **figure 32**, other fragment ions were appeared at m/z 179 (100%) [M-H-caffeoyl-rhamnosyl]-, m/z 161[M-H- caffeoyl- rhamnosyl- H2O]- and m/z 135 [caffeic acid-CO_2] (**Shi *et al.*, 2013; Ado *et al.*, 2016; Abdel-Hady *et al.*, 2018**). Cistanoside F was proposed as structure taking into account the molecular formula provided, the presence of caffeic acid in the structure and the naturally occurrence of this compound in *Lippia alba* (**Quirantes-Pine´ *et al.*, 2009; Hennebelle *et al.*, 2008)**. This compound has a vasorelaxant activity (**Ono *et al.*, 2008)**, as well as radical scavenging capacity against DPPH and Ferric-reducing power, acetylcholinesterase, α-glucosidase and tyrosinase inhibitory activities (**Ado *et al.*, 2016**). It was previously identified in *Aloysia triphylla* extracts (**Sánchez-Marzo *et al.*, 2019**).

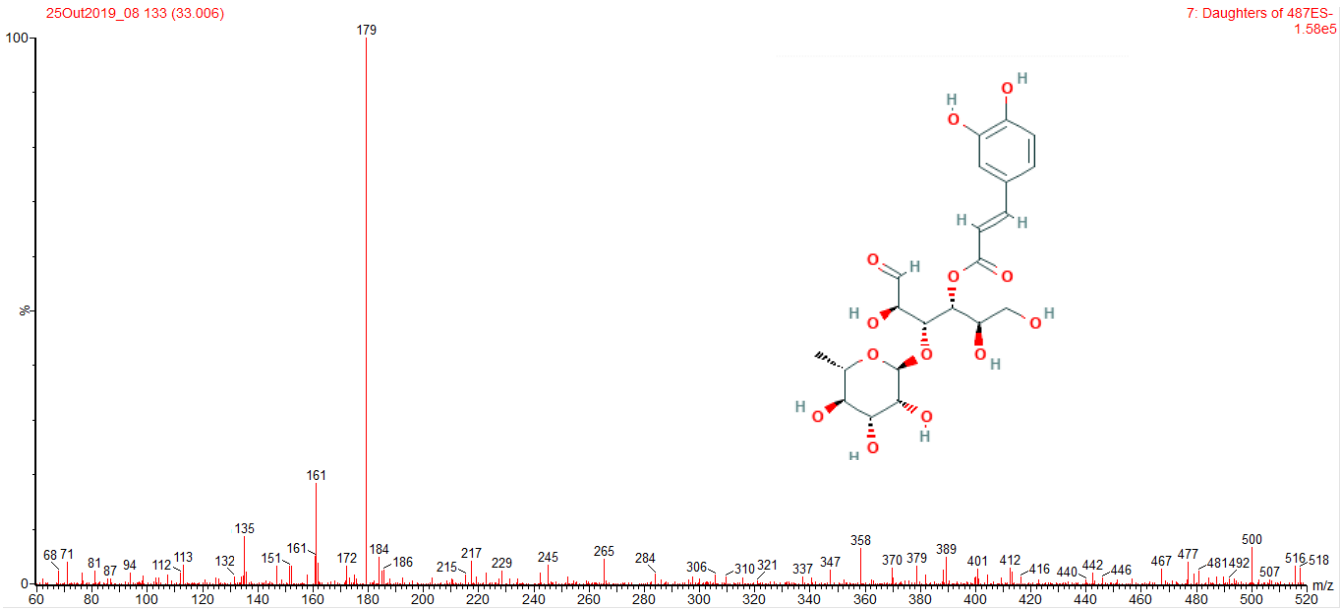

Figure 32: ESI-MS/MS spectra of Cistanoside F

Compound 11 with a precursor ion [M-H]- at m/z 639 was identified as β-hydroxyverbascoside (Acteoside). The MS/MS fragmentation of the molecular ions at m/z 639 yielded the major daughter ion at m/z 621. This compound was corresponding to the loss of water, and three minor fragments at m/z 529, corresponding to the loss of a catechol unit, m/z 459 corresponding to the loss of a caffeine or rhamnose group, and m/z 179 and 161 m/z, attributed to fragmentation of the caffeine group (**Figure 33**). This fragmentation is typical of verbascoside derivatives as reported in the literature (**Ryan et *al.*, 2002).**

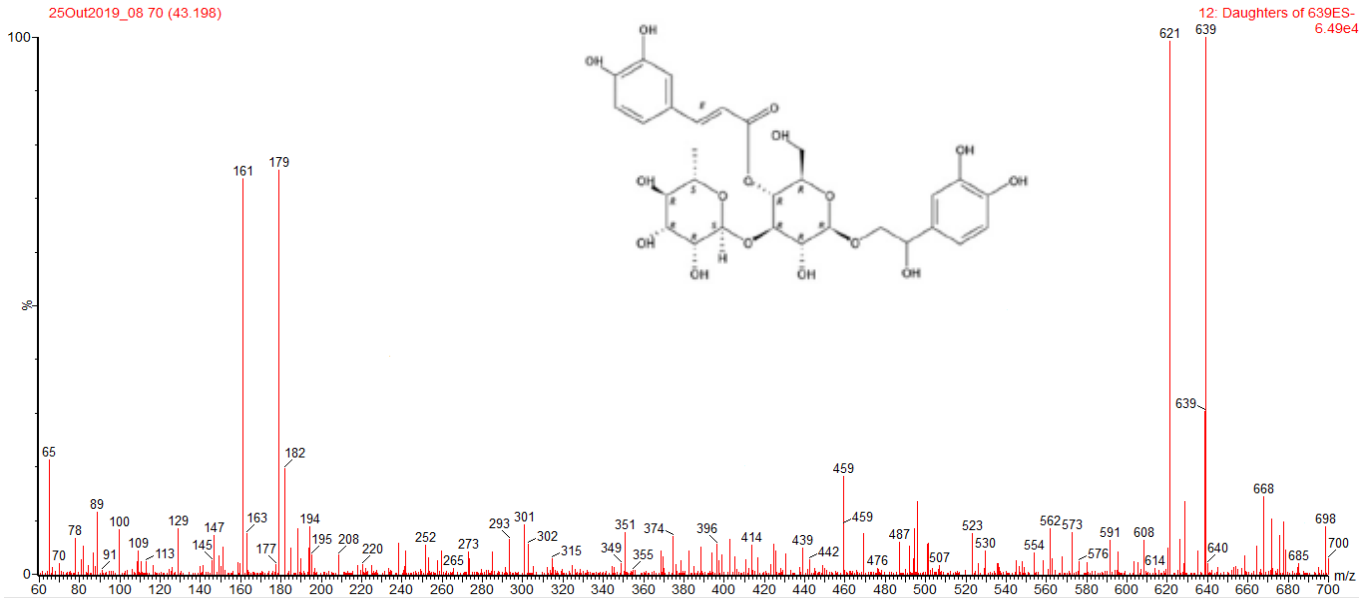

Figure 33: ESI-MS/MS spectra of β-hydroxyverbascoside

Compound 13, a phenylethanoid glycosides derivatives ([M-H]- at m/z 637) was identified as eukovoside (**Figure 34**) using the fragmentation described by **Smiljković *et al.* (2018)** and **Ado *et al.* (2016)**. It was previously identified in the studied plant (**Quirantes-Pine´ *et al.*, 2009)**. According to **Ado *et al.* (2016)**, Cistanoside F and eukovoside were contributed to the biological activity of the extracts like antioxidant, Tyrosinase inhibitory, α-Glucosidase inhibitory and Acetylcholinesterase (AChE) inhibitory activities.

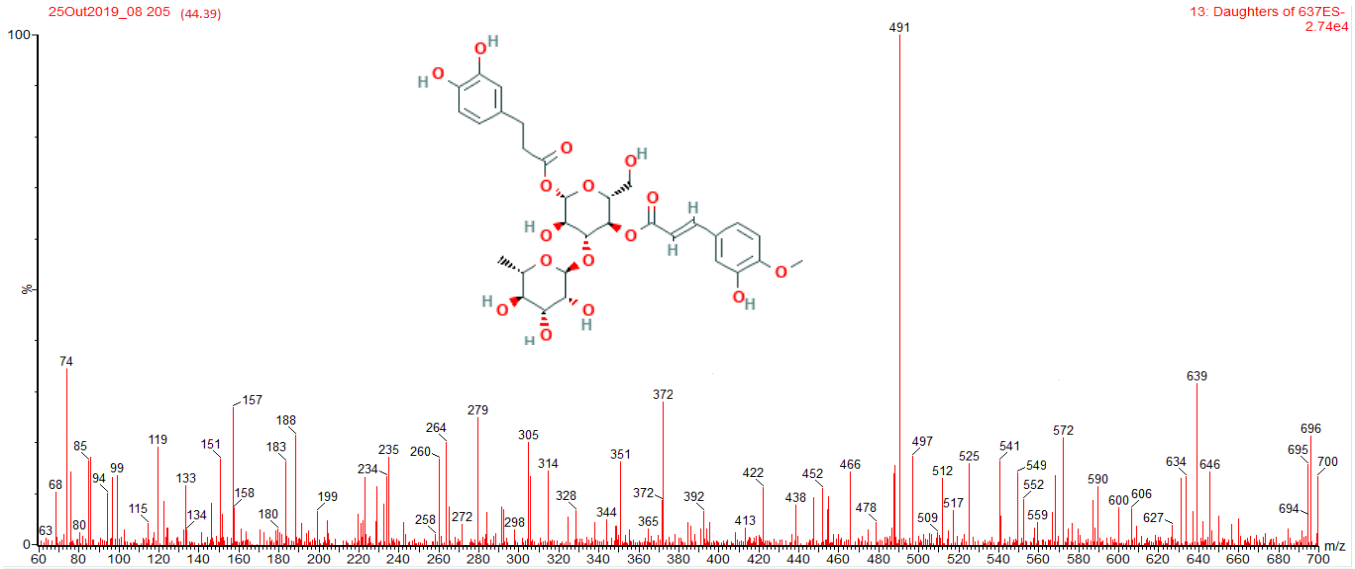

Figure 34: ESI-MS/MS spectra of eukovoside

Compound 17 (R_t= 48.07), with a precursor ion [M-H]- at m/z 637, has been tentatively characterized as Cistanoside C, relying on the MS and MS/MS fragmentation pattern that showed product ions at m/z 461 ($C_{20}H_{29}O_{12}^-$), 193, and 161 ($C_9H_5O_3^-$) (**Han *et al.*, 2012; Abdel-Hady *et al.*, 2018**) as illustrated in the **figure 35**. This compound showed an antioxidant activity, it can be effective in the treatment of renal deficiency syndrome (**Zhang *et al.*, 2019**). To the best of our knowledge, this compound is described for the first time in *Aloysia triphylla* leaves.

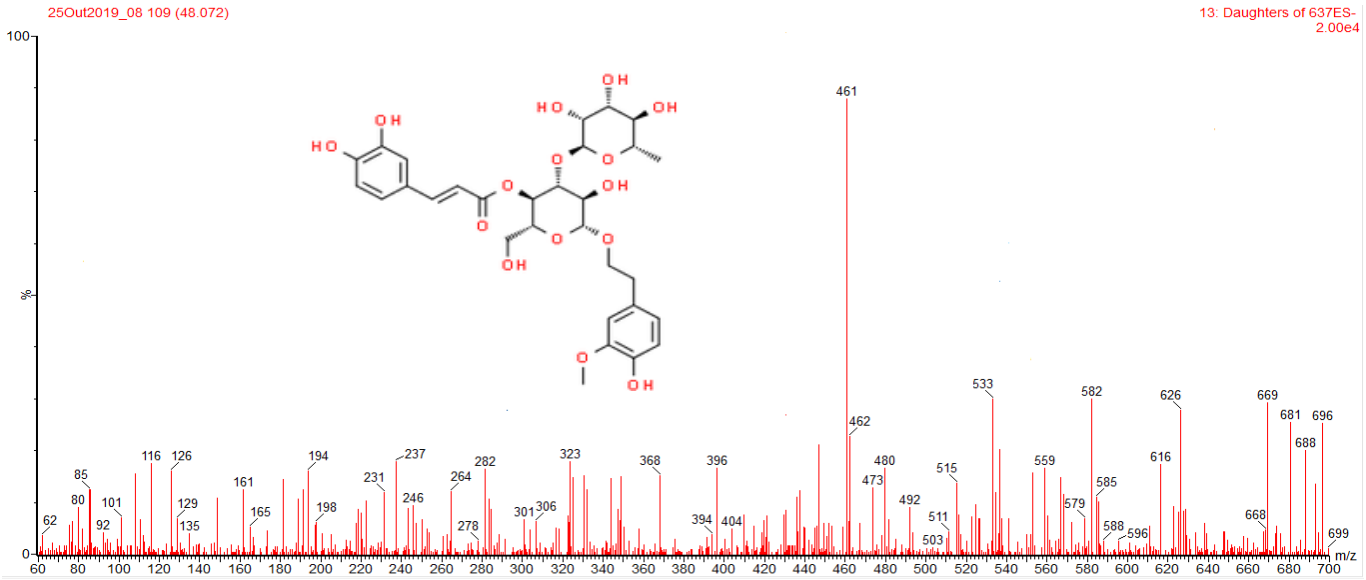

Figure 35: ESI-MS/MS spectra of Cistanoside C

Compound19, another verbascoside (Glycosylated Verbascoside) (t_R = 48.30) was identified with [M-H]- ion at m/z 623 and a product ion at m/z 161, 461 (**Figure 36**).

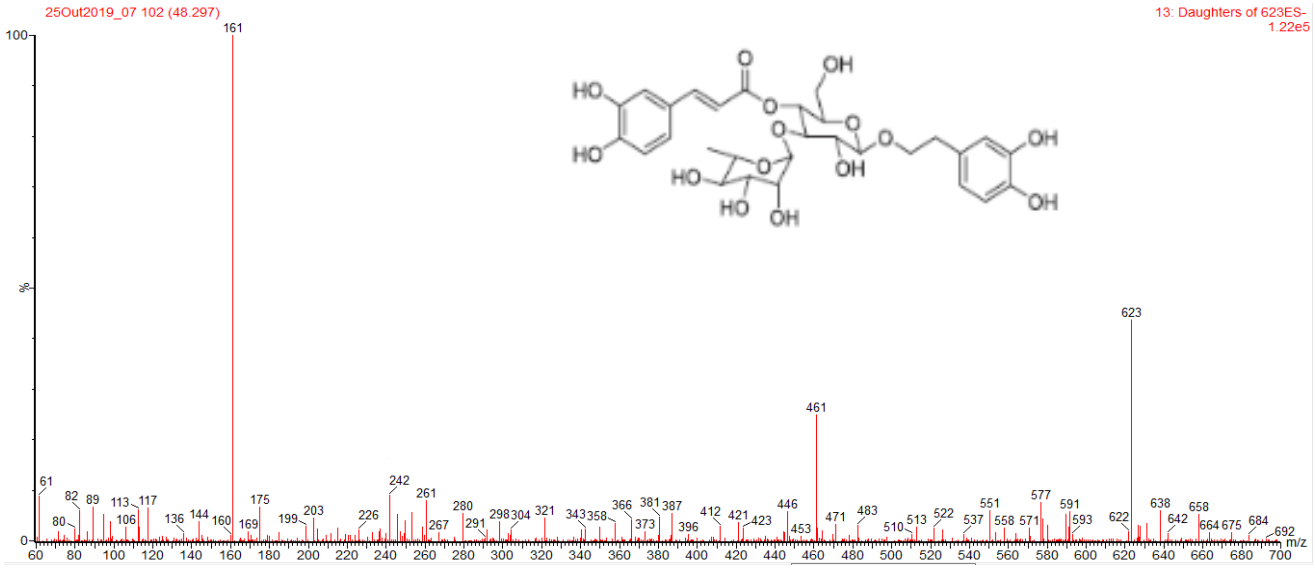

Figure 36: ESI-MS/MS spectra of Glycosylated Verbascoside

The fragments found at m/z 461 corresponded to the loss of the caffeoyl moiety (**Quirantes-Piné *et al.*, 2009; Quirantes-Piné *et al.*, 2010**). Experimental evidence has indicated that *Aloysia triphylla* extract verbascoside has antidepressant potential and possesses an important antioxidant activity (**Sánchez-Marzo *et al.*, 2019; Gomes *et al.*, 2022**).

Derhamnosyl verbascoside is a phenylpropanoid with precursor ion [M-H]- at m/z 477 and fragment ions [M-H]- at m/z 315 and 285. It was previously identified in aerial parts of *Aloysia triphylla* infusion (**Abderrahim *et al.*, 2011**). However, derhamnosyl verbascoside has been found in other herbs belonging to the *Verbenaceae* family like *Lantana camara* L. and *Lippia alba* (**Hennebelle *et al.*, 2008**).

V.1.4. Flavonoids compounds

A total of 15 flavonoids were tentatively identified. Most of them were identified in previous works (**Quirantes-Piné *et al.*, 2010; de la Luz Cádiz-Gurrea *et al.*, 2018; Hong *et al.*, 2019 and Leyva-Jiménez *et al.*, 2020**).

IV.1.4.1. Homoisoflavone

Compound 30 was eluted late in the chromatogram, with its [M-H]- at 339, its produced ions m/z 311 $[M-H-CO]^-$ was also observed (**Figure 37**). According to the literature, it was identified as methylophiopogonone A (**Hong *et al.*, 2019**). This compound showed scavenging effect on superoxide anion, hydroxyl radical and hydrogen peroxide (**Lin *et al.*, 2010**). It was already reported in *Camellia oleifera* (Theaceae) (**Hong *et al.*, 2019**), but for the first time in *Aloysia triphylla* plant.

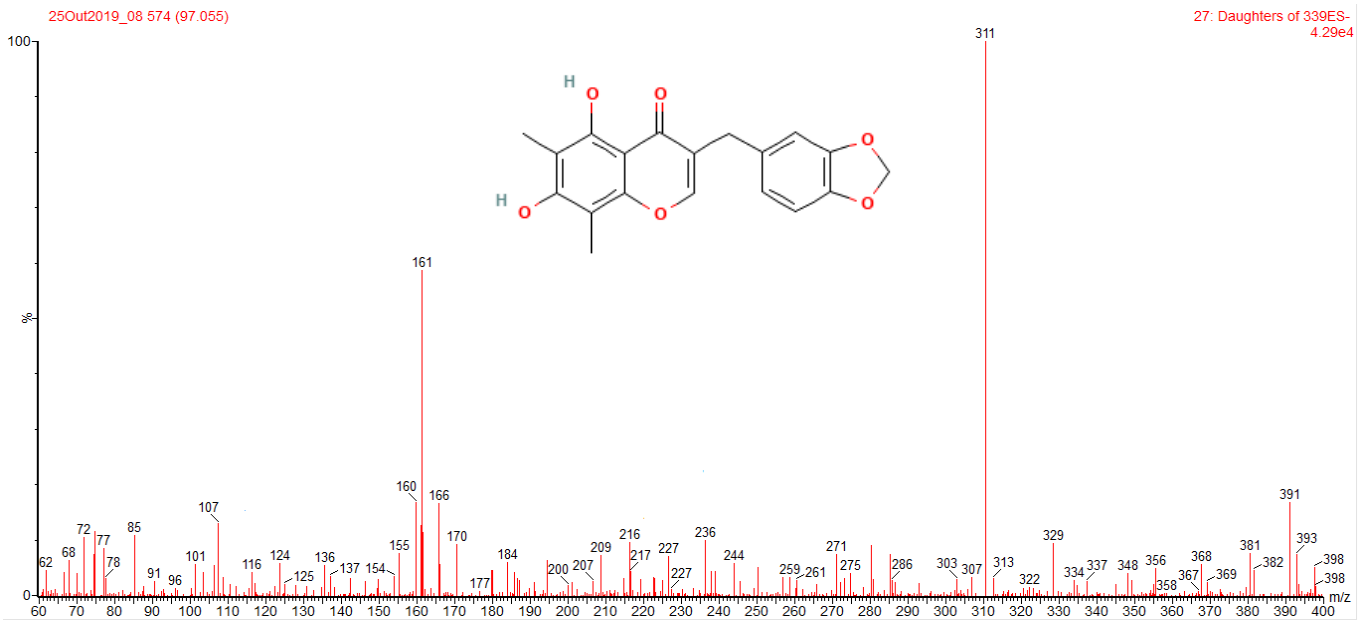

Figure 37: ESI-MS/MS spectra of methylophiopogonone A

V.1.4.2.Flavone

MS/MS fragmentation of **compounds 12** (Luteolin-7-diglucoronide) presented a fragment ion at m/z 351 which is a characteristic of diglucuronide group after water elimination and at m/z 285, corresponding to luteolinaglycone (**Quirantes-Piné *et al.*, 2009**), as shown in the **Figure 38**. It is represented by an intense peak; proving its presence with an important proportion in the two extracts. This compound has been well reported in *Aloysia triphylla* (**Bilia *et al.*, 2008; Quirantes-Piné *et al.*, 2009; de la Luz Cádiz-Gurrea *et al.*, 2018; Leyva-Jiménez *et al.*, 2020b**). Luteolin-7-diglucoronide possesses a wide range of biological activities, involved in the prevention and treatment of many diseases, including antioxidant, anti-inflammatory, antimicrobial and anticancer activities (**López-Lázaro, 2009**).

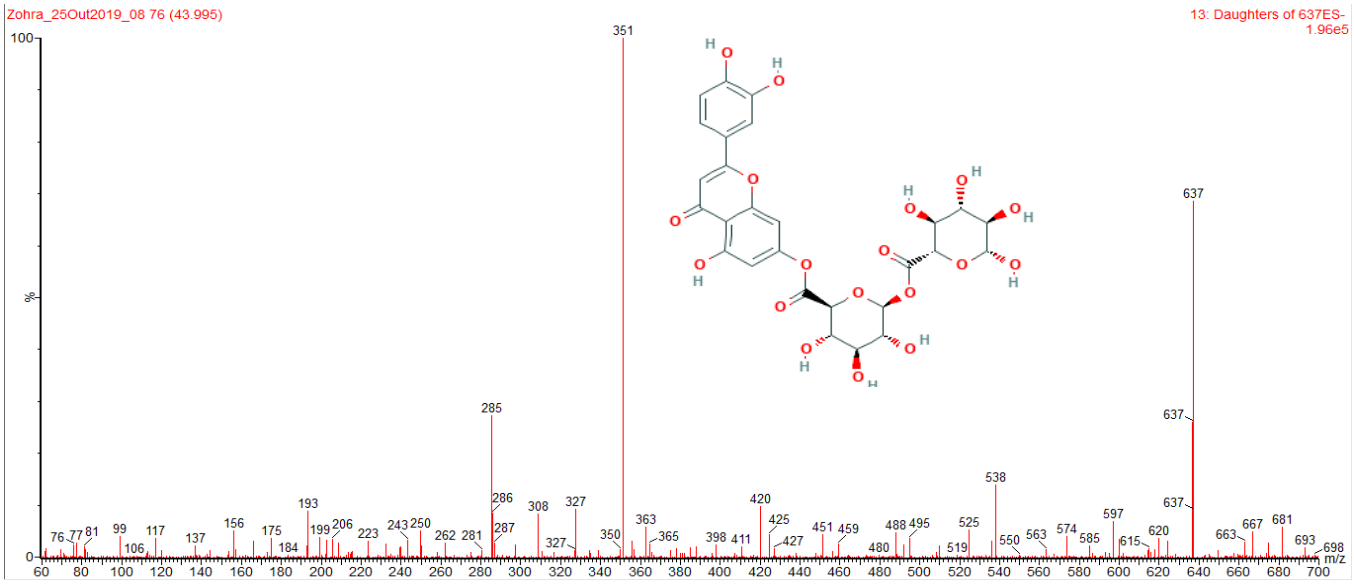

Figure 38: ESI-MS/MS spectra of Luteolin-7-diglucoronide

MS-MS fragmentation of compounds 12, 15, 16 and 23 presented the same ion at m/z 351 which is characteristic of diglucuronide group after water elimination. As well, all these compounds showed similar UV-visible spectra typical of flavonoids. According to their molecular formulas, with a parant ion at m/z 621 (**Figure 39**), **compound 15** was identified as apigenin-7-diglucuronide (Clerodendrin) **(Quirantes-Piné *et al.*, 2009)**. This compound has been previously well described in *Aloysia triphylla* leaves (**Bilia *et al.*, 2008; Quirantes-Piné *et al.*, 2009; Felgines *et al.*, 2014; Mehrparvar *et al.*, 2016**).

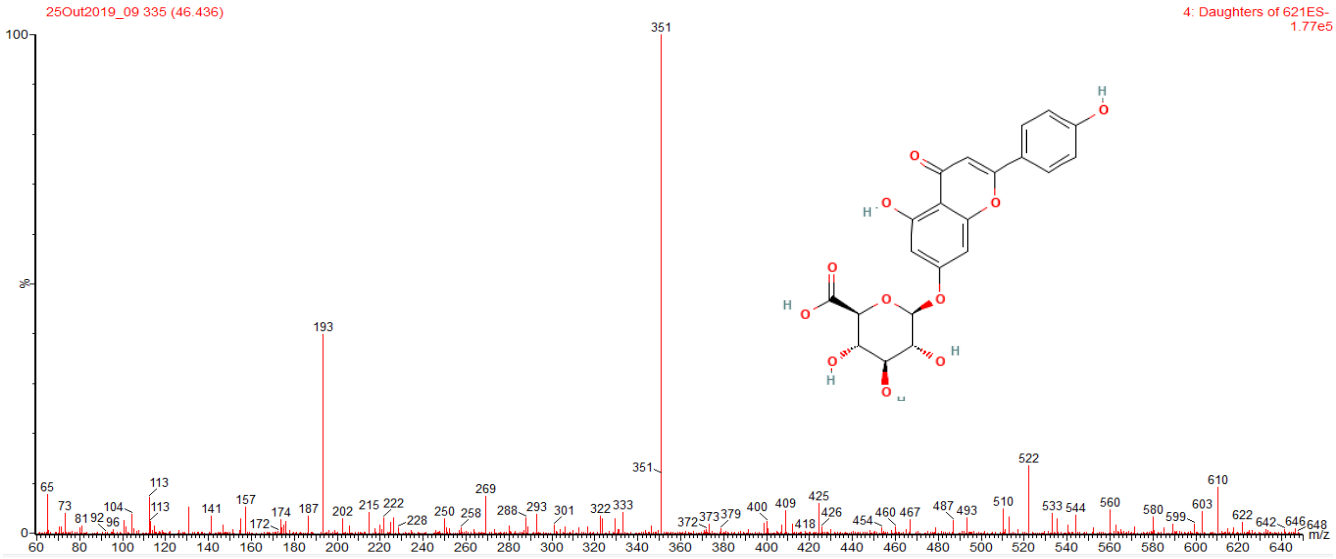

Figure 39: ESI-MS/MS spectra of apigenin-7-diglucuronide

Compound 16 (Chrysoeriol-7-diglucoronide) with a base ion [M-H]- at m/z 651 and MS/MS fragmentation with the ion at m/z 351 (**Figure 40**) is characteristic of the diglucuronide group after water removal, this compound was previously found in *Aloysia triphylla* extract (**Quirantes-Piné *et al.*, 2009; Leyva-Jiménez *et al.*, 2020b**).

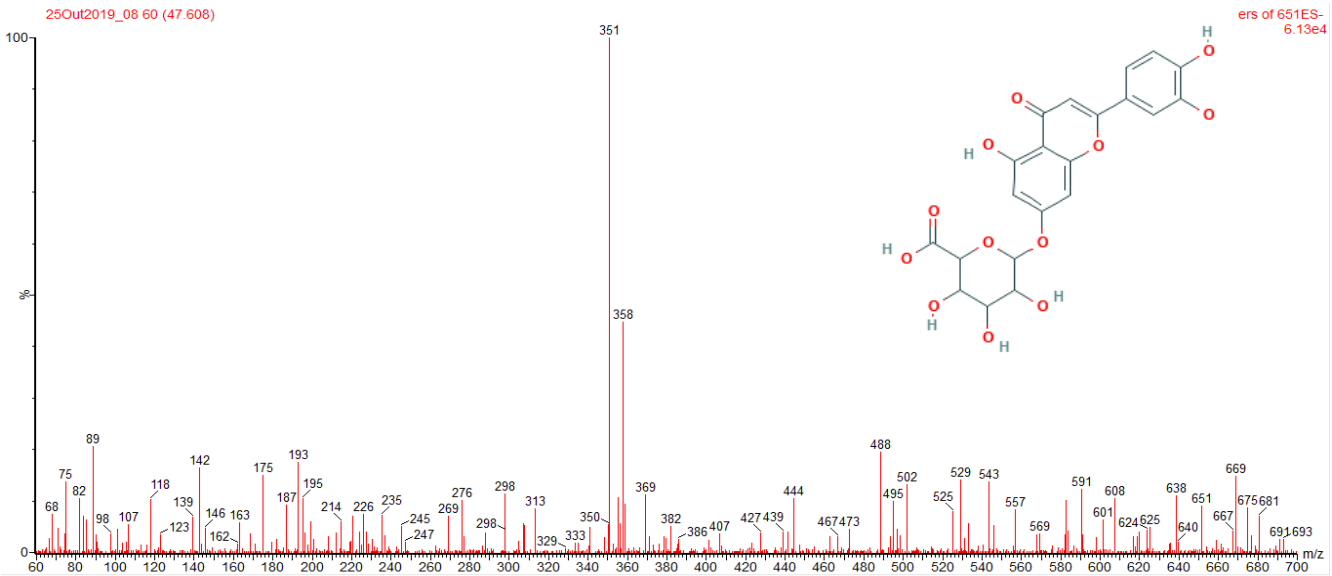

Figure 40: ESI-MS/MS spectra of chrysoeriol-7-diglucoronide

Compound 18 exhibit a fragment ion at m/z 285 (**Figure 41**). It was caused by the loss of a CH_2O in the molecular ion [M - H]- at m/z 447. Therefore, this compound was identified as astragalin (**Hao *et al.*, 2021**). Astragalin is considered to be ubiquitous in the plant kingdom such as *Gymnosperma glutinosum* (**Morado-Castillo *et al.*, 2016**). To our knowledge, no studies have been published on the presence of astragalin in the extract of *Aloysia triphylla*. Researchers have reported multiple pharmacological applications of astragalin in various diseases. In fact, this compound has anti-inflamatory, antibacterial, antiallergic, antioxidant, antidiabetic, hepatoprotective and antitumor properties (**Riaz *et al.*, 2018**).

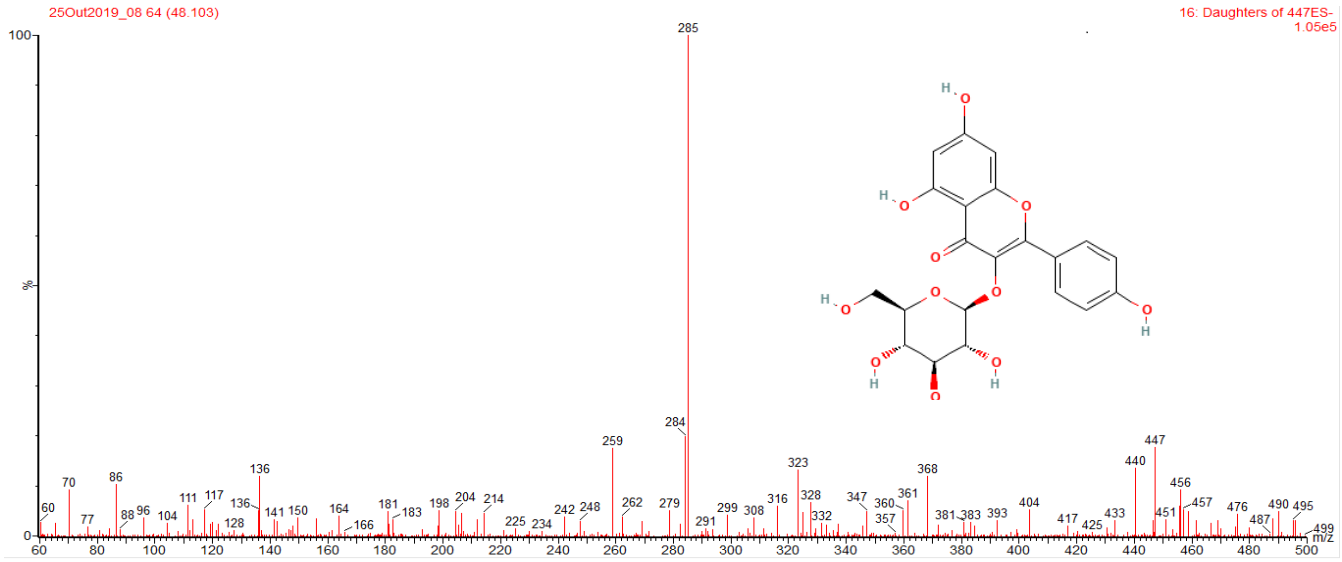

Figure 41: ESI-MS/MS spectra of astragalin

Compound 21 confirmed as scutellarin.The fragmention of m/z 285 in its MS mass spectrum suggested the loss of a glucuronic acid residue in the molecular ion [M - H]- at m/z 461. Further dissociation of the glycone m/z 285 ion yielded a series of fragments, 193, 113 (**Figure 42**), in comparison with the reference compound and literature (**Zhang *et al.*, 2015; Hao *et al.*, 2021**). This compound has been described in *Verbena officinalis* (**Kubica *et al.*, 2020**), but this was characterized in *Aloysia triphylla* leaves for the first time. Scutellarin exerts vasoprotective properties and many activities such as: anti-oxidative, anti-inflammatory, anti-ischemic, cardioprotective and anti-hypertensive (**Ozarowski *et al.*, 2019**).

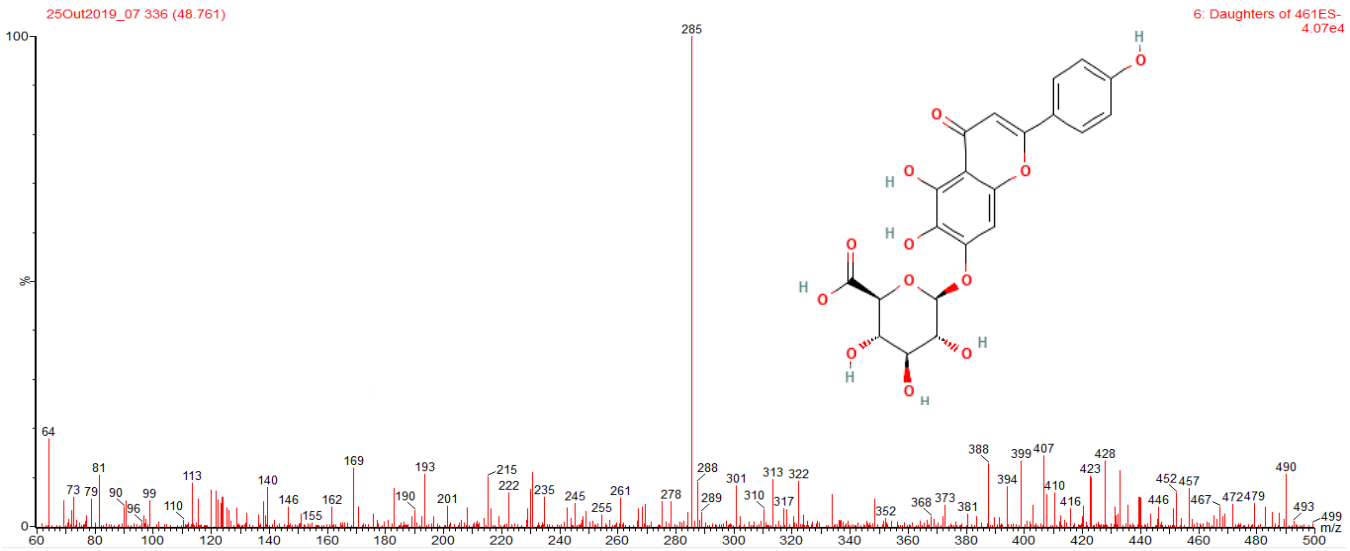

Figure 42: ESI-MS/MS spectra of scutellarin

Compound 22, with R_t= 51.37 min at m/z 445 showed product ions at m/z 269, m/z 113 and m/z 85 (**Figure 43**). It was identified as baicalin. This compound has shown to have a high bioactive potential, as anxiolytic activity which could be antagonized by a GABAA-specific antagonist (**Kuroda *et al.*, 2012**). It was previously isolated from *Scutellaria lateriflora* L. (Lamiaceae), *Lavandula stoechas* and *Mentha pulegium* **(Razavi *et al.*, 2017; Baali *et al.*, 2022)**.This compound possesses antiviral **(Majumder *et al.*, 2020)** and anxiolytic activities **(Kuroda *et al.*, 2012)**. It has already been identified in *Aloysia triphylla* (**Rashid *et al.*, 2022**).

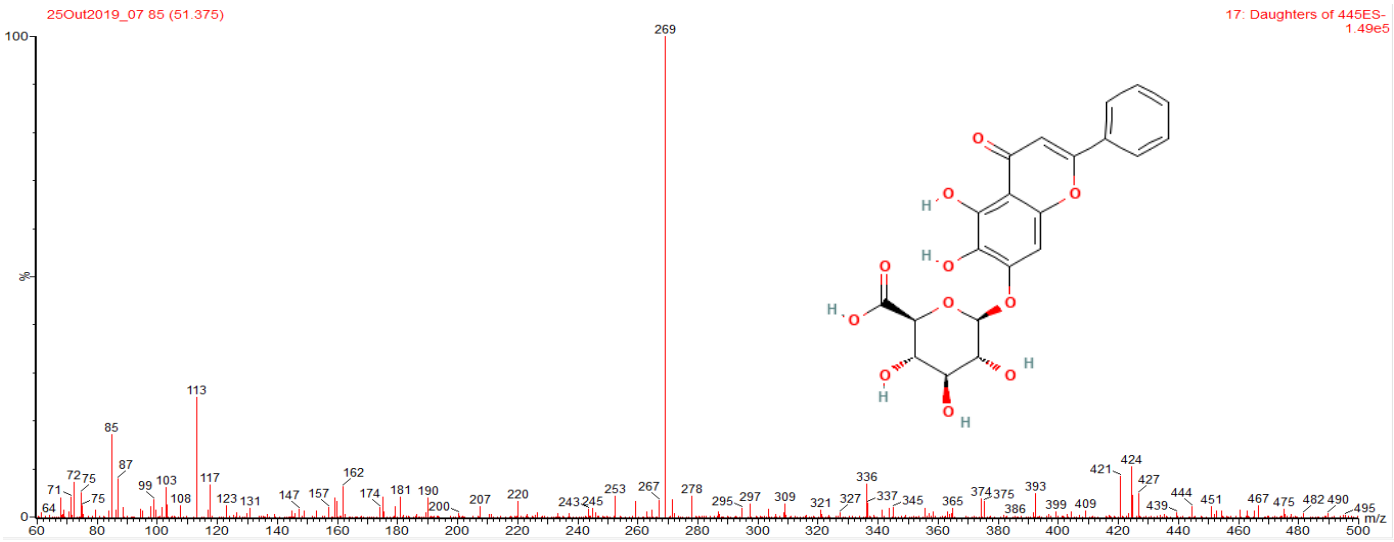

Figure 43: ESI-MS/MS spectra of baicalin

Compound 23 has been found in a previous study on *Aloysia triphylla* extract (**Quirantes-Pine´ *et al.*, 2009; Quirantes-Pine´ *et al.*, 2010**). This compound was tentatively characterized as acacetin-7-diglucoronide (**Quirantes-Piné *et al.*, 2010**), presenting a precursor ion [M-H]- at m/z 635 and product ions at m/z 351 **(Figure 44)**.This fragment is characteristic of diglucuronide group after water elimination (**Quirantes-Pine´ *et al.*, 2009)**.

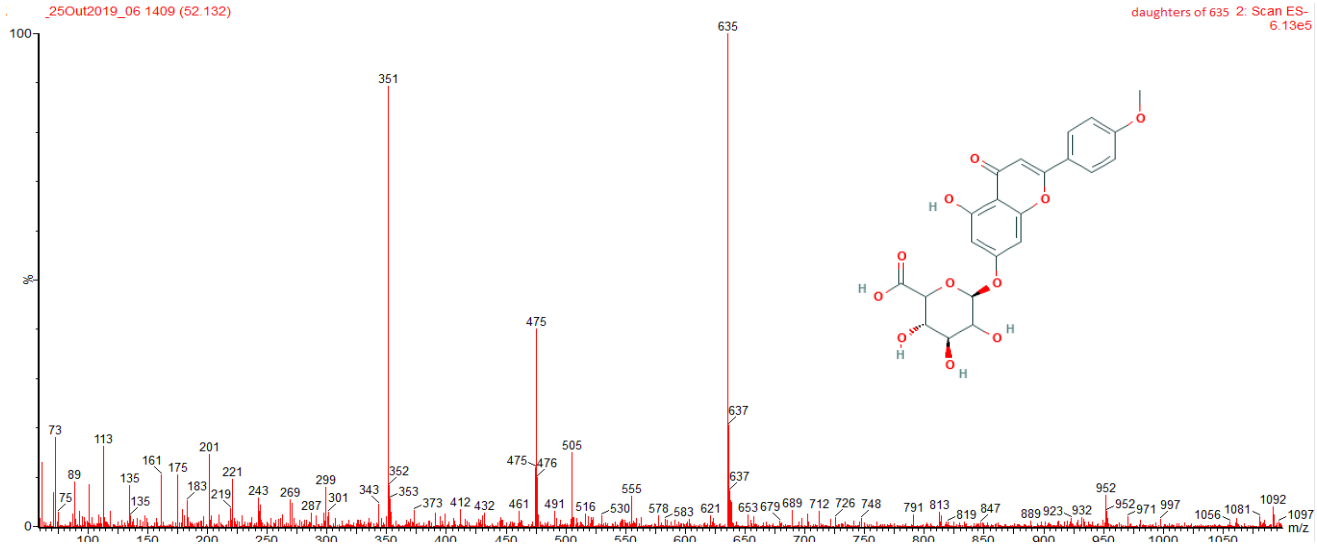

Figure 44: ESI-MS/MS spectra of Acacetin-7-diglucoronide

Peak 27 (R_t= 68.60 min) generates a precursor ion $[M-H]^-$ at m/z 269. In the MS/MS spectrum, the fragment ion at m/z 151 was formed by the removal of C_8H_6O during the pyrolysis of the flavonoid, then, one molecule of CO_2 was removed by ion m/z 151 to obtain fragment ions of m/z 107. Therefore, compound 27 (**Figure 45**) was putatively characterized as apigenin (4',5,7-trihydroxyflavone) (**Hao *et al.*, 2021**). Many studies have revealed that apigenin has a cytostatic and cytotoxic effects on the various cancer cells, an anti-stress effect, prevents the atherogenesis, hypertension, cardiac hypertrophy, inhibits the asthma, bleomycin-induced pulmonary fibrosis, type 2 diabetes and its complication, osteoporosis, and collagen-induced arthritis (**Iwashina *et al.*, 2011; Zhou *et al.*, 2017**), treat Alzheimer's disease, amnesia and depression (**Salehi *et al.*, 2019**). This compound was previously identified in *Aloysia triphylla* aerial parts (**Tammar *et al.*, 2021**).

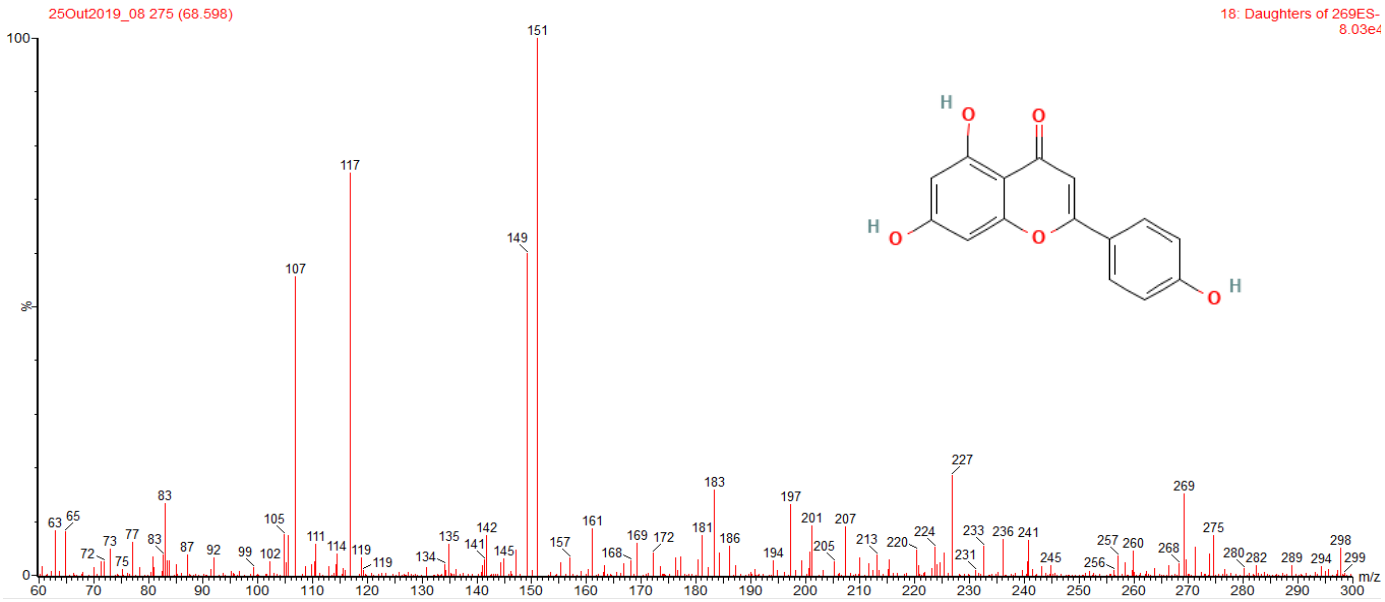

Figure 45: ESI-MS/MS spectra of apigenin

O-methylated flavones (**Compound 28**) was identified as diomestin (5,7,3′-trihydroxy-4′-methoxyflavone). It has a precursor ion [M-H]- at m/z 299 (**Figure 46**). The MS/MS spectra showed the loss of a methyl group [M-H-CH_3]-originating m/z 284 (**Kheyar-Kraouche *et al.*, 2018**). This compound also known as methylluteolin is a bioflavonoid distributed in different plant species (**Victor *et al.*, 2021**) and it was previously identified in *Aloysia triphylla* (**Felgines *et al.*, 2014; Tammar *et al.*, 2021**). Recently, extensive study had indicated that diosmetin has

been found to be active in treatment and management of multitude of chronic disorders, including anticancer activity. Indeed, it exerts significant cytotoxic effects apoptosis on human breast cancer (**Wang *et al.*, 2019**) and acute myeloid leukemia (AML) (**Roma *et al.*, 2018**). In addition, diosmetin has antidiabetic (**Chan *et al.*, 2013**), antimicrobial, anti-inflammatory, antioxidant, antilipolytic, analgesic and oestrogenic activities (**Androutsopoulos and Spandidos, 2013; Victor *et al.*, 2021; Garg *et al.*, 2022**).

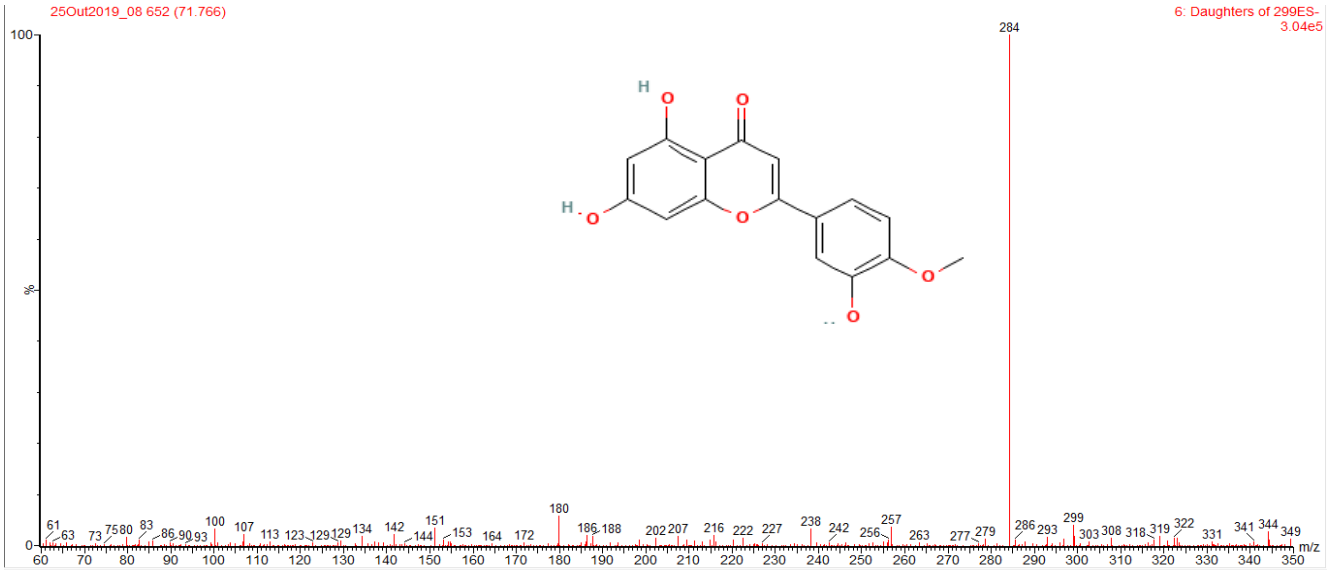

Figure 46: ESI-MS/MS spectra of diomestin

Compound 29 (R_t=86.42 min) with a precursor ion [M-H]- at m/z 283 was putatively identified as genkwanin, based on the data obtained by MS and MS/MS, and literature already cited (**Kheyar-Kraouche *et al.*, 2018**). It showed a product ion at m/z 268 (100%) (**Figure 47**). This compound was previously identified in verbenaceae family like *Lantana camara* (**Bangou *et al.*, 2019**), *Vitex peduncularis* (**Haque *et al.*, 2018**) and *Lippia graveolens* HBK (**Cortés-Chitala *et al.*, 2021**). To the best of our knowledge, this is the first report of genkwanin in *Aloysia triphylla* plant.

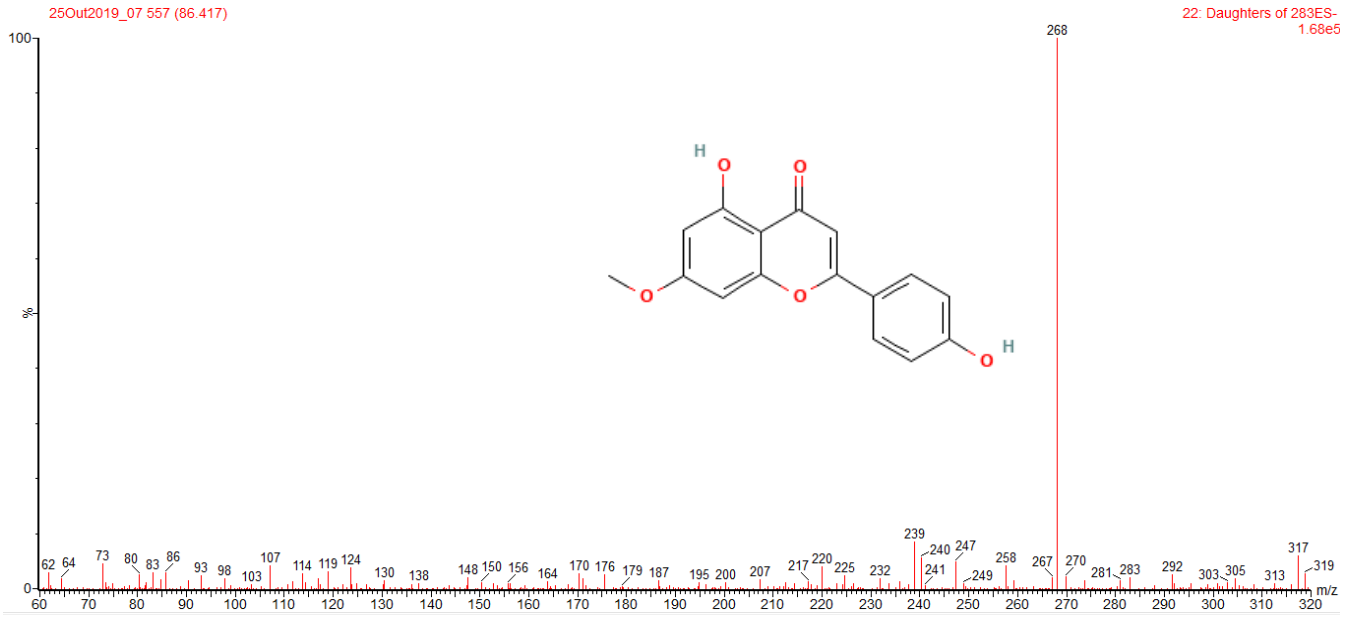

Figure 47: ESI-MS/MS spectra of genkwanin

V.1.4.3. Flavonol

Compound 14 generated an [M - H]- ion at m/z 463. There was a fragment ion peak of m/z 301 in the secondary mass spectrum, which was the ion peak of aglycon quercetin. From the mass spectrometry information, it can be inferred that m/z 463 lost one molecule of glucose to obtain aglycon ion m/z 301. Therefore, compound 14 was identified as quercetin-3-*O*-glucoside (**Hao *et al.*, 2021**). Its structure is illustrated in the **figure 48**. This compound was previously characterized in *Amorphophallus titanium* (Araceae) (**Iwashina *et al.*, 2015**), *Nelumbo nucifera* Gaertn (Nelumbonaceae) (**Guo *et al.*, 2017**), *Lactuca indica* (Asteraceae) (**Hao *et al.*, 2021**). To the best of our knowledge, this is the first report of quercetin-3-*O*-glucoside compounds in *Aloysia triphylla*. It exhibits a strong anticancer action (**Sudan and Rupasinghe, 2015**). It is also known as an antioxidant, anti-inflammatory, cardioprotective, and anti-obesity compound (**Ulusoy and Sanlier, 2019**).

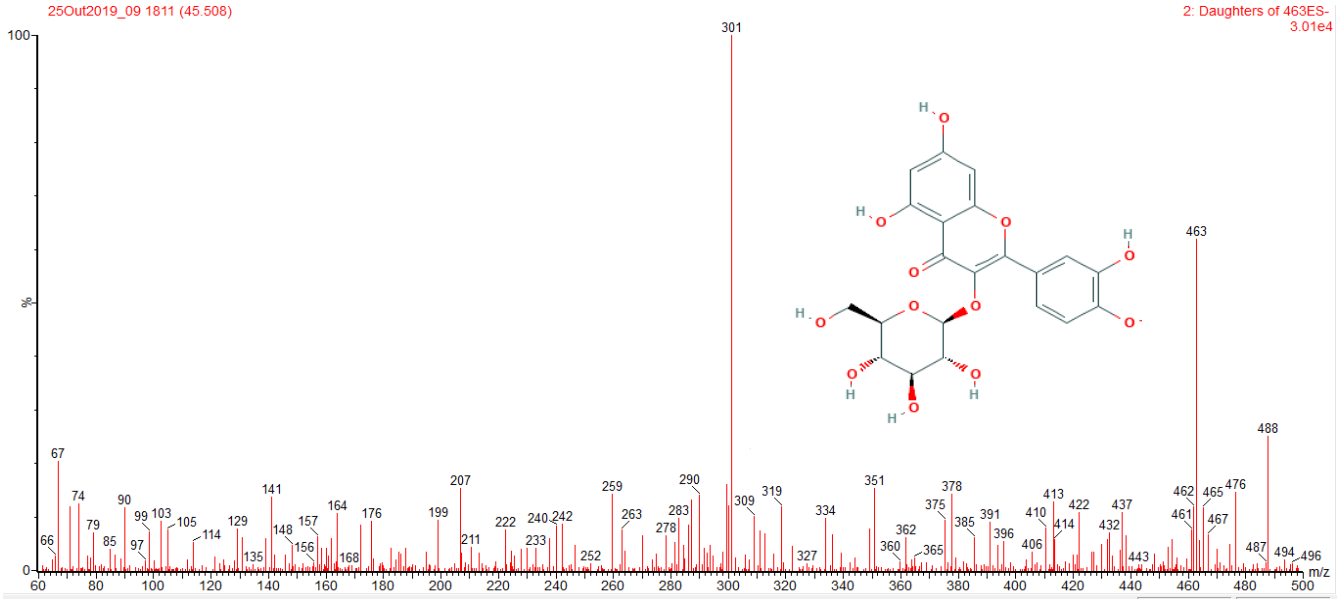

Figure 48: ESI-MS/MS spectra of quercetin-3-O-glucoside

Compound 24 (R_t= 59.70 min) showed similar product ions as isorhamnetin (**Boukhris *et al.*, 2016**), its structure is shown in **figure 49**. This peak can correspond to nepetin (**Kheyar-Kraouche *et al.*, 2018**). This compound was previously identified in Verbenaceae family like *Phyla Nodiflora* (**Jabeen *et al.*, 2015**), *Stachytar phetacayennensis* (**Onofre *et al.*, 2016**) and in aerial parts of *Aloysia triphylla* (**Zhang *et al.*, 2015a**). It is known for its antiproliferative activity (**Talib *et al.*, 2012**).

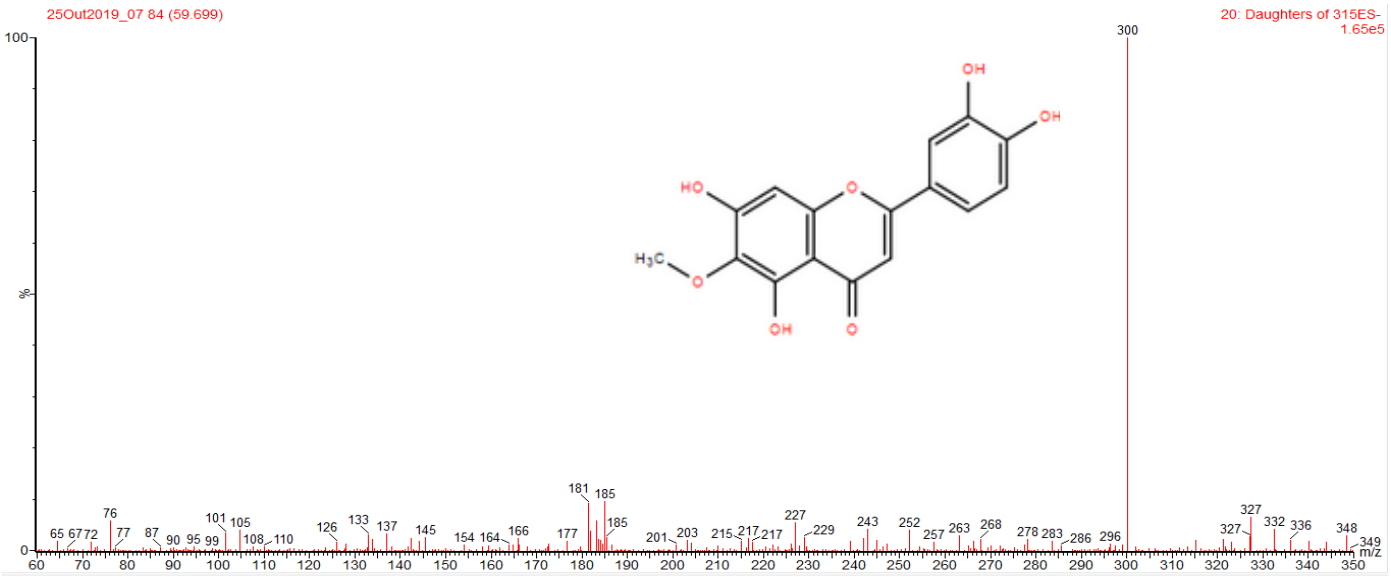

Figure 49: ESI-MS/MS spectra of nepetin

Compound 25 was identified as luteolin (**Figure 50**), with precursor ion [M-H]- at m/z 285 and product ions at m/z 133 $[M-H-152]^-$ and 151$[M-H-134]^-$ (**Shi *et al.*, 2013**). With verbascoside, luteolin is the main compounds in *Aloysia triphylla*. It possesses a variety of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial and anticancer. The ability of luteolin to inhibit angiogenesis, to induce apoptosis, to prevent carcinogenesis in animal models, to reduce tumor growth *in vivo* and to sensitize tumor cells to the cytotoxic effects of some anticancer drugs suggests that this flavonoid has chemopreventive and chemotherapeutic potential (**López-Lázaro, 2009**). Luteolin was already noted as having activity against SARS-CoV. This flavone is active against a range of other viruses, including enteroviruses, coxsackieviruses, and hepatitis B (**Bai *et al.*, 2016; Yarnell, 2016**). It has also an anti-stress activity (**Iwashina *et al.*, 2011**).

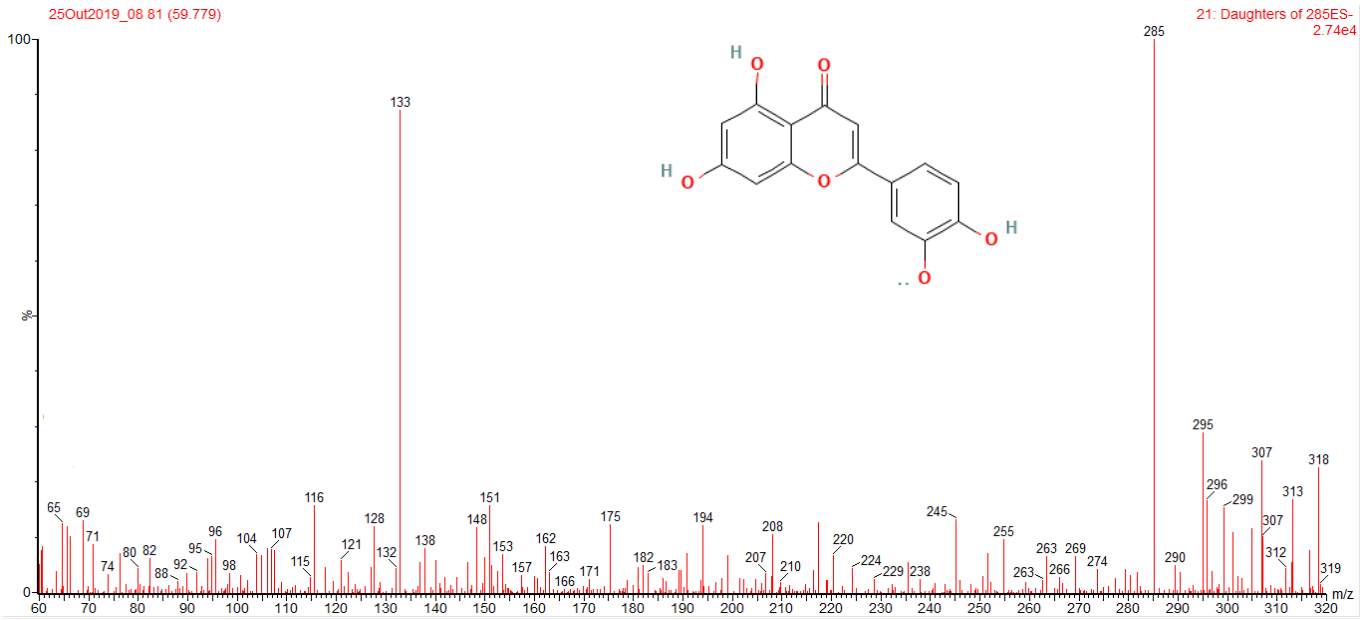

Figure 50: ESI-MS/MS spectra of luteolin

Compound 26 (R_t= 59.92 min) was identified as isorhamnetin (**Aguado *et al.*, 2016**). Its precursor ion at m/z 315, generated the product ion at m/z 300 (**Figure 51**). Collision-induced dissociation of the aglycone, yielded ions at m/z 300 which were evidently produced by the loss of the methyl group. In the case of isorhamnetin, this was the most prominent fragment (**Schiebe *et al.*, 2002**). Isorhamnetin possesses extensive pharmacological activities, such as cardiovascular and cerebrovascular protection, anti-tumor, anti-inflammatory, antioxidant, organ protection, prevention of obesity and antimicrobial activities (**Aguado *et al.*, 2016; Gong *et al.*, 2020)**. This compound is identified for the first time in *Aloysia triphylla*.

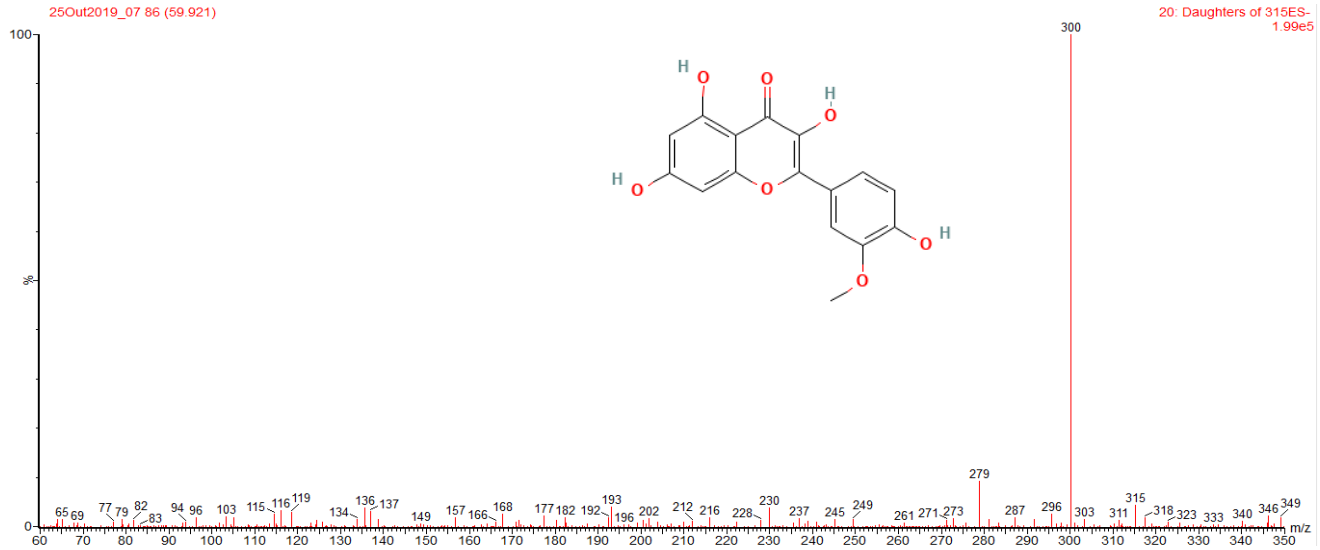

Figure 51: ESI-MS/MS spectra of isorhamnetin

V.1.5. Fatty acyl glycosides

Compound10 was identified as 12-hydroxyjasmonic acid glucoside (Tuberonic acid glucoside). Its structure is illustrated in **figure 52**. This compound was previously isolated from leaves of *Aloysia triphylla* (**Quirantes-Pine´ *et al.*, 2010; Leyva-Jiménez *et al.*, 2020)**. Its MS/MS spectrum showed a precursor ion [M-H]- at m/z 387 and product ions at m/z 185,101, 207, 163, 341 (**Marzouk *et al.*, 2018; GAŠI´C *et al.*, 2021**), the fragment ion at m/z 207, is consistent to the loss of a saccharidic moiety as 180 Da. The decarboxylation (loss of CO_2 (44 Da)) of the fragment ion at m/z 207 generated the ion at m/z 163 (**Pacifico *et al.*, 2015; GAŠI´C *et al.*, 2021**).

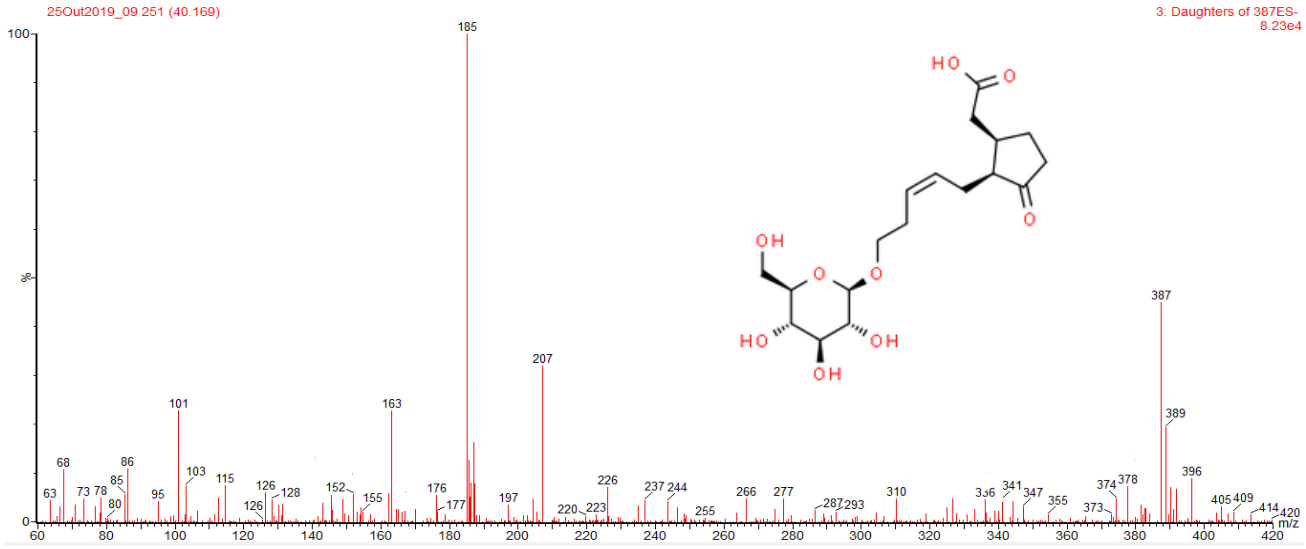

Figure 52: ESI-MS/MS spectra of 12-hydroxyjasmonic acid glucoside

V.2. Characterization with the positive mode

As shown in **Table IX**, the characterization in positive ionization mode had resulted in an identification of 29 compounds by LC-DAD-ESI-MS/MS experiments with their retention times (R*t*), UV absorption maxima, precursor ion, MS/MS product ions and literature references, these compounds were numbered according to their elution order.

Table IX: Characterization of phenolic compounds in the *Aloysia triphylla* conventional and MAE extracts by LC–DAD–ESI–MS/MS in positive ionization mode

Peak	t_R (min)	UV (λmax)	[M+H]+ m/z	HPLC–DAD–ESI MS/MS m/z (% base peak)	Tentative identification	Compound group	Reference
1	35.43	230,283,321,338	391	**151 (100),** 123 (33), 95 (20)	Theveside ($C_{16}H_{22}O_{11}$)	Iridoid Glycosides	Cittadini *et al.*, 2017
2	40.92	291,334	595	**457 (100),** 355 (88),559 (68), 337 (67), 464 (60),339 (56), 295(35), 307 (32) , 505 (29)	Genistein-C-glucosylglucoside ($C_{27}H_{30}O_{15}$)	Isoflavones	Hong *et al.*, 2019

3	44.06	270, 327	639	**287 (100)**	Luteolin-7-diglucuronide ($C27H_{26}O_{18}$)	Flavones	Rehecho *et al.*, 2011
4	45.57	230, 275, 332	479	**303 (100),** 137 (8) , 284 (5), 153 (3)	Quercetin-7-O-glucuronide ($C_{21}H_{18}O_{13}$)	Flavonols	Carazzone *et al.*, 2013
5	46.42	231, 267, 338	623	**271 (100)**	Apigenin-7-diglucuronide ($C_{27}H_{26}O_{17}$)	Flavones	Timoteo *et al.*, 2008 ; Guo *et al.*, 2011
6	46.97	280, 327,352	325	**163 (100)**	Ester of caffeic acid	Phenolic acids	Atoui *et al.*, 2005
7	47.67	230, 275, 335	465	**303 (100)**	Quercetin 3-O-glucoside (Isoquercetin) ($C_{21}H_{20}O_{12}$)	Flavonols	Lin *et al.*, 2008
8	47.74	230, 270, 340	653	**301 (100),** 477 (8), 353 (1)	Chrysoeriol-7-diglucuronide (C_{28} $H_{28}O_{18}$)	Flavones	Timóteo *et al.*, 2015
9	48.25	230, 280, 332	463	**287 (100)**	Kaempferol-(2”-O-methyl)-4'-O-α-D-glucopyranoside	Flavonols	Rezende *et al.*, 2019
10	48.29	230, 270, 330	449	**287 (100),** 471 (10)	Kaempferol-3-glucoside (astragalin) ($C_{21}H_{20}O_{11}$)	Flavonols	Atoui *et al.*, 2005
11	48.84	230, 275, 330	479	**317 (100),** 302 (24)	Isorhamnetin-3-O-glucoside ($C_{15}H_{10}O_6$)	Flavonols	Yao *et al.*, 2012
12	49.81	230, 280, 329	339	**177 (100),** 145 (60)	Demethoxycurcumin ($C_{20}H_{18}O_5$)	curcuminoids	Mass Bank, Bonifacio *et al.*, 2018
13	50.86	230, 267, 330	433	**271 (100)**	Apigenin-7-O-glucoside ($C_{21}H_{20}O_{10}$)	Flavones	Atoui *et al.*, 2005 Abu-Reidah *et al.*, 2015
14	51.22	230, 267, 330	447	**271 (100)**	Apigenin 4′-O-glucuronide	Flavones	Timoteo *et al.*, 2008 ; Xiao *et al.*, 2016
15	51.66	332	271	**153 (21),** 119 (11)	Apigenin ($C_{15}H_{10}O_5$)	Flavones	Mahfoudhi *et al.*, 2014
16	51.73	329	301	**286 (100)**	Kaempferid ($C_{16}H_{12}O_6$)	Favonols	Mahfoudhi *et al.*, 2014
17	51.89	332	449	**287 (100)**	Luteolin 7-O-glucoside ($C_{21}H_{20}O_{11}$)	Flavones	N’gaman-Kouassi *et al.*, 2016 ; Olate-Gallegos *et al.*, 2019
18	52.04	230, 275, 330	507	**331 (100),** 491 (10), 316 (20)	Tricin-7-O-glucuronide ($C_{23}H_{22}O_{13}$)	Flavones	Timoteo *et al.*, 2015
19	52.13	230, 270, 332	637	**285 (100)**	Acacetin 7-diglucuronide ($C_{28}H_{28}O_{17}$)	Flavones	Olivares-Vicente *et al.*, 2019 ; Sánchez-Marzo *et al.*, 2019 ; Cádiz-Gurrea *et al.*, 2019

20	52.27	230, 275, 330	477	**301 (100),** 286 (4)	5,6,7-trihydroxy-8-methoxy flavone-7-O-glucuronide ($C_{22}H_{21}O_{12}$)	Flavones	Hu *et al.*, 2012
21	58.62	230, 270, 330	285	**270 (100),** 242 (71)	Acacetin ($C_{16}H_{12}O_5$)	Flavones	Mass bank, Eissa *et al.*, 2020
22	59.52	230, 267, 338	287	**153 (100),** 241 (5)	Luteolin ($C_{15}H_{10}O_6$)	Flavones	Mass Bank, Gao *et al.*, 2017
23	59.82	230, 270, 330	317	**302 (100),** 168 (16)	Isorhamnetin aglycone ($C16H_{12}O_7$)	Flavonols	Mass Bank; Boukhris *et al.*, 2012
24	68.21	230, 267, 338	271	**153 (74),**119 (36)	Apigenin ($C_{21}H_{20}O_{10}$)	Flavones	Mass Bank, Rafi *et al.*, 2020
25	70.07	230, 270, 330	301	**286 (100),** 258 (4)	Diosmetin (C_{16} $H_{12}O_6$)	Favonols	Mass Bank ; Ding *et al.*, 2021
26	72.04	230, 270, 330	301	**286 (100),** 258 (29)	Isokaempferide ($C_{16}H_{12}O_6$)	Flavonols	Hao *et al.*, 2021
27	73.36	339	331	**316(100),**301(9),168(9), 273(7)	Rhamnazine. 3,5,4′-Trihydroxy-7,3′-dimethoxyflavone ($C_{17}H_{14}O_7$)	Flavonols	El Karkouri *et al.*, 2020
28	83.39	230, 275, 350	345	**312 (100),** 330 (42), 284 (53), 269 (6)	Penduletin ($C_{18}H_{16}O_7$)	Flavones	Atoui *et al.*, 2005
29	84.51	291, 321,338	375	**359 (100),** 343 (4), 217 (2)	3-O-methylrosmarinic acid ($C_{19}H_{18}O_8$)	Phenolic acids	Mastino *et al.*, 2018

V.2.1. Phenolic acids

Compound 6 (Rt= 46.97 min) presented the precursor ion [M+H]+ at m/z 325 and product ion at m/z 163 (**Figure 53**) corresponding to the loss of a hexose moiety (**Fischer *et al.*, 2011**). This compound was tentatively assigned as ester of caffeic acid (**Atoui *et al.*, 2005**). This compound may play a role in the body's defense against carcinogenesis (**Li *et al.*, 2012**).

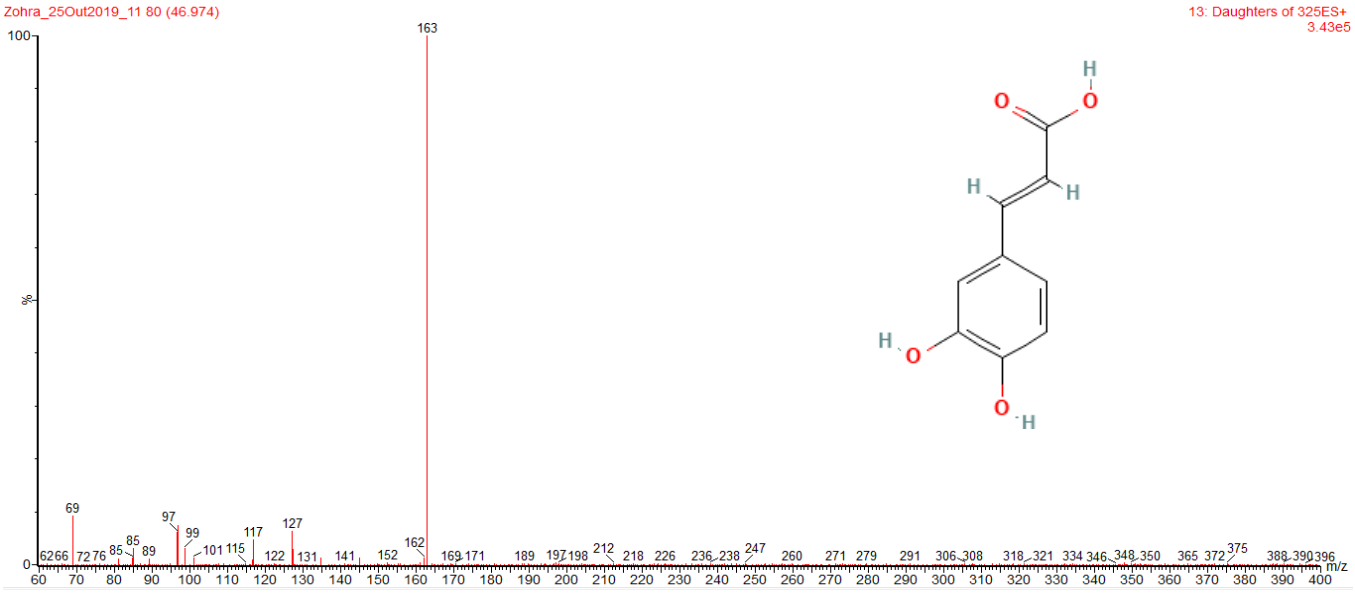

Figure 53: ESI-MS/MS spectra of ester of caffeic acid

Compound 29 (Rt= 84.51 min) was identified as 3-*O*-methyl rosmarinic acid. In its mass-spectra, we can observe the ion at m/z 375 as the base peak and a fragment at m/z 359, 343 and 217 (**Figure (54)**, which allowed its tentative identification according to the literature as 3-*O*-methylrosmarinic acid. This compound has been reported for its anti-inflammatory properties (**Mastino *et al.*, 2018**).

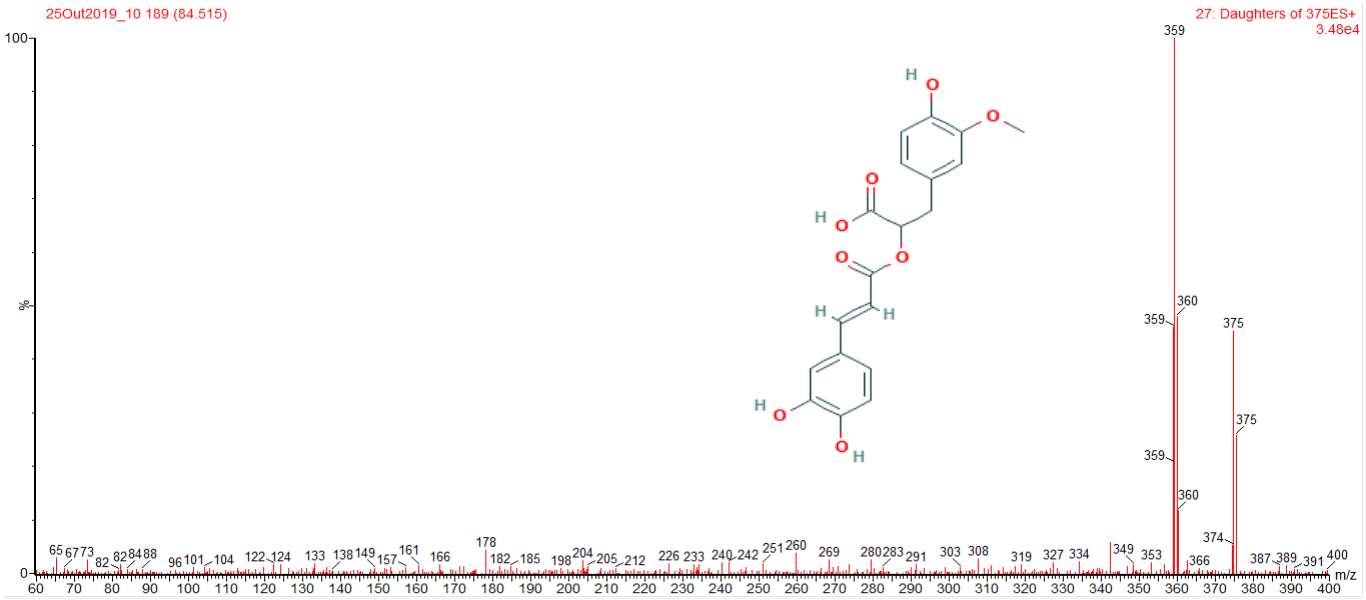

Figure 54: ESI-MS/MS spectra of 3-O-methylrosmarinic acid

V.2.2. Iridoids compomds

Compound 1 eluted at 35.43 min exhibited a protonated ion [M+H] + at m/z 391, confirmed the molecular formula ($C_{19}H_{18}O_9$). This compound has already been identified by the negative mode. Based on the literature and its UV spectra (230 nm), this compound was tentatively assigned as theveside (**Figure 55**). Theveside was previously identified in *Lantana grisebachii* and *Aspido spermaquebracho-blanco* (**Cittadini *et al.*, 2017**), and for the first time in *Aloysia triphylla* species. It has anti-inflammatory, neuroprotective, hepatoprotective and cardioprotective activities. This compound has shown promising efficacy in vitro and in vivo studies with minimal adverse effects and has been shown to be effective in several target organs (**Gorantla *et al.*, 2014**).

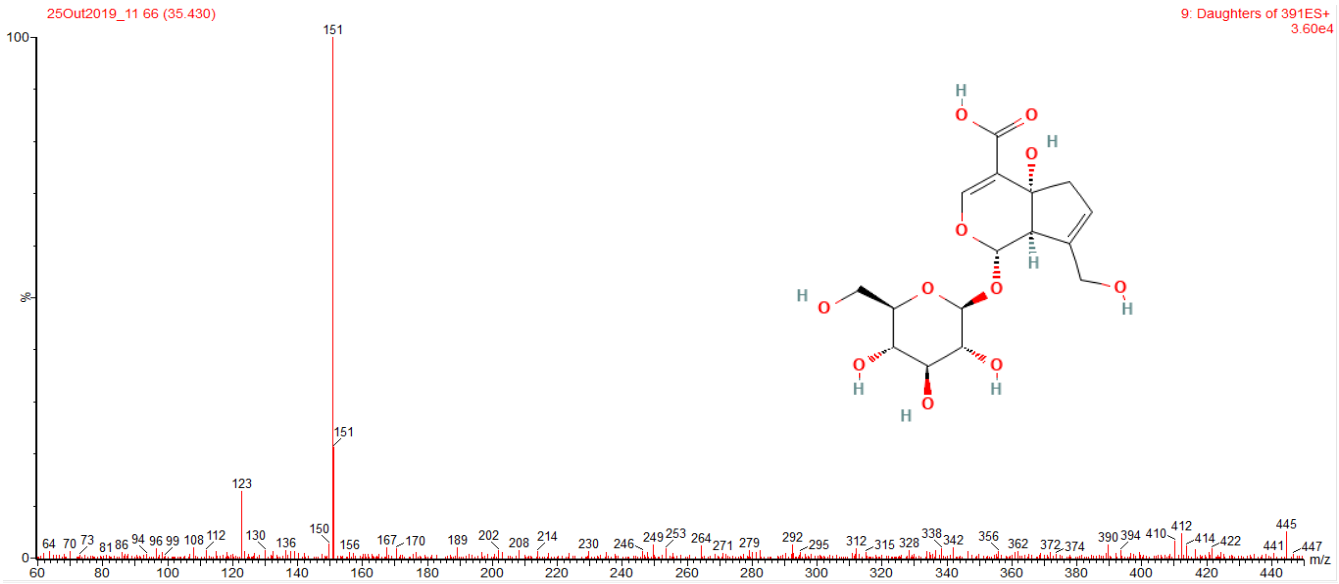

Figure 55: ESI-MS/MS spectra of theveside

V.2.3. Flavonoids compounds

V.2.3.1. Flavones

Flavones always afforded obvious band I (300-380 nm) and band II (240-280 nm) in the UV spectra, in response to the B-ring cinnamoyl conjugated system and A-ring benzoyl system, respectively (**Abad-García *et al.*, 2009**), its structure is shown in the **figure 56**.

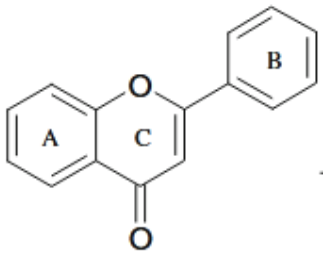

Figure 56: Chemical structure of flavone **(Seo *et al.*, 2010)**

As shown in **Table IX,** 14 flavones were identified with the positive mode.

Compouns 2 (isoflavone) was present with an important peak at R*t*= 40.92 min. It showed a precurssor ion [M+H]+ at m/z 595 and fragment ions at m/z 457, 355, 559, 337, 464, 339, 295,

307 and 427 (**Figure 57**) . It was tentatively identified as genistein-C-glucosylglucoside (**Hong *et al.*, 2019**).

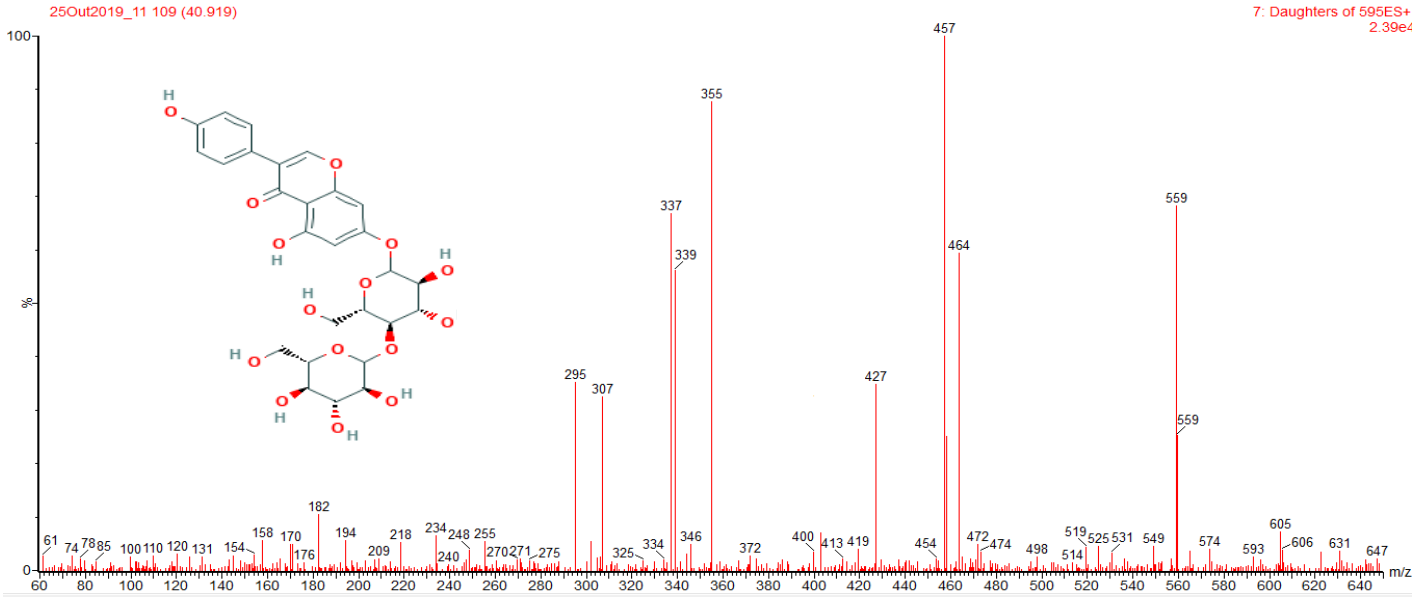

Figure 57: ESI-MS/MS spectra of genistein-C-glucosylglucoside

Genistein is a phytoestrogen with diverse biological activities. It is a potent antioxidant and antibrowning agent *in vivo* and *in vitro*. This molecule acts as a preventative and therapeutic effects for cancers, postmenopausal syndrome, osteoporosis and cardiovascular diseases in animals and humans (**Mazumdera and Hongsprabhas, 2016**).

Already identified by the negative mode, **compound 3** (R*t*= 44.06) was tentatively assigned as luteolin-7-diglucuronide (m/z 639), with a fragment ion at m/z 287 (100 %) (**Figure 58**). It was previously reported in *Aloysia triphylla* leaves (**de la Luz Cádiz-Gurrea *et al.*, 2018; Diez-Echave *et al.*, 2020**). This compound is known by its antioxidant potential (**Sánchez-Marzo *et al.*, 2019**).

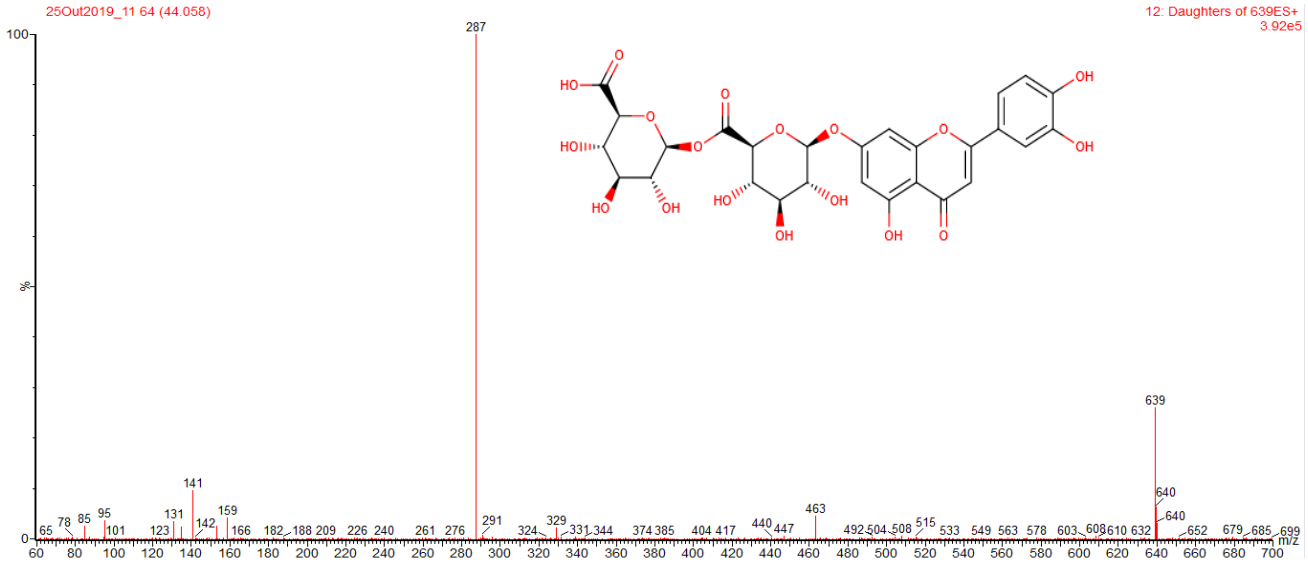

Figure 58: ESI-MS/MS spectra of luteolin-7-diglucuronide

Represented by an intense peak, **compound 5** was identified as apigenin-7-diglucuronide (**Atoui *et al.*, 2005**). This compound presents a precursor ion [M+H]+ at m/z 623 and MS/MS product ions at m/z 271 (100%) (**Figure 59**), it was already characterized in the negative mode. Apigenin-7-diglucuronide (A7DG), is present in a variety of medicinal plants. This compound provides remarkable photoreceptor protection in mice exposed to bright light, attenuates photoreceptor apoptosis, reduces oxidative stress, and reduces pro-inflammatory gene expression in retinas exposed to bright light (**Bian *et al.*, 2017**).

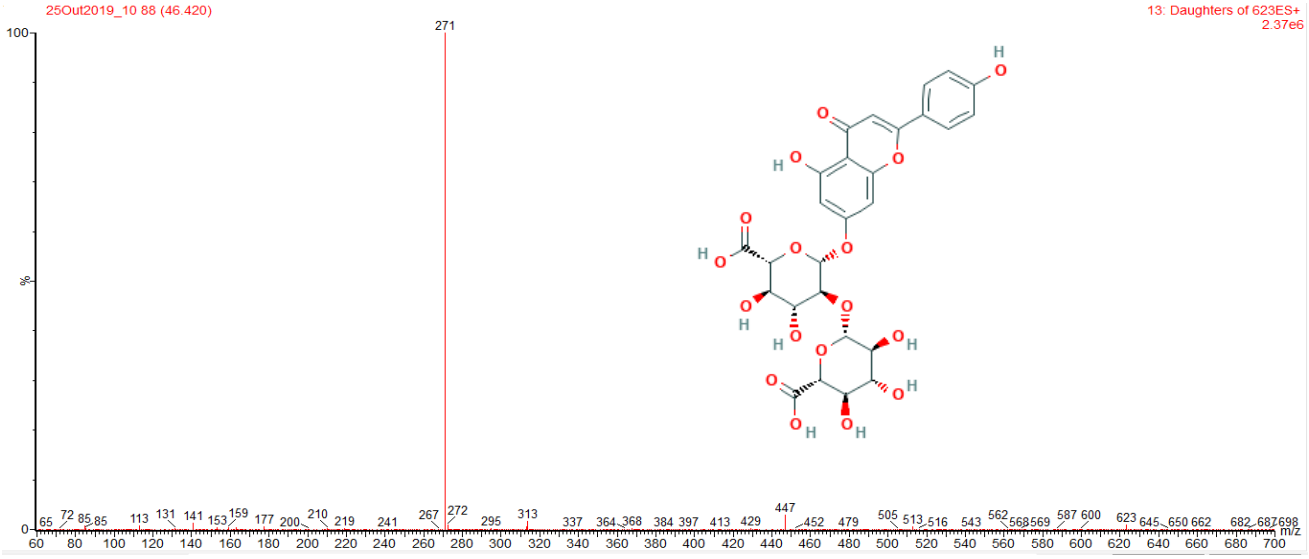

Figure 59: ESI-MS/MS spectra of apigenin-7-diglucuronide

Compound 7 was represented with an intense peak. It was tentatively assigned to quercetin 3-*O*-glucoside (**Figure 60**), with a parent ion at m/z 465 and a quercetin aglycone fragment ion at m/z 303 which was due to the loss of glucose **(Wittig *et al.*, 2001; Lin *et al.*, 2008**). This molecule possesses antimicrobial and antioxidant effects (**Razavi *et al.*, 2009**).

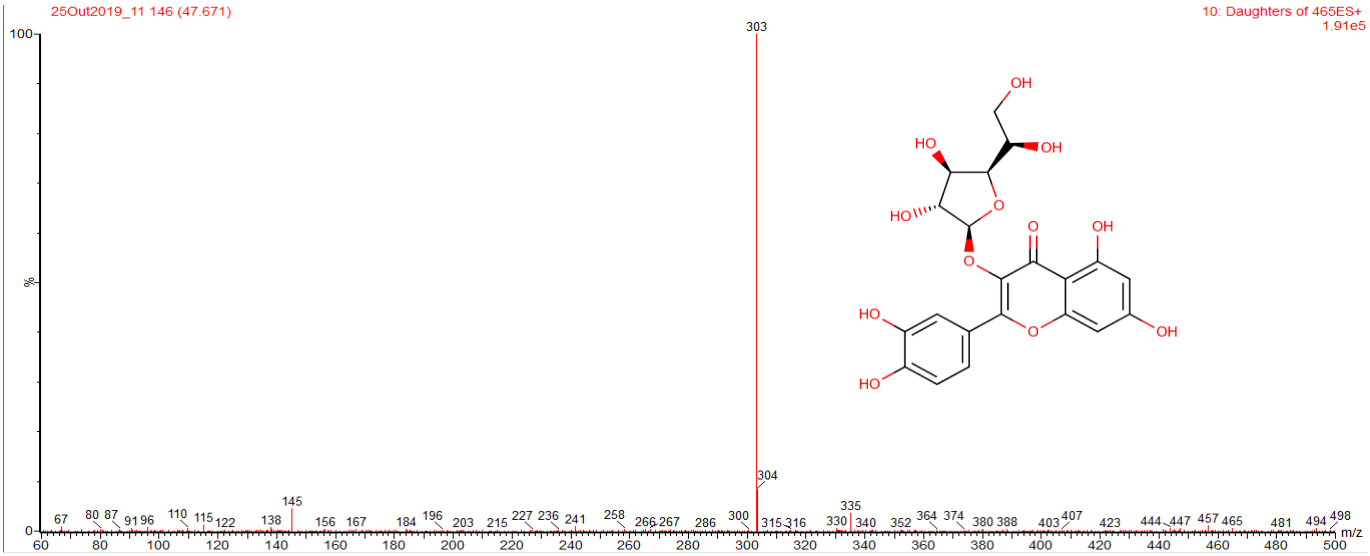

Figure 60: ESI-MS/MS spectra of quercetin 3-O-glucoside

Compound 8 is a flavonoid compound with base ion at m/z = 653 [M+H]+. It gives a fragment ion at m/z 301 [Aglycone-H]+ (**Figure 61**), indicating the presence of chrysoeriol-7-diglucuronide ($C_{28}H_{28}O_{18}$) (**Timóteo *et al.*, 2015)**. Already characterized with the negative mode, this flavonoid has been previously identified in the aerial parts of *Aloysia triphylla*. (**Quirantes-Piné *et al.*, 2009; de la Luz Cádiz-Gurre *et al.*, 2018; Leyva-Jiménez *et al.*, 2019; Sánchez-Marzo *et al.*, 2019**), and in species belonging to the verbenaceae *f*amily like *Lippia alba* (**Timóteo *et al.*, 2015**).

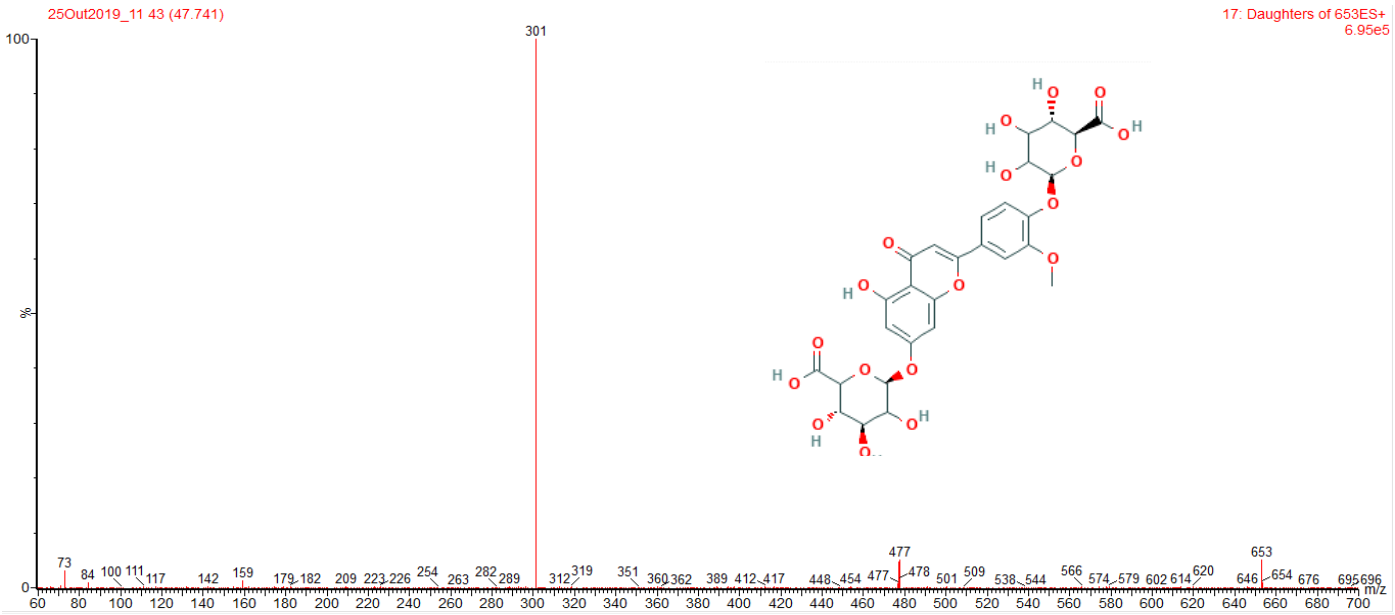

Figure 61: ESI-MS/MS spectra of chrysoeriol-7-diglucuronide

Compound 13 was detected and tentatively characterized as apigenin-7-*O*-glucoside (Apigetrin) (t_R= 50.86 min), with parent ion at m/z 433. According to its MS/MS spectra, this compounds had the fragment ion at m/z 271 (**Figure 62**), indicating the existence of apigenin in the structure (**Abu-Reidah *et al.*, 2015**). Detailed pharmacological studies have proven the biological importance of apigetrin in medicine for its anticancer activity and treatment of neurodegenerative disorders. In addition, apigetrin significantly enhances the expression of antioxidant enzymes by increasing radical scavenging activity and shows a significant antibacterial activity against *Escherichia coli* (**Patel and Patel, 2021).**

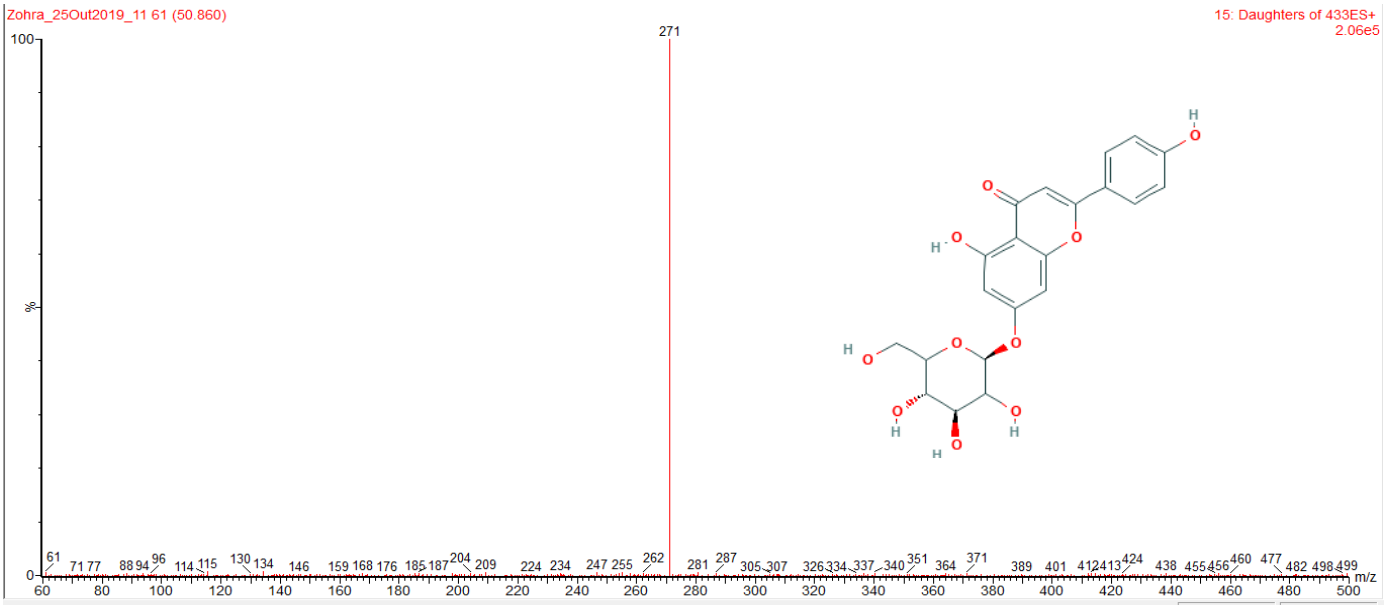

Figure 62: ES-/MS/MS spectra of apigenin-7-O-glucoside

Compound 14 was represented by an intens peak at 51.22 min. It showed a precursor ion [M+H]+ at m/z 447 and produced a prominent ion at m/z 271 (**Figure 63**), due to loss of glucuronyl ([Aglycone+H]+). When compared with literature, this compound was characterized as apigenin 4′-*O*-glucuronide (**Xiao *et al.*, 2016**). It is used in the clinic for the treatment of cerebral diseases (**Taupi, 2009**).

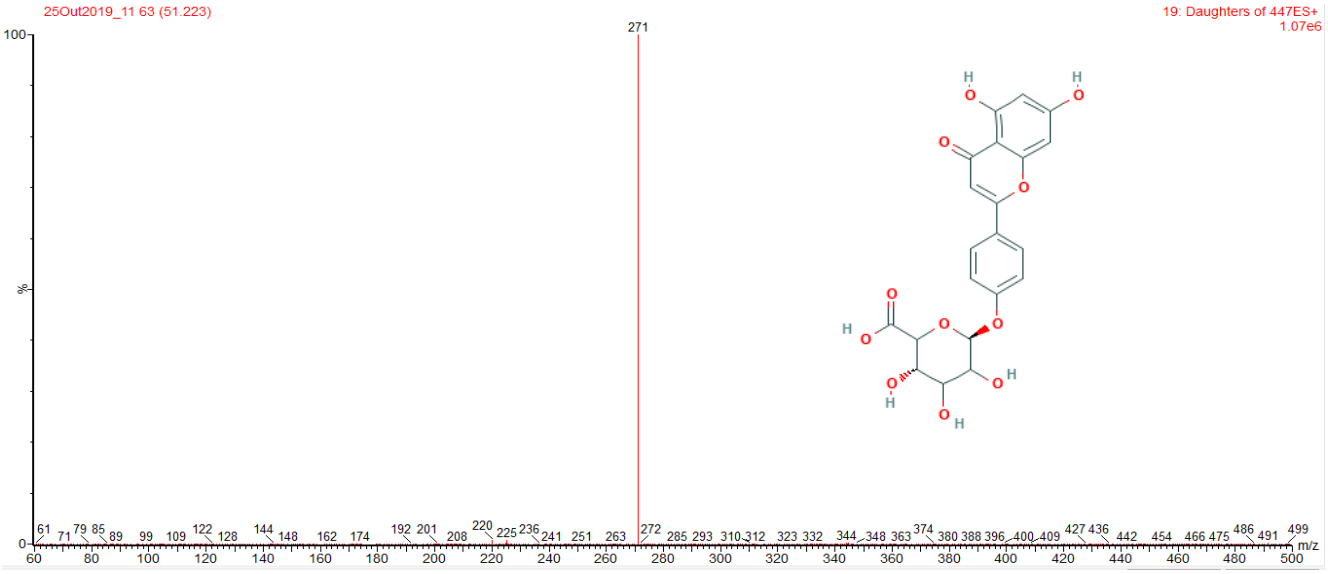

Figure 63: ESI-MS/MS spectra of apigenin 4′-O-glucuronide

Compound 15 gave the [M+H]+ ion at m/z 271, the positive ion mode showed an intense peak at R*t*= 51.66 min, with a product ion at m/z 153 (**Figure 64**) corresponding to the missing of ring A ($[^{1,3}A]+$) and m/z 119 corresponding to the missing of the ring B from the flavonoids ($[^{1,3}B]_+$). Previously characterized by the negative mode, this compound was unambiguously identified as apigenin (4',5,7-trihydroxyflavon) by comparison with data from the literature (**Mahfoudhi *et al.*, 2014; Rafi *et al.*, 2020**). Apigenin was also recorded at R*t*= 68.21 min with fragment ions at m/z 153 and 119 (**Compound 24)**. Apigenin is one of the most common compounds in the plant kingdom, found in vegetables, fruits and beverages, it is used for the treatment of photoreceptor degeneration through regulation of microglial activities (**Chumsakul *et al.*, 2021)**. It is also known for its anti-cancer therapeutic potential (**Ahmed *et al.*, 2021**), antioxidant, anti-inflammatory and antiviral activities **(Yan *et al.*, 2017**).

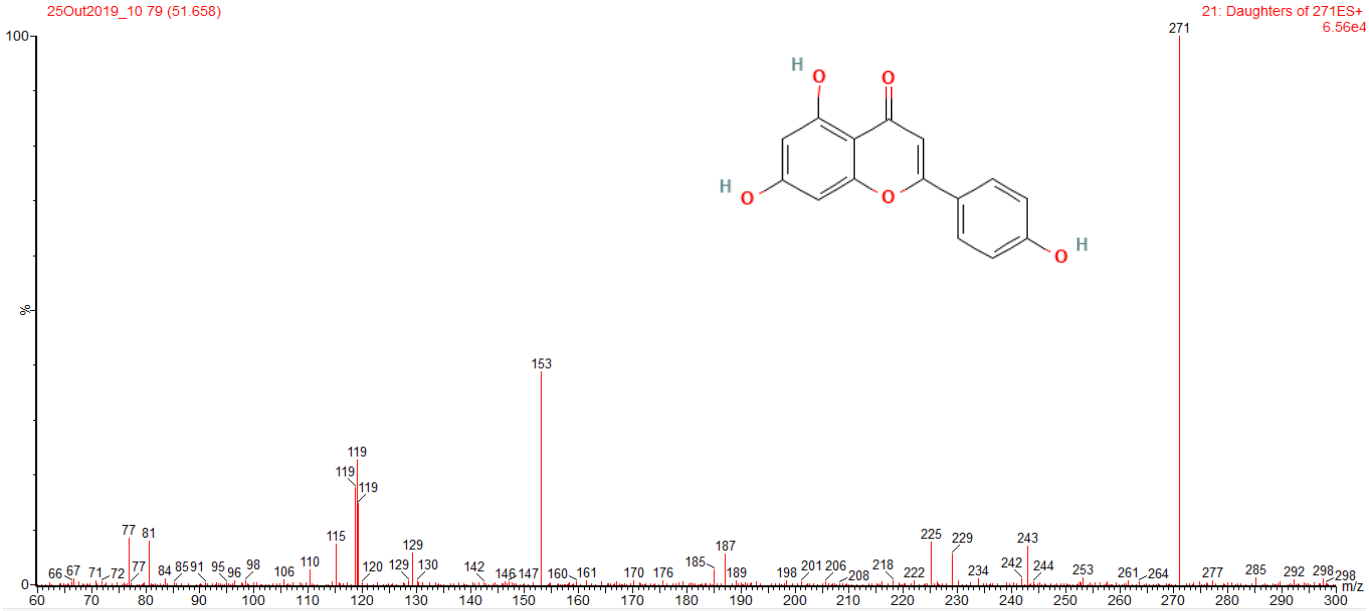

Figure 64: ESI-MS/MS spectra of apigenin

Compound 17, was represented by an intens peak at R*t*= 51.89 min, the presence of luteolin 7-*O*-glucoside has been demonstrated by [M+H]+ ion at m/z 449 which gives $[M+H\text{-hexose}]^+$ ion at m/z 287 as shown in the **figure 65** (**N'gaman-Kouassi *et al.*, 2016**). According to **Bilia *et al.* (2008)**, luteolin 7-glucoside possesses an antioxidant activity.

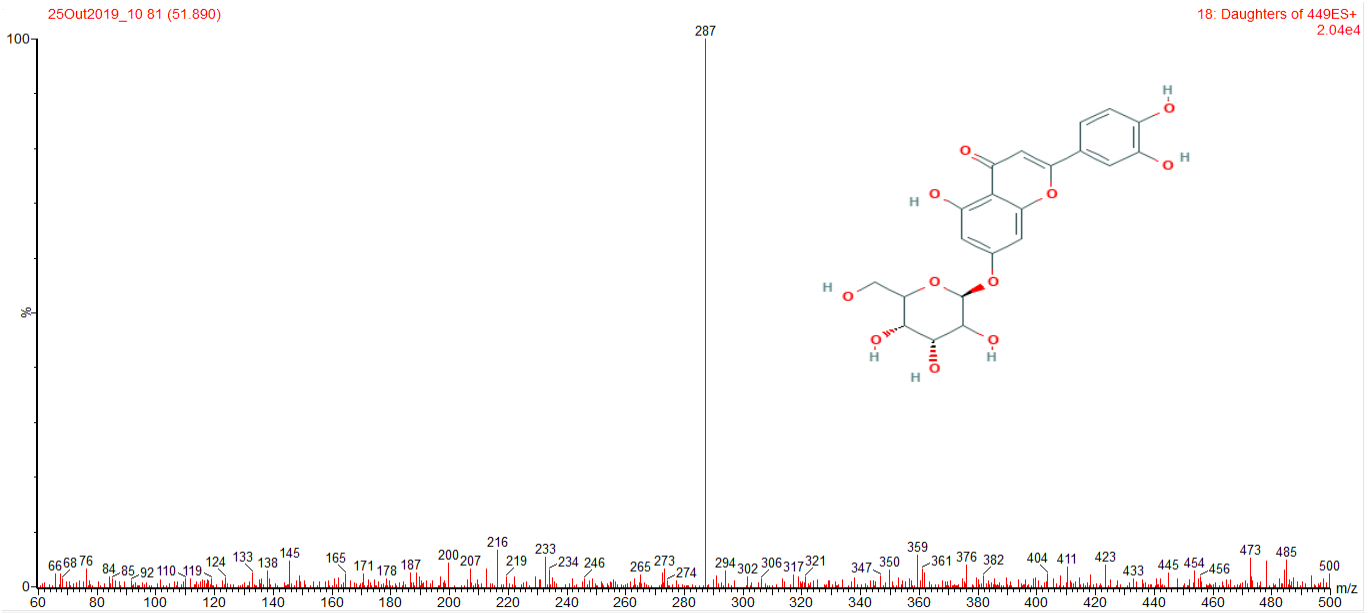

Figure 65: ESI-MS/MS spectra of luteolin 7-O-glucoside

Represented with an intense peak at R*t*= 52.04 min, **Compound 18** was tentatively identified as tricin-7-*O*-glucuronide. It showed a base ion [M+H]+ at m/z 507, and a fragment ion at m/z 331 (**Figure 66**), due to the loss of aglycone **(Timóteo *et al.*, 2015)**. This compound could have antioxidant and anti-inflammatory properties (**Ayoub *et al.*, 2022**).

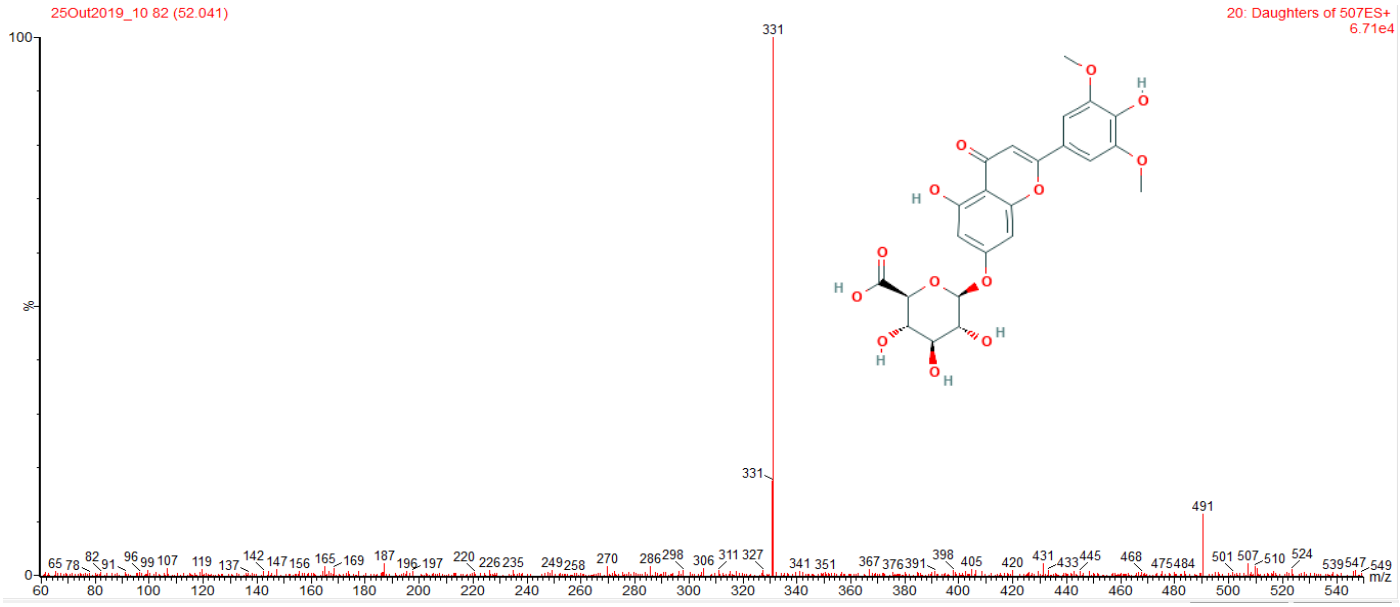

Figure 66: ESI-MS/MS spectra of tricin-7-O-glucuronide

Compound 19 showed a [M + H]+ base ion at m/z 637, with a fragment ion peak at m/z 285 in the secondary mass spectrum (**Figure 67**). Therefore, according to the literature, this compound was tentatively identified as acacetin 7-diglucuronide. As for luteolin-7-diglucuronide (peak 3, m/z 639), apigenin-7-diglucuronide (peak 4, m/z 623), chrysoeriol-7-diglucuronide (peak7, m/z 653), glucuronidation occurs in plants for enhancing the flavonoid solubility in water and favoring its accumulation in vacuoles (**Sánchez-Marzo *et al.*, 2019**). This compound was previously reported in *Aloysia triphylla* (**de la Luz Cádiz-Gurrea *et al.*, 2018; Olivares-Vicente *et al.*, 2019; Sánchez-Marzo *et al.*, 2019**).

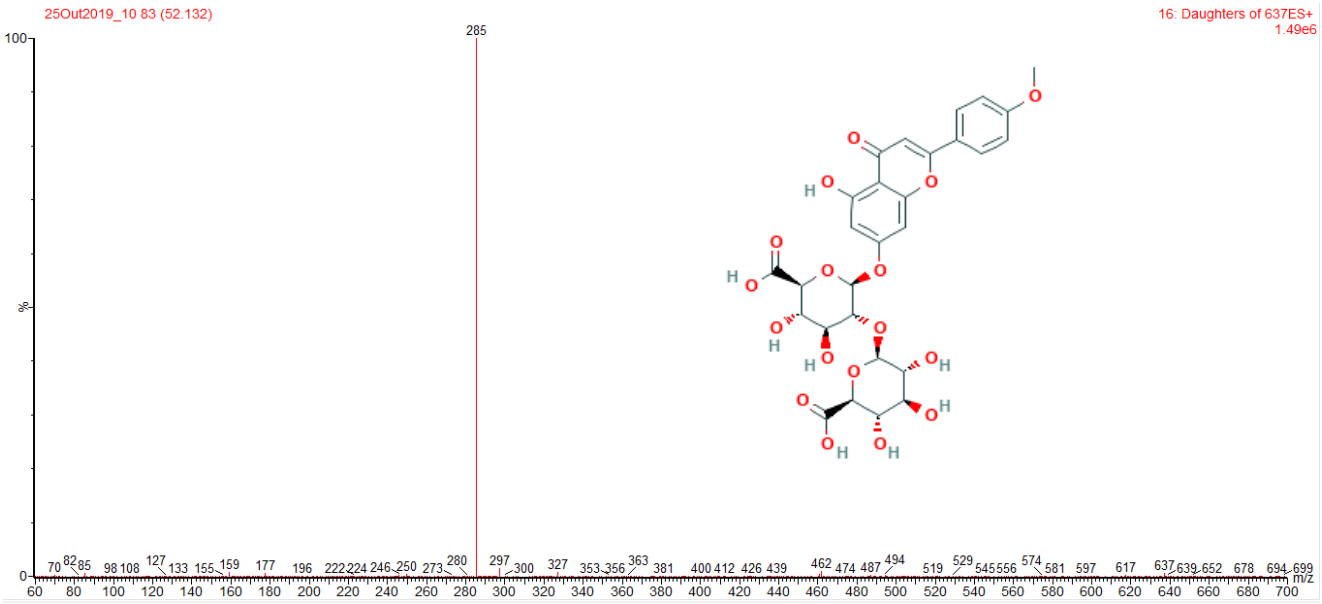

Figure 67: ESI-MS/MS spectra of acacetin 7-diglucuronide

According to **Hu *et al.* (2012)**, **compound 20** whose m/z 477, with a product ion at m/z 301 and 286 (**Figure 68**) was tentatively identified as 5,6,7-trihydroxy-8-methoxy flavone-7-*O*-glucuronide. It was presented with an intense peak.

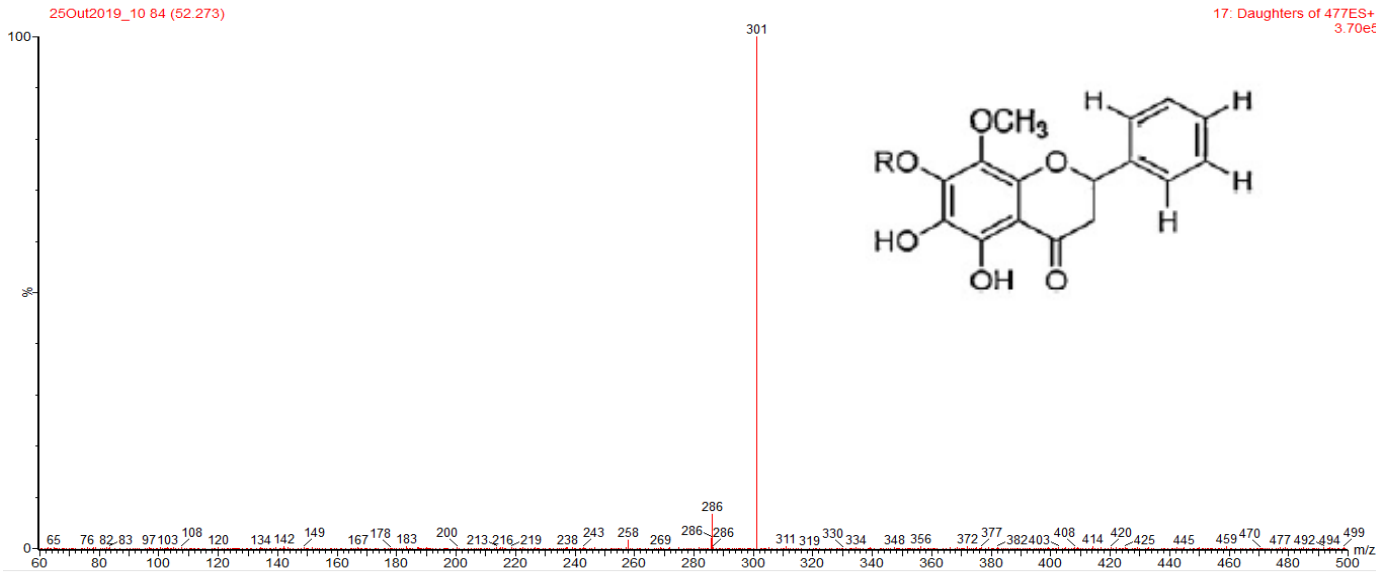

Figure 68: ESI-MS/MS spectra of 5,6,7-trihydroxy-8-methoxy flavone-7-O-glucuronide

Compound 21 (m/z 285; $C_{16}H_{13}O_5$+), shown in **figure 69** was identified as methyl apigenin, known as acacetin or linarin (5, 7-dihydroxy-4-methoxyflavon) (**Eissa *et al.*, 2020**). It is a natural flavonoid, known to possess many pharmacological properties, including neuroprotective, cardioprotective, anticancer, anti-inflammatory, antidiabetic and antimicrobial activities. This molecule is widespread throughout the plant kingdom and has been shown to have strong inhibitory effects on glutathione reductase, cyclooxygenase and acetylcholinesterase (**Semwal *et al.*, 2019**).

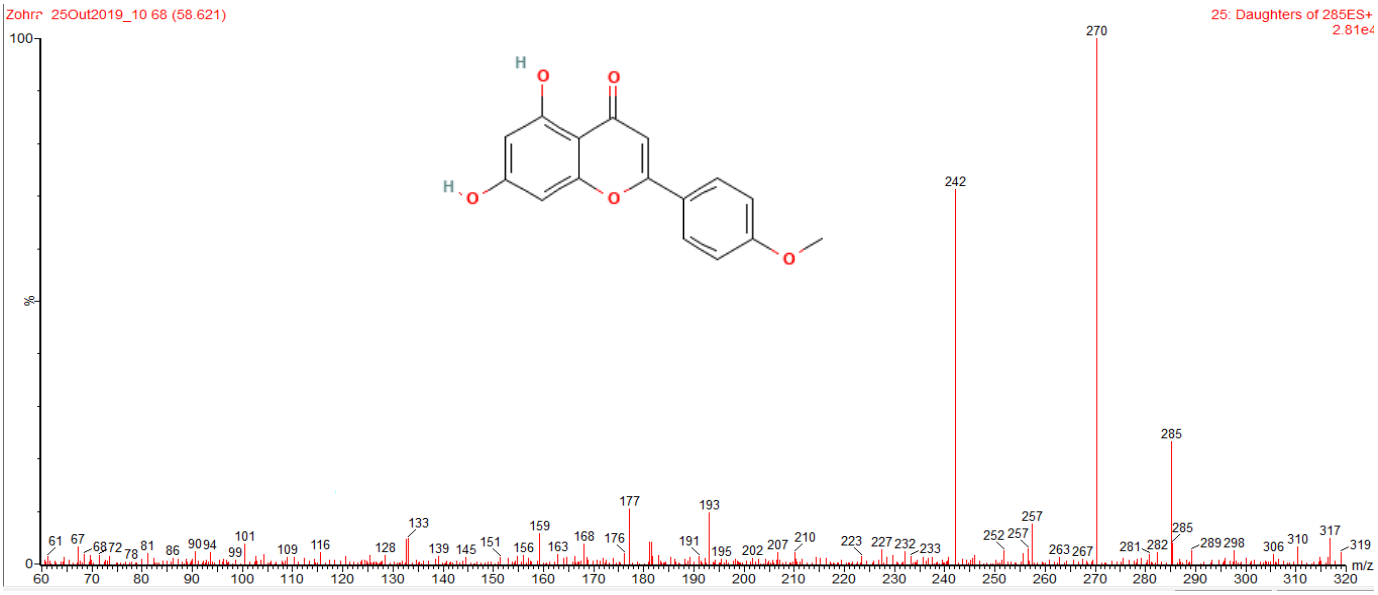

Figure 69: ESI-MS/MS spectra of acacetin

Compound 22 eluted at 59.52 min generated an [M+H]+ ion at m/z 287, which is consistent with the molecular weight of a [luteolin+H]+ ion. As shown in the **figure 70**, it gave a first signals at m/z 241, due to the loss of -CO and H_2O and a second signal at m/z 153 (**Gao *et al.*, 2017**). Luteolin has been showing numerous therapeutic activities such as anti-cancer, anti-inflammatory, antioxidant, and antimicrobial activities (**Manzoor *et al.*, 2019**).

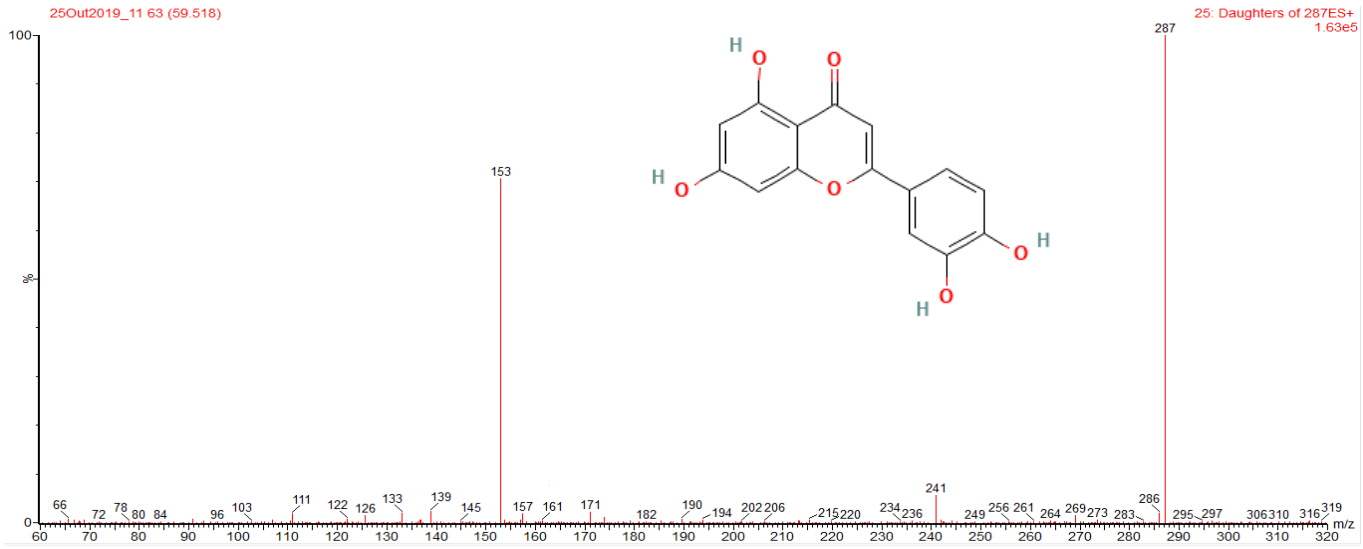

Figure 70: ESI-MS/MS spectra of luteolin

Compound 28 was represented by an important peak. It showed a precursor ion at m/z 345 [M+H]+ and fragment ions at m/z 312, 284 and 269 (**Figure 71**). It was identified as penduletin (5,4′-Dihydroxy-3,6,7-trimethoxyflavone) (**Atoui *et al.*, 2005; El-Gazar *et al.*, 2022**). According to **Lo *et al.* (2022)**, this compound possesses antibacterial and anti-cancer activities (**Vo *et al.*, 2022**).

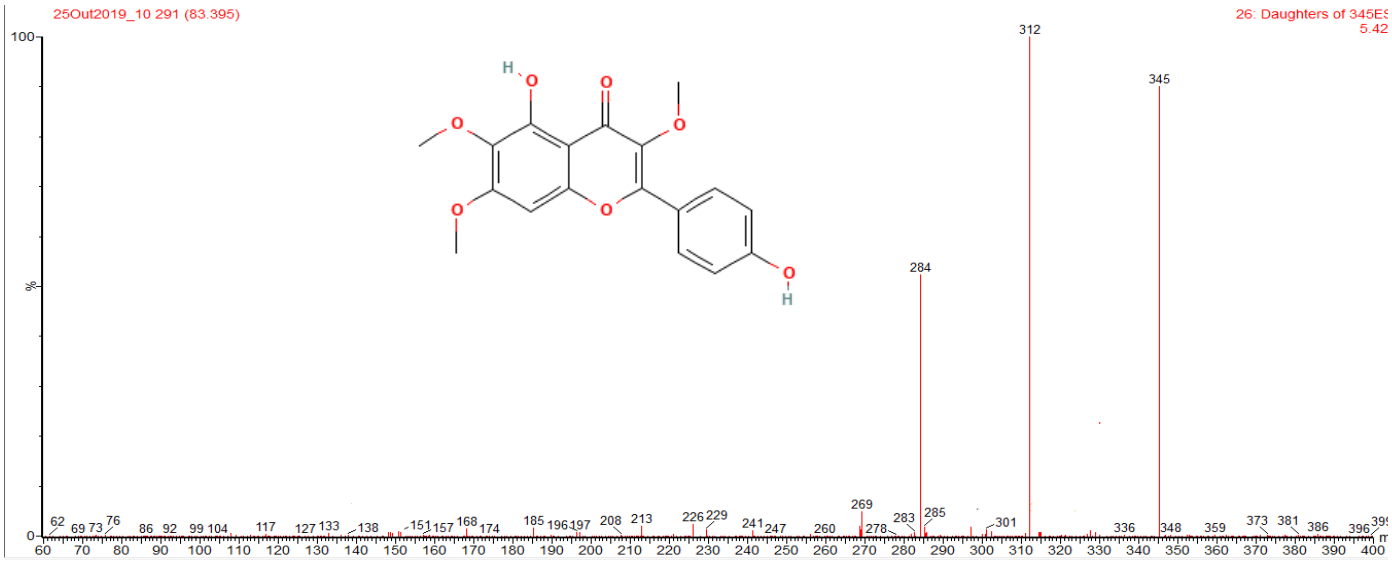

Figure 71: ESI-MS/MS spectra of penduletin

V.2.3.2. Flavonols compounds

As illustrated in the **figure 72**, the flavonol glucosides quercetin-7-*O*-glucuronide (**Compound 4)**, was identified by the ion [M+H]+ at m/z 479 and its fragment ion at m/z 303 [$C_{15}H_{10}O_7$+H]+ (**Carazzone *et al,* 2013; Wu *et al.,* 2018**). This compound is an important derivative of quercetine, known for its antioxidant, anti-inflammatory activities (**Hai *et al.,* 2020**).

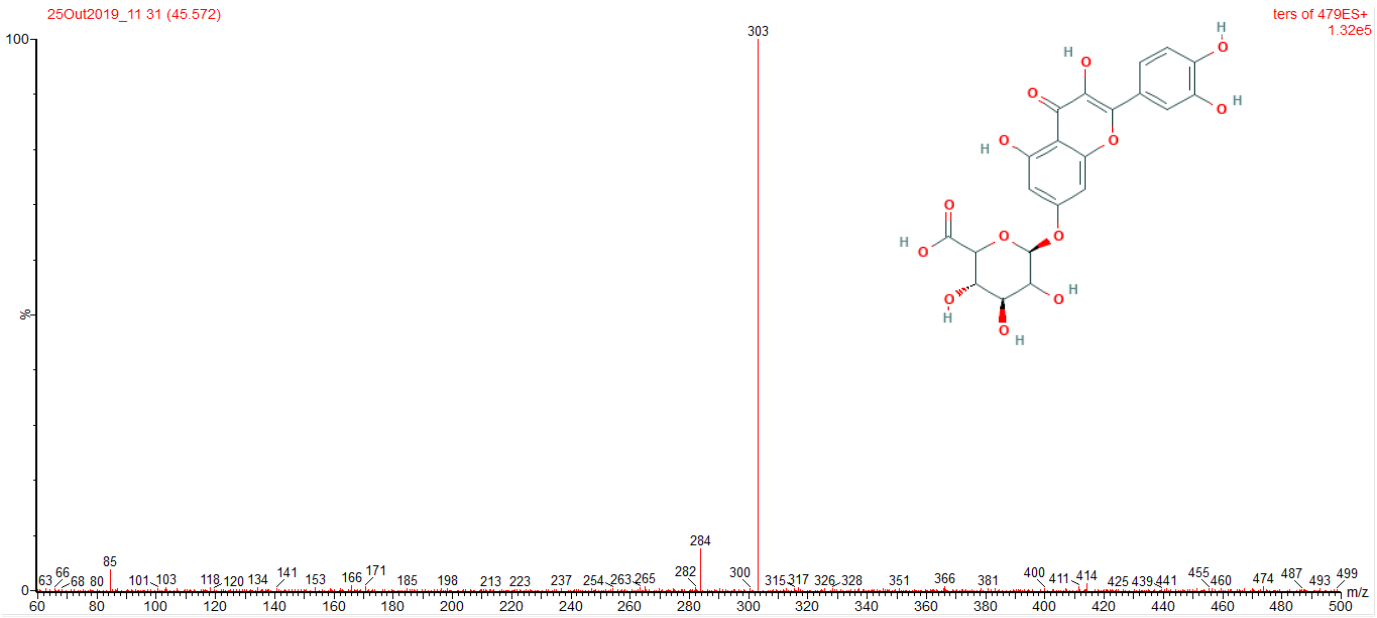

Figure 72: ESI-MS/MS spectra of quercetin-7-*O*-glucuronide

At R*t*= 48.25 min, **Compound 9** with a base ion [M+H] + at m/z 463 was represented with an intense peak, it was tentatively identified as Kaempferol-(2”-*O*-methyl)-4'-*O*-α-D-glucopyranoside, a kaempferol derivatives (**Figure 73**). This molecule exhibited the loss of 162 amu during MS analysis, indicating the presence of a hexose, probably a galactosyl or a glucosyl group, showing a fragment ion at m/z 287 [M -176]+ (**Rezende *et al.,* 2019**). Many preclinical studies have shown that kaempferol and some glycosides of kaempferol have extensive pharmacological activities, including antioxidant, anti-inflammatory, anticancer, antimicrobial, cardioprotective, neuroprotective, antidiabetic, anti-osteoporotic,

estrogenic/antiestrogenic, anxiolytic, analgesic and antiallergic activities (**Villers and Fougere, 2013**).

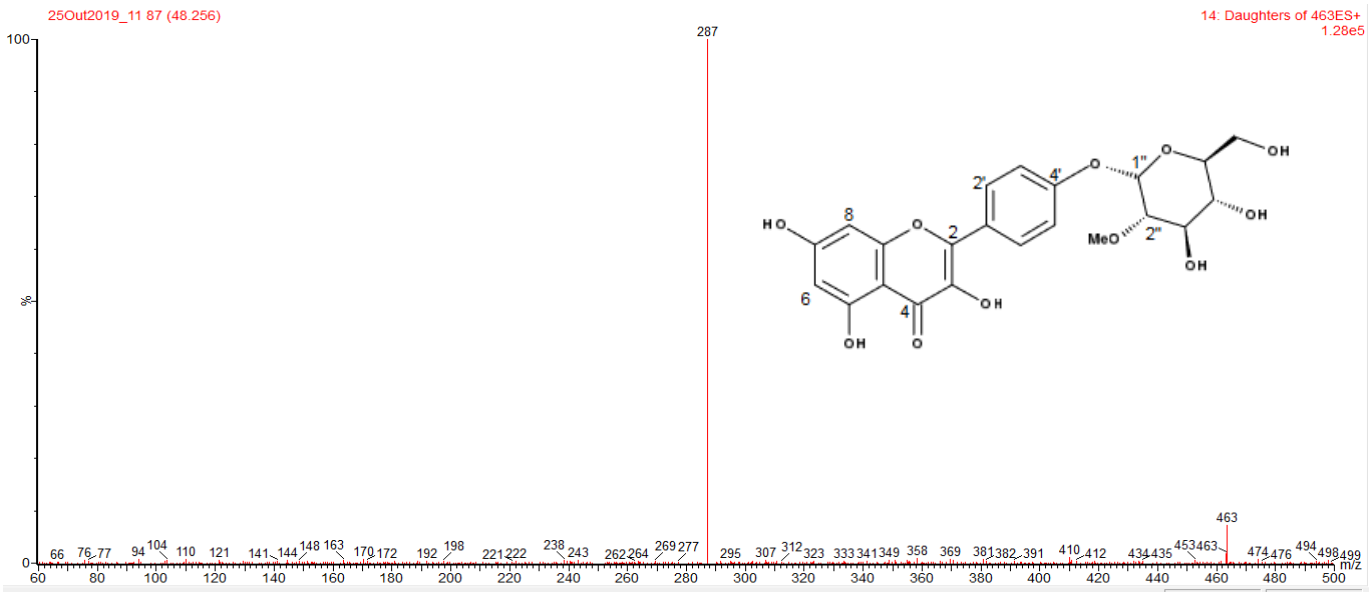

Figure 73: ESI-MS/MS spectra of Kaempferol-(2”-*O*-methyl)-4'-*O*-α-D-glucopyranoside

Present with an intense peak, **compound 10** shows the [M+ H]+ ion at m/z 449 and the main fragment ion at m/z 287 [M+ H− Glu]+ (**figure 74**), to indicate an aglycon moiety. The UV-DAD profile (330 nm) is typical of kaempferol. This compound was confirmed by the comparison with the literature reports (**Carini *et al.*, 2001**) as the Kaempferol-3-*O*-glucoside (astragalin). Astragalin is a bioactive constituent of various traditional medicinal plants such as Litchi pericarp; *Cuscuta chinensis; Azadirachtaindica* (**Lin *et al.*, 2014; Riaz *et al.*, 2018; Rao *et al.*, 2018**). It is well known for its diversified pharmacological applications such as anti-inflammatory, antioxidant, neuroprotective, cardioprotective, antiobesity, antiosteoporotic, anticancer, antiulcer, antimicrobial and antidiabetic properties (**Riaz *et al.*, 2018; Ivanov *et al.*, 2020**).

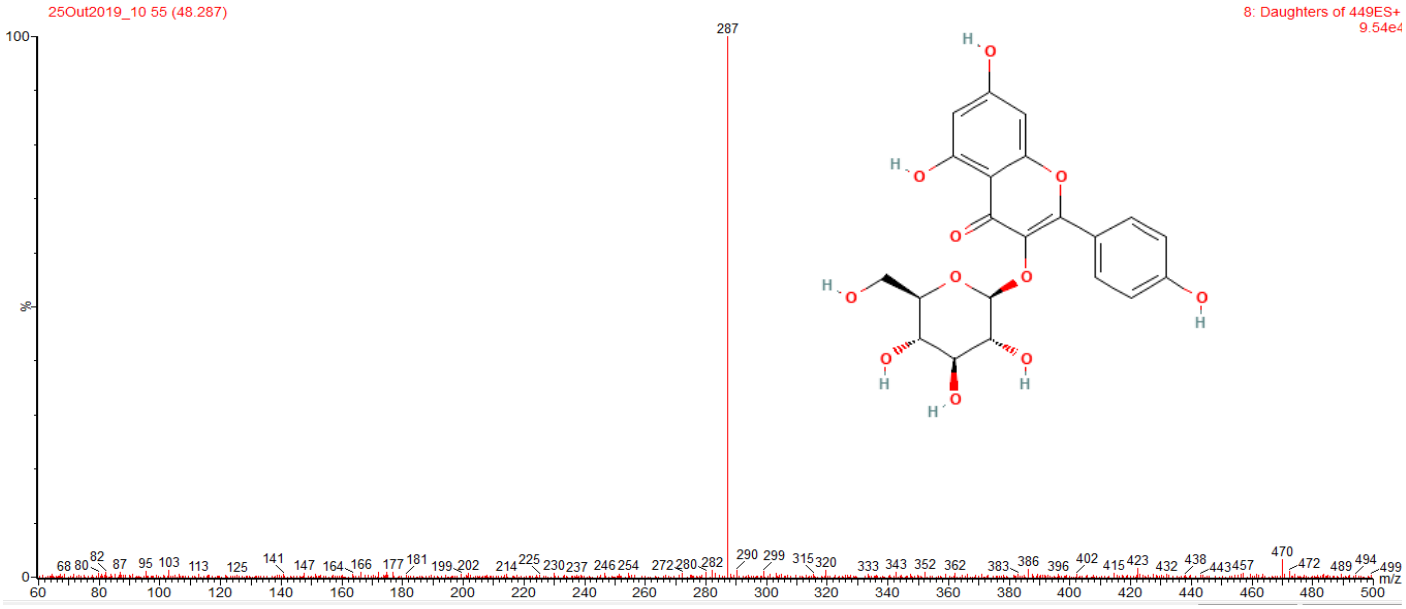

Figure 74: ESI-MS/MS spectra of astragalin

C**ompound 11** (R*t*= 48.84 min) was present with a significant peak. It has been tentatively characterized as isorhamnetin-3-*O*-glucoside, the (+) ESI-MS provided a precursor ion [M+H]+ at m/z 479. MS^2 spectrum, showed fragment ions at m/z 317 ([M+H-162]+) corresponding to the loss of $C_6H_{10}O_5$ (glucosyl), and at m/z 302 ([M+H-162-15]+) (**Figure 75**), which was relatively corresponding to the loss of CH_3 (**Lee *et al.*, 2012; Yao *et al.*, 2012**). Isorhamnetin-3-*O*-glucoside is known to exert various pharmacological activities such as antiproliferative and antioxidant activities (**Hadj Salem *et al.*, 2011**).

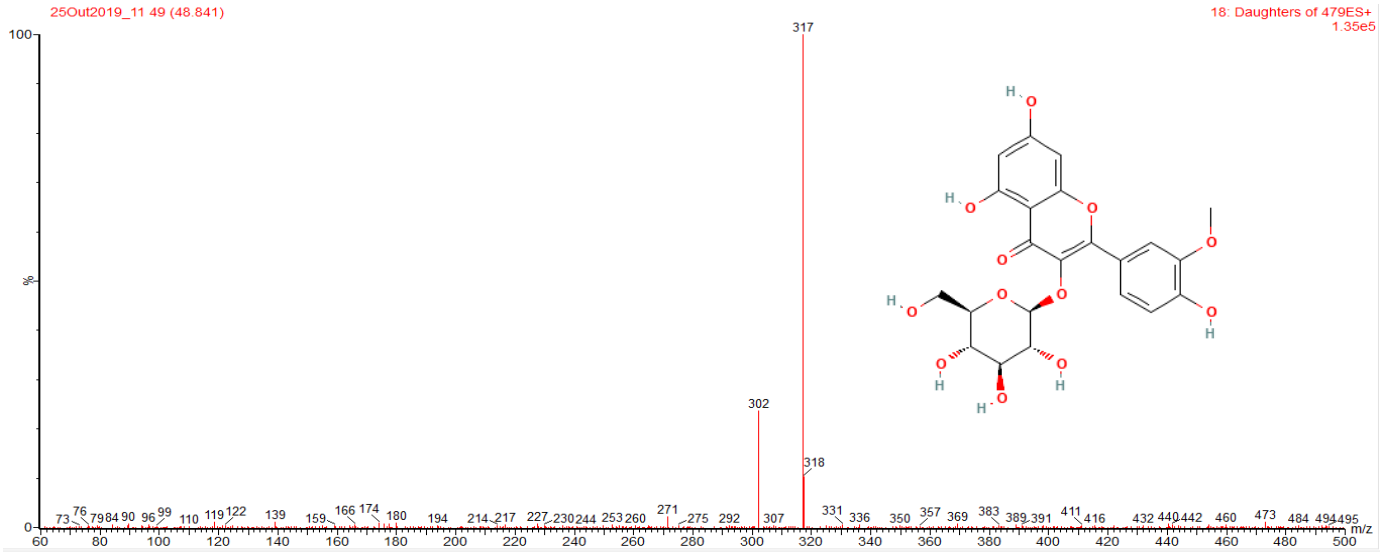

Figure 75: ESI-MS/MS spectra of isorhamnetin-3-O-glucoside

Compound 16 (R*t*= 51.73 min) was recognized as the methylated flavonol kaempferide. The loss of a methyl group (-15 amu) generated a relevant product ion at m/z 286 [M+H-CH3]+ in the MS^2 experiments (**Mahfoudi *et al.*, 2014**), as illustrated in the **figure 76**.

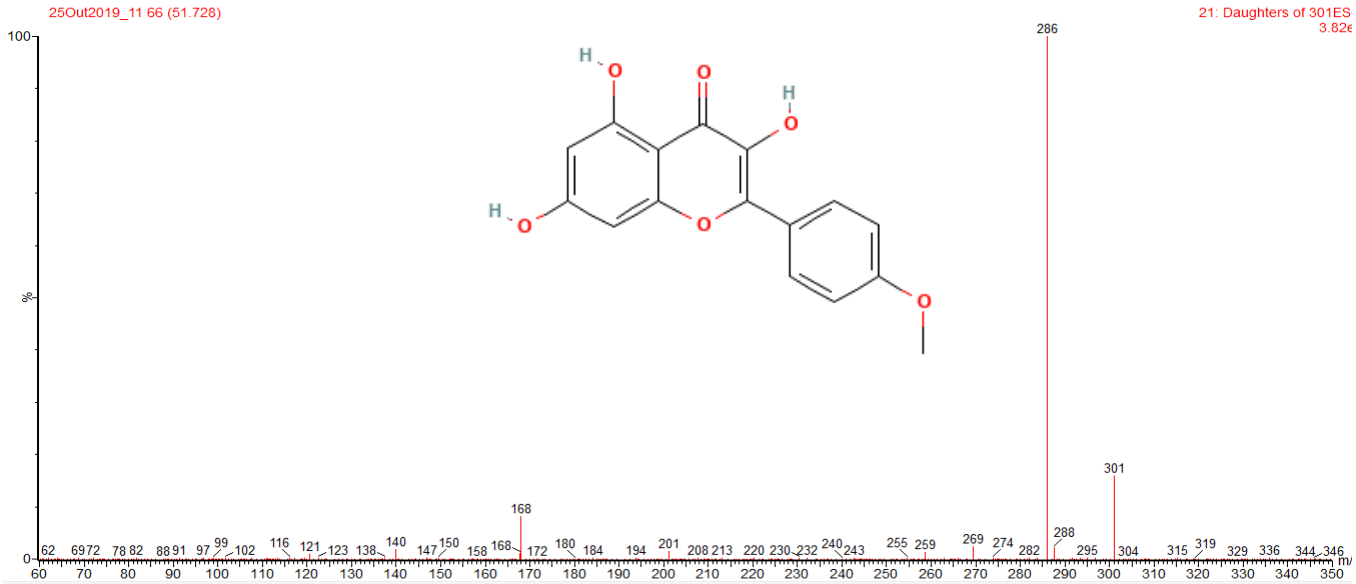

Figure 76: ESI-MS/MS spectra of kaempferide

Isorhamnetin (**compound 23**), was represented by a significant peak. It was confirmed by its MS^2 spectra in positive ionization mode (**Figure 77**). MS^2 of [M+H]+ at m/z 317 showed a peak at m/z 302, which corresponds to the loss of methyl group (CH_3). Further fragmentation showed ions at m/z 301 (loss of H) and 228 [M+H-CH_3-C_2O_2H-OH]+. This suggests that this compound was the isorhamnetin aglycone (**Boukhris *et al.*, 2012;Vuković *et al.*, 2017)**. The aglycone compounds were more active than their glycosylated derivatives. Isorhamnetin aglycone presented high antiproliferative and antioxidant properties (**Salem *et al.*, 2011**).

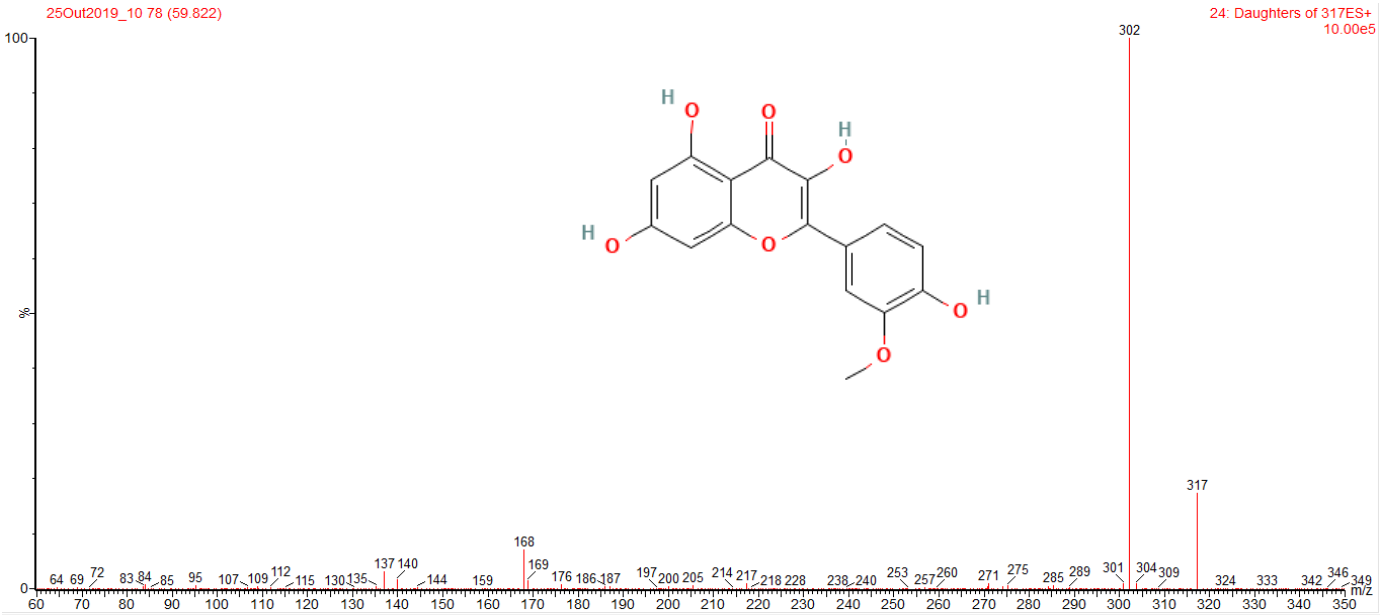

Figure 77: ESI-MS/MS spectra of Isorhamnetin

Compound 25 showed in the **figure 78,** was tentatively identified as diosmetin (3', 5, 7-trihydroxy-4'-methoxyflavone). It was protonated in the positive ion mode to produce the ion m/z 301 [M+H]+, then lost methyl ([M + H-CH_3]+), to obtain m/z 286, and further lost the CO group ([M+H-CH_3-CO]+) to obtain m/z 258 [M + H–CH_3-CO]+ (**Ding *et al.*, 2021**). This compound has been reported to exhibit various pharmacological activities, including anti-cancer, anti-inflammatory and antioxidant activities effect on central nervous system,

oestrogenic and antimicrobial activities, especially against *Helicobacter pylori* (**Pate et *al.*, 2013; Wang *et al.*, 2019**).

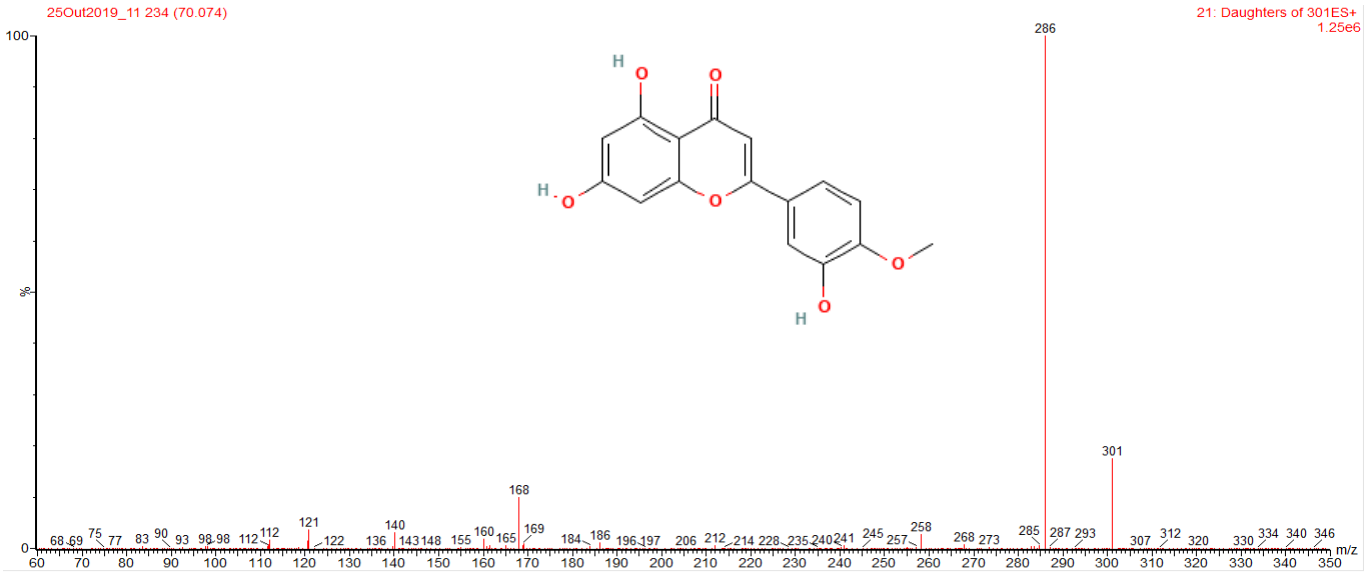

Figure 78: ESI-MS/MS spectra of diosmetin

The kaemperide isomer (**compound 26**, [M+ H]+ ion at m/z 301) was detected at 72.04 min. According to **Hao *et al.* (2021)**, this compound is isokaempferide (**Figure 79**). In vitro studies have shown that this compound has hepatoprotective, antimicrobial, and antiproliferative activities with pharmacological safety (**Leal *et al.*, 2008**).

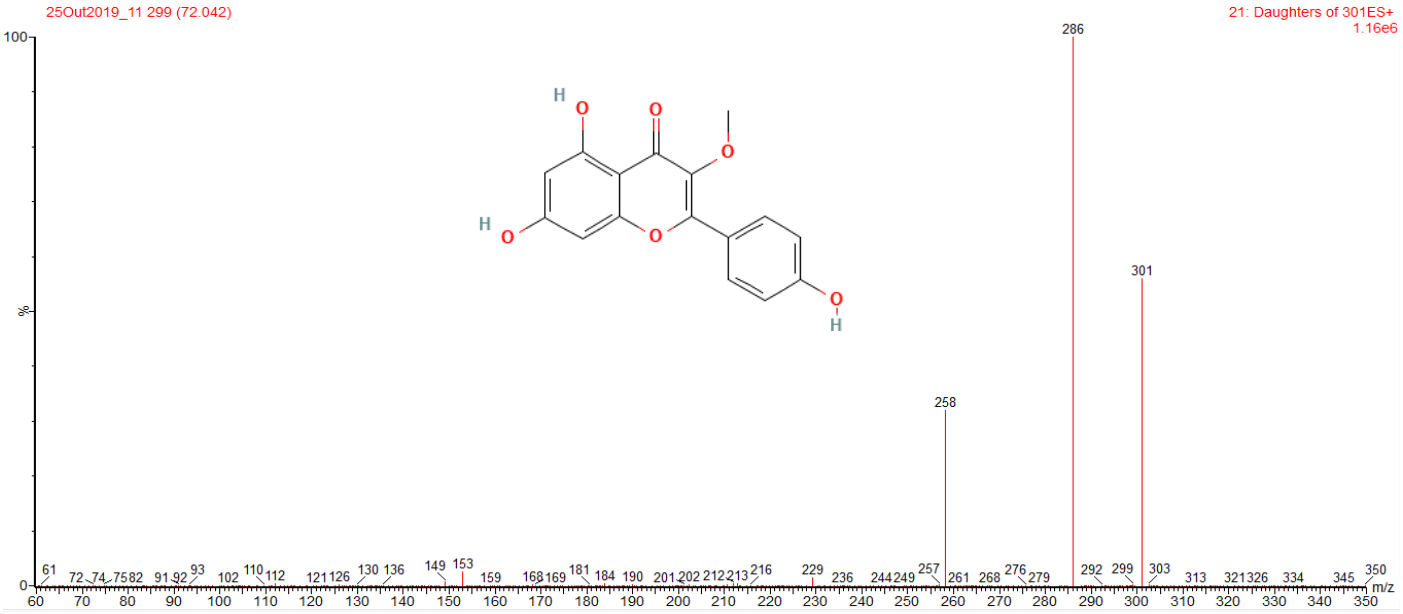

Figure 79: ESI-MS/MS spectra of Isokaempferide

Presented with an intense peak, **compound 27** gave a protonated ion [M+H]+ at m/z 331,with fragment ions at m/z 316 [M+H-CH3]+, m/z 301 [M+H-2CH3]+, and m/z 273 [M+H-CO-2CH_3]+, as shown in the **figure 80**. This compound was tentatively assigned as rhamnazine ($C_{17}H_{14}O_7$) (**Subhadhirasakul and Jankeaw, 2003; El-Karkouri *et al.*, 2020**). This molecule possesses an antiproliferative and apoptogenic effects, and inhibit oxidative stress and inflammation (**Philchenkov and Zavelevych, 2015; Yang *et al.*, 2021**).

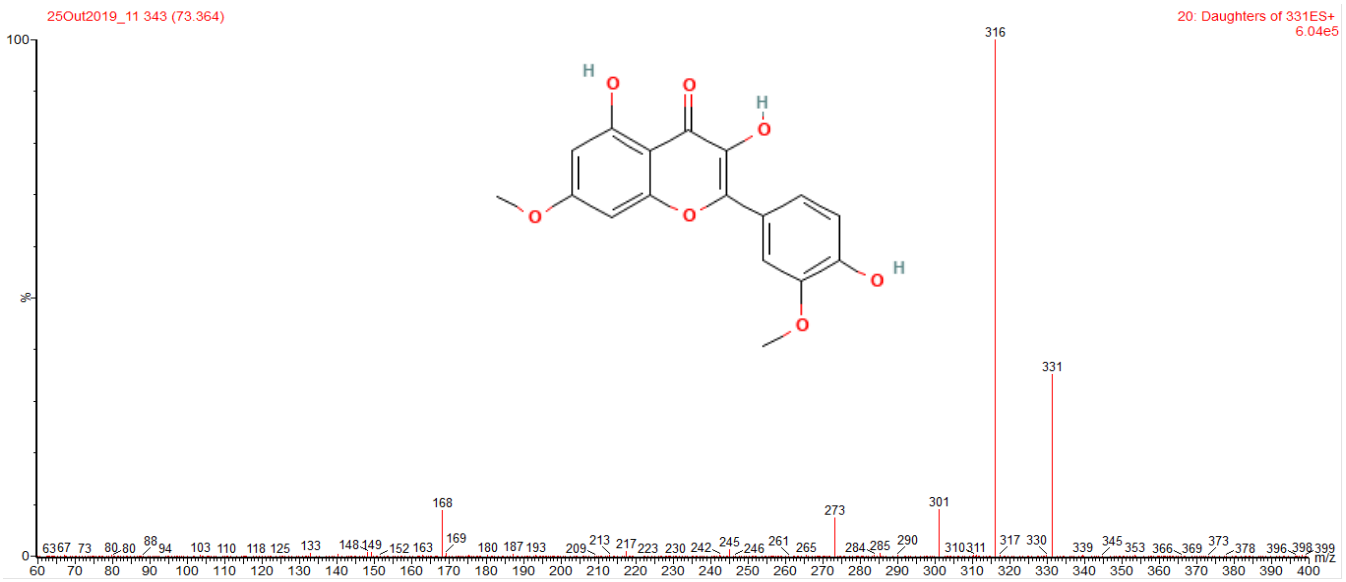

Figure 80: ESI-MS/MS spectra of rhamnazine

V.2.4. Curcuminoids

Showed in the **figure 81**, the **compound 12** eluted at 49.81 min showed in its MS spectrum, a molecular ion species [M+H]+ at m/z 339, which is consistent with the structure of demethoxycurcumin (**Bonifacio *et al.*, 2018**). This molecules possesses superoxide anion scavenging activity (**Niranjan and Prakash, 2008**), and active for the treatment of degenerative diseases (**Sabir *et al.*, 2021**).

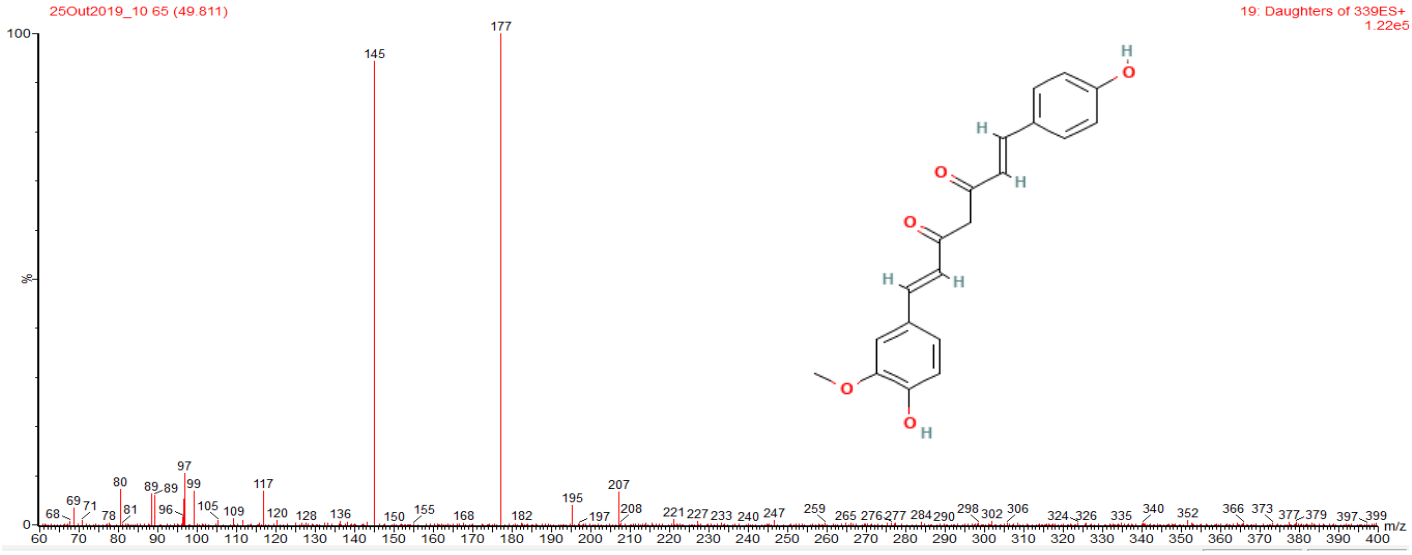

Figure 81: ESI-MS/MS spectra of demethoxycurcumin

VI. Biological activities

VI.1. Antioxidant activities

Aloysia triphylla leaf extract obtained under optimal conditions has been tested for several antioxidant activities, which are, DPPH test, reducing power, ABTS test, total antioxidant activity, ORAC activity and HOSC capacity. Several reports have suggested that there is a correlation between the total phenol content and the antioxidant activity of plant extracts (**Amaral *et al.*, 2009; Céspedes *et al.*, 2010; Guemghar et al., 2020**).

VI.1.1. DPPH radical-scavenging activity

The sample scavenging activity was concentration-dependent, and the DPPH activity of Gallic acid ($50.98\pm8.719_a$ µg/mL) was significantly lower ($p<0.05$) than the two extracts of *Aloysia triphylla*. The DPPH test had shown that MAE was the better technology due to the significantly lower IC_{50} ($p<0.05$) which was $139.65\pm1.02_b$ µg/mL, compared with that of conventional extraction CE ($229.50\pm9.77_c$ µg/mL). This result shows that the ethanolic extract of the *Aloysia triphylla* leaves had a high scavenger effect, which was related to the phenolic compounds. Polyphenols are indeed known to be effective hydrogen atom donors to the DPPH radical due to their ideal chemical structures. Studies have proven that phenolic compounds and especially flavonoids are responsible for the scavenger effect of free radicals (**Amessis-Ouchemoukh *et al.*, 2014; Zhang *et al.*, 2018)**. Indeed a direct correlation exists between DPPH radical-scavenging and TPC and DPPH radical-scavenging and flavonoids (**Uribe *et al.*, 2016**).

According to **Cheurfa and Allem (2016)**, the CI_{50} found with the hydro-ethanolic extract of *Aloysia triphylla* was 23.52 ± 0.035 mg/mL compared to that of BHT which was 6.96 ± 0.1 mg/mL. **Rashid *et al.* (2022)**, had shown that the ethanolic extract of fresh leaves of *Aloysia triphylla* had a DPPH activity with an IC50's of 22.85 mg/mL. This difference in values could be due to the extraction methods and standards used. In addition, phenols and flavonoids content vary during the different stages of plant growth **(Aldeen *et al.*, 2015)**.

VI.1.2. Dosage of ABTS cations$^+$

Proton radical scavenging is a fundamental function of antioxidants. At concentration of 339.33 µg/ mL, *Aloysia triphylla* extracts showed 97.37 ± 0.78%, 56.54± 1.1% inhibition of the $ABTS^+$ radical for optimized and conventional extracts, respectively, compared to Gallic acid which shows 100% inhibition for the same concentration. The IC_{50} of *Aloysia triphylla* extracts

was168.35 and 293.17±1.1 µg/ mL, for microwave and conventional extracts. These values were significantly ($p<0.05$) higher than that of Gallic acid, which had an IC_{50} of 37.16±0.95 µg/ mL. The work of **Tiwari and Tripathi (2007)** carried out on the ethanolic extract of *Vitex negundo* Linn. (*Verbenaceae*), showed that at the concentration of 300 µg/ mL, the extract showed an inhibition of 55.5 ± 1.3% and that CI_{50} was 235 µg/mL. At a concentration of 150 µg/mL, the ethanolic extract of *Gmelina arborea* (*Verbenaceae*), showed an inhibition of 88.33% (**Chellappan and Pemiah, 2014)**. This could be explained by the fact that the extract used in this work was crude, contains other compounds other than polyphenols, contrary to the standards which were pure phenolic compounds.

It should be noted that IC_{50} of DPPH scavenging activities of optimized and conventional leaves extracts gave linear result with their IC_{50} of ABTS scavenging activities.

VI.1.3. Ferric reducing antioxidant power assay (FRAP assay)

The IC_{50} of Gallic acid, optimized and conventional extracts were $21.67\pm0.48_a$ µg/mL, $47.62\pm0.32_b$ µg/mL and $66.79\pm0.23_c$ µg/mL, respectively. The work carried by **Cheurfa and Allem (2016**), showed that the hydro-alcoholic macerate of *Aloysia triphylla* has an antioxidant potential of 6.63±0.10 moles Fe (II)/g of extract. **Rezig *et al*. (2019)** showed an IC_{50} values for the reducing power of 209.33 µg/mL and 37.33 µg/mL for pure methanolic extract and ascorbic acid, respectively. **Bangou *et al.* (2012)** have found that the IC_{50} calculated for *Lippia chevalieri* and *Lantana camara* belonging to the *Verbenacae* family were 15.16-± 0.11mmol ascorbic acid equivalent (AAE/g) and 8.17 ± 0.04 mmol AAE/g, respectively. Suggesting an association between the reducing capabilities with the phenol composition complexity of the extracts. Indeed, the reducing power of this plant could be due to the presence of hydroxyl groups in phenolic compounds that can serve as electron donors, and the number of phenolic hydroxyl groups may play an important role in the free radical scavenging activity. As a result, antioxidants are considered to be reducing and inactivating oxidizing agents (**Justino *et al.*, 2004; Balasundram *et al.*, 2006; Siddhuraju and Becker, 2007**).

VI.1.4. Evaluation of total antioxidant capacity by phosphomolybdate (TAC)

The TAC of the optimized *Aloysia triphylla* extract was 99.88 ± 0.0021 mg / 100 g Ms, this value was significantly lower than the TAC of Gallic acid (154.39 ± 0.006 mg Eq AG / 100 g Ms). The work of **Cheurfa and Allem (2016)** had shown a total antioxidant capacity of aqueous extract of Lemon verbena, which was 173.50 ± 0.044 mg, Eq AA/g extract, while the

hydroalcoholic extract had shown a total antioxidant capacity of 270.14 ± 0.1 mg Eq ascorbic acid/g extract. This significant antioxidant capacity could be attributed to its composition in phenolic compounds (**Bangou *et al.*, 2012; Cheurfa and Allem, 2016).**

According to **Parikh and Patel. (2018),** TAC showed positively strong and significant correlation with TPC as well as flavonoid content, indicating that phenolic compounds are the major contributors to the antioxidant properties of this plant, which explains the difference of TAC activity between the optimized and the conventional extracts.

VI.1.5. ORAC activity (Oxygen Radical Absorbance Capacity)

The antioxidant capacity of the sample is quantitated using the area under the fluorescence decay curve (AUC). A standard curve of Trolox concentration versus AUC is generated and used for comparison of antioxidant samples.

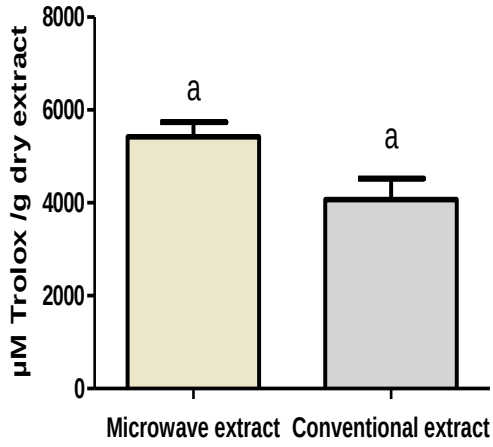

Figure 82: ORAC assay of microwave and conventional extracts

A calibration curve was obtained by plotting the area under the curve (AUC) versus the concentrations of Trolox in the range 5-40 µmol. The equation of the calibration curve was y = 0.3706 x + 0.4612 with a good correlation coefficient (r^2 = 0.994) (**Annexe 2**).

It was mentioned earlier that the TPC content of the extract obtained by MAE was higher than that of the conventional extract. According to **Bangou *et al.* (2012)**, there is an association between antioxidant activity and the complexity of the phenolic composition of the extracts.

The extracts obtained by the MAE and CE methods showed no statistical difference for ORAC activities, using ANOVA and Turkey's posthoc test ($P < 0.05$) **(Figure 82)**. As for all other antioxidant activities, the conventional extract had shown lower antioxidant activity. It was $5424.29 \pm 545.10_a$ µM TEAC /g dry extract for the microwave extract, and $3624.83 \pm 187.84_a$ µM TEAC /g dry extract for the conventional extract.

Figure 83 shows the time course of the reaction of fluorescein with AAPH in the presence of both extracts. The conventional extract had shown the lowest fluorescence intensity during the test compared to the extract obtained by microwave and trolox. Beyond 10 min, the fluorescence signal had decreased rapidly in the presence of Trolox, however for the extracts obtained by MAE and CAE, the steep decreases in fluorescence had been recorded after 7 min and 5 min respectively.

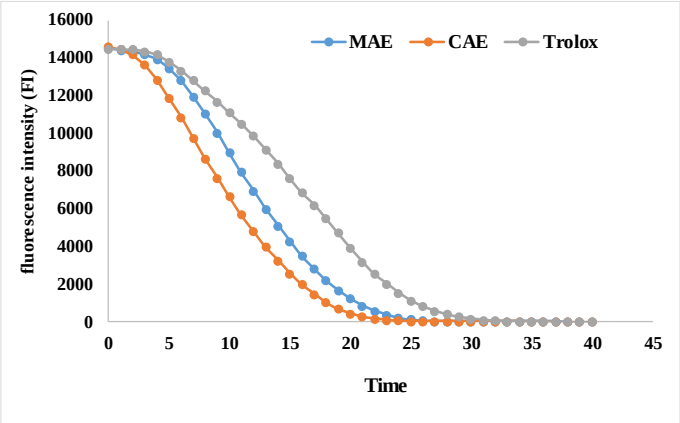

Figure 83: Effect of MAE and CAE extracts on fluorescence (FL) Intensity decay

The lower antioxidant activity of the conventional extract could be explained by the fact that the conventional extraction was performed at room temperature by maceration. In contrast to the microwave extraction, knowing that high temperature improves the extraction yield of natural compounds (**Aspé *et al*, 2011**). Microwave power intensity, controls the amount of energy supplied to the sample that is converted into thermal energy in the dielectric material to raise its temperature. It affects the interactions and distribution of analytes between sample and solvent **(Ma *et al*., 2009)**. The increase of microwave extraction yields is due to a synergistic combination of the two phenomena of mass and heat transfer. Indeed, in microwave extraction,

thanks to the volumetric heating effect, rapid temperature increases can be obtained, depending on the microwave power and the dielectric loss factor of the irradiated material (**Dahmoune *et al.*, 2015)**.

On the other hand, the optimal extraction time required for maximum ORAC activity using MAE was just 180 seconds vs. 3 days for conventional extraction, which may be explained by the fact that a longer extraction time could negatively influence the recovery of bioactive compounds. The MAE process has the ability to extract more bioactive compounds in a very short time compared to other extraction methods. The presence of water in extraction solvent (40 % ethanol) caused dielectric heating due to the dipolar nature of water. Microwave heating can also occur due to the oscillatory migration of ions in materials that generates heat in the presence of a high-frequency oscillating electric field (**Chandrasekaran *et al.*, 2013**), when the microwave energy is absorbed and converted into heat, the water tends to evaporate, which leads to the rupture and bursting of the cell wall, improving the extraction yield.

The ORAC activity is attributed to the presence of iridoid in *Aloysia triphylla* leaves. Indeed, some studies have explored the antioxidant capacity of iridoids, but the mechanism and structure-activity relationships are not well understood (**Sánchez-Marzo *et al.*, 2019)**. The antioxidant capacity of the isolated iridoid as peruloside was investigated using ORAC test and the result demonstrates an ORAC value of 179 ± 18 µmol eq TE/ g as peruloside. **Sánchez-Marzo *et al.* (2019)** reported a high antioxidant potential, around 180 ± 18 µmol eq TE/ g, of a iridoid-rich fraction of *Aloysia triphylla*. It is important to note that the leaf extracts obtained by the two methods contain several irridoids namely loganic acid, gardoside, shanzhiside, theveside, which would be responsible for the ORAC activity of the *Aloysia triphylla* extracts.

This potential could also be due to a synergic effect of the various types of compounds present besides iridoids, such as the phenylethanoids (**Bridi *et al.*, 2020**).

VI.1.6. HOSC activity

The HOSC activity of the MAE and CE extracts presented in the **Figure 84** were very interesting, and Tukey's multiple comparison test indicated that there was no statistically significant difference ($P > 0.05$) between the HOSC activity of the two extracts. It was 3888.66 ± 208.43 and 2963.03 ± 358.09 µM TEAC/g DW, for the optimized and conventional extracts, respectively.

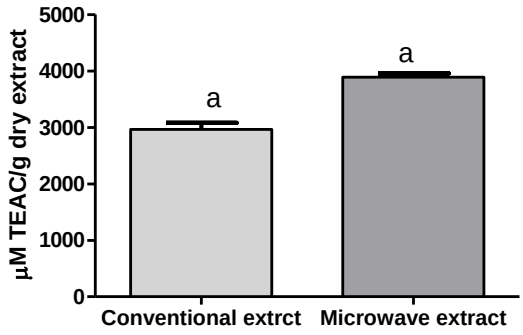

Figure 84: HOSC assays of microwave and conventional extracts

As shown in the **Figures 82** and **84**, there is a strong correlation between ORAC and HOSC activities for the optimized and conventional extracts. These results corroborate with those of **Moore *et al.* (2006**), they had shown a strong correlation between the HOSC and ORAC values of five phenolic antioxidant acids, such as *p*-coumaric, caffeic, ferulic, syringic, and 4-hydroxybenzoic. Indeed, the higher of Trolox equivalent per micromole value of these phenolic acids, the higher the HOSC or ORAC value. These results are in agreement with those reported by **Zhou *et al.* (2005)**, who studied the ·OH scavenging activities of coumaric, ferulic, syringic, and vanillic acids using an ESR spin-trapping method and found coumaric acid to have the strongest activity. The lack of significant difference in the values of these two antioxidant activities is probably due to the presence of an equivalent concentration of phenolic compounds with HOSC and ORAC activity, in both extracts.

The antioxidant activities of the optimized and conventional extracts of *Aloysia triphylla* leaves, is attributed to the different phenolic compounds identified by HPLC-DAD-ESI-MS/MS. These compounds are; phenolic acids (caffeic acid), irridoids (gardoside, loganic acid), phenylthanoids (Cistanosid F, eukovoside, Cistanoside C, verbascoide and its derivatives), flavonoids (methylophiopogonone A, luteolin-7-diglucuronide, astragalin, scutelarin, diosmetin, quercetin-3-O-glucoside, isorhamnetin, genistein-C-glycosylglucoside, apigenin-7-diglucuronide, apigenin, luteolin-7-O-glucoside, Tricin-7-O-glucuronide, luteolin, quercetin-7-O-glucuronide, kaempferol and its derivatives, astragalin, isorhamnetin-3-O-glucoside, rhamnazine) and demethoxycurcumin.

It is worth noting that verbascoside and isoverbascoside, identified with by HPLC-DAD-ESI/MS, are the main compounds responsible for the antioxidant effects evidenced by lemon

verbena extracts. The number of hydroxyl groups and their position in relation to the carboxyl functional group influences the antioxidant activity of phenolic compounds. Indeed, verbascoside and β-hydroxy(iso)verbascoside showed the most powerful antioxidant activity than that of isoverbascoside, reflecting the influence of the extra hydroxylation and the caffeoyl moiety position in verbascoside isomers which was more notable for their capacity to transfer a hydrogen atom (**Sánchez-Marzo *et al.*, 2019**).

VI.2. Antibacterial activity

The results of the inhibition zones in millimeters obtained with the different strains are shown in the **Table X**.

Table X: Antibacterial activity of optimized and conventional extracts of *Aloysia triphylla* leaves

Antibacterial activity							
Inhibition diameter (mm)							
Strains	***Aloysia triphylla* extracts**		**Antibiotics**				
	Optimized extract	**Maceration extract**	**P**	**C**	**K**	**NOV**	**OFX**
P. aeruginosa	11.50±0.71	/	/ (R)	6 (R)	/ (R)	6 (R)	27.00 (S)
E. coli	/	/	/ (R)	30.50 (S)	23.00 (S)	/ (R)	33.50 (S)
B. subtilis	5.00±1.41[a]	4.50±0.71[b]	5 .00(R)	31.00 (S)	22.50(S)	23.50 (S)	27.00 (S)
S. aureus	13.50±0.71[a]	5.00±1.41[b]	33.50 (S)	34.00(S)	35.50 (S)	33.50 (S)	28.00 (S)
Minimum inhibitory and bactericidal concentrations (MIC and MBC) (mg/mL)							
Strains	**Microwave extraction**			**Conventional extraction**			
	MIC	**MBC**	**MBC/MIC**	**MIC**	**MBC**	**MBC/MIC**	
P. aeruginosa	3.13[b]	/	6.00	1.56[a]	25.00[b]	16.00	
E. coli	/	/	2.50	1.56[a]	25.00[b]	16.00	
B. subtilis	1.56[a]	1.56[a]	1.00	3.13[b]	9.37±4.42[b]	3.00	
S. aureus	0.20[a]	1.56 [a]	8.00	0.20[a]	25.00[b]	128.00	

P: Penicillin, C: Chloramphenicol, K: Kanamycin, NOV: Novobiocin, OFX: Ofloxacin, S: Sensible,

R: Resistant.

Values are mean ± standard deviation.

Values with different letters (a-b-c) were significantly different (Tukey, $p<0.05$).

The inhibition zones obtained with ethanol extracts are generally lower than those obtained with reference antibiotics. However, it should be noted that these extracts with antibacterial activity are not pure products, and further purification may yield more potent compounds (**Fabry *et al.*, 1998**).

According to the antibiogram profile, *S. aureus*, *B. subtilis* and *E. coli* were qualified to be sensitive strains, whereas *P. aerugenosa* was a resistant one. The *Aloysia triphylla* leaves extracts showed a moderate antibacterial activity, which were concomitant with the findings of **Kumar *et al.* (2008)**. The extract obtained by MAE had the best antibacterial activity comparing to the CE. In fact, it exhibited a considerable antibacterial activity against *P. aeruginosa* and *S. aureus*. However, no activity was shown towards *B. subtilis* and *E. coli* strains. These results corroborate with those of **Mirzaie *et al.* (2016)**. For *P. aeruginosa*, the extract obtained by MAE showed antibacterial activity (11.5±0.71mm) which was greater than that obtained with Chloramphenicol, Kanamycin and Novobiocin.

This bacterium was resistant due to several antimicrobial agents, such as pyocyanin pigment, which plays an important role in the virulence of this bacterium (**Lau *et al.*, 2004**). Hence its involvement in hospital-acquired infections (**Mann *et al.*, 2000**).

Several studies proved the antibacterial effect of polyphenols on Gram-positive and Gram-negative bacteria, **Funes *et al.* (2010)** showed that verbascoside, which is the major polyphenol in this plant, disrupts the structure of the phospholipid membrane. Indeed, the extract obtained with MAE is richer in polyphenols and flavonoids (67.86±0.92 mg GAE/g DW and 37.27±1.62 mg CE/g DW, respectively), than the extract obtained by CE (34.55± 0.90 mg GAE/g DW; and 23.25±1.47 mg CE/g DW, respectively). This explains the highest antibacterial activity of the MAE extract. According to **Tsuchiya *et al.* (1996)** and **Tian *et al.* (2009)**, polyphenols can alter bacterial cell walls; interact with membrane proteins through hydrogen bonds *via* their hydroxyl groups. Consequently, this induces changes in membrane permeability and cell destruction, and disrupts the co-aggregation of microorganisms **(Naz *et al.*, 2007)**.

The characterization of *Aloysia triphylla* extracts in negative and positive ionization mode, had indeed allowed to identify several molecules in addition to the verbascoside which are endowed with antibacterial activity, such as luteolin-7-diglucuronide, caffeic acid, astragalin, diosmetin, isorhamnetin, quercetin-3-O-glucoside, apigetrin, acacetin, penduletin, kaempferol and its derivates, astragalin and diosmetin

VI.2.1. MIC and MBC

The obtained values of MIC and MBC are reported in **Table X**. The results show that these values vary depending on the germ and the extraction method. The MIC and MBC values ranged from 0.19 to 3.12 mg/mL and 1.56 to 25.00 mg/mL, respectively. A MBC/MIC ratio less than or equal to 4, indicates the existence of a bactericidal effect of the tested extracts against the different germs (**Mamadou *et al.*, 2014**). The extract obtained by MAE showed a bactericidal effect against *E. coli* and *B. subtilis* with MBC/MIC ratio of 2.5 and 1, respectively, which confers antibiotic power on these strains. However, the macerated extract had a bactericidal effect only on the *B. subtilis* with a MBC/MIC ratio of 3. Comparing the two modes of extraction, it can be seen that the MAE extract has better antibacterial activity with low MBC levels (1.56 to 18.75 mg/mL), compared to the MBC of the extract obtained by CE, with higher concentrations (9.37 to 25.00 mg/mL). This effect could be due to the higher concentration of phenolic compounds in the extract obtained by MAE (**Bouarab-Chibane *et al.*, 2019; Efenberger-Szmechtyk *et al.*, 2021**).

According to these results, Gram-negative bacteria are more resistant by registering higher values of MIC and MBC than Gram-positive bacteria. These results were in agreement with those of **Efenberger-Szmechtyk *et al.* (2021)**. This difference is due to the distinct cell wall structure between Gram-positive and Gram-negative bacteria (**Cushnie and Lamb, 2011**). Indeed, a Gram-positive bacterium has a single-layer cell wall structure, whereas Gram-negative bacteria has a multi-layer structure containing an outer cell membrane (**Mamadou *et al.*, 2014; Ghedadba *et al.*, 2015**). This outer membrane is composed of lipopolysaccharides, phospholipids and proteins that form an impermeable barrier to most molecules (**Yakhlef *et al.*, 2011**). In the another hand, both extracts' effect on Gram+ bacteria may be due to flavonoids which are good inhibitors of sortases who are enzymes composing the cytoplasmic membrane of Gram-positive bacteria that catalyze surface proteins, such as adhesins and internalins (**Cushnie and Lamb, 2011**).

VI.3. Anti-acetylcholinesterase activity of optimized and conventional extracts

The optimized and conventional extracts of *Aloysia triphylla* leaves were tested for their acetylcholinesterase inhibitory activities *in vitro* by the spectrophotometric Ellman method. Results obtained were shown in **Table XI**.

Table XI: Acetylcholinesterase inhibition by microwave and conventional extracts of *Aloysia triphylla* leaves.

Concentration	Inhibition activity (%)	
	Conventional extract	Microwave extract
0.1 mg/ Ml	5.261± 0.001[a]	0
0.2 mg/ Ml	19.90±1.62[a]	27.04± 0.73[a]
0.35 mg/ mL	28.55±2.72[a]	31.58± 0[a]
0.5 mg/ Ml	55.35±2.63[b]	66.66± 0[a]
0.6 mg/ mL	66.78 ±2.84[b]	78.95± 0[a]
0.75 mg/ mL	81.48± 3.20[b]	90.74± 2.82[a]

According to the classification for acetylcholinesterase reducing potential of crude extracts, weak inhibitors present inhibitory value below 30%; moderate inhibitors present 30 to 50% inhibition, and potent inhibitors show over 50% of inhibition enzyme (**Vinutha *et al.*, 2007**). **Figure 85** showed that the extracts of *Aloysia triphylla* inhibit acetylcholinesterase in a dose-dependent manner, the percentage of inhibition increases with the increase of the extract concentration.

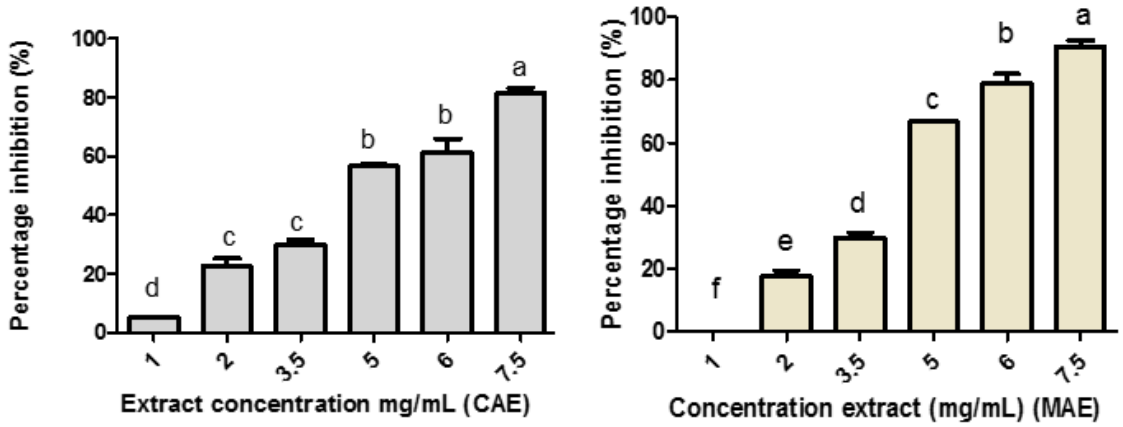

Figure 85: A dose-dependent inhibition of Acetylcholinesterase activity by microwave and conventional extracts

Indeed, at a concentration of 0.1 mg/mL, the inhibitory activity was low for the extract obtained by the conventional method (5.26 ± 0.003 %), it was null for the extract obtained by microwave at the same concentration.

At a concentration of 0.2 mg/mL, both extracts had shown a weak inhibitory activity, 19.9±1.62 % and 27.04±0.73 % for conventional and microwave extracts, respectively.

At a concentration of 0.35 mg/mL, the inhibitory activity of both conventional and microwave extracts were moderate and were not significantly different ($p < 0.05$) with 28.55± 2.72% and 31.58± 0% respectively. At concentrations of 0.5 mg/mL, 0.6 mg/mL, 0.75 mg/mL, the microwave and the conventional extracts of *Aloysia triphylla* leaves had shown goods inhibitory activities with significantly difference (P>0.05). Indeed, microwave extract showed better anti-acetylcholinesterase activity (0.5 mg/mL, 66.66±0 %; 0.6 mg/mL, 78.95±0%; 0.75 mg/mL, 90.74±2.82%) compared to the conventional extract (0.5 mg/mL, 55.35±2.63%; 0.6 mg/mL, 66.78±2.84%; 0.75 mg/mL, 81.48±3.20%).

These results corroborate with those of **Ricardo *et al.* (2015)**, they had shown that *Lantara camara* (*Verbenaceae*) essential oil showed a good acetylcholinesterase inhibition, which had

reached 77.15 % and **Oliveira *et al.* (2019)** found that *Lippia hirta* (*Verbenaceae*) organic extract has an excellent acetylcholinesterase activity.

Flavonoids are well-known natural compounds that attract increasing attention due to a wide range of pharmacological properties related to a variety of neurological disorders, like neuroprotective effect (**Schroeder *et al.*,2001**), AChE inhibitory activity (**Uriarte and Calvo, 2011; Khan *et al.*, 2018)**. According to **Santos *et al.* (2018**) and **Khan *et al.* (2018**), flavonoids are currently considered as prominent source of anti-AD (Alzheimer's disease) compounds because of their potential AChE inhibitory activity allied to the well-known antioxidant activity and low toxicity (**Uriarte and Calvo, 2011**). Therefore, it is evident that the AChE inhibition observed for *Aloysia triphylla* extracts can be, at least attributed to these metabolites.

This inhibitory potential may have been highlighted by the synergism of the several chemical constituents present in the extracts. Indeed, **Ricardo *et al.* (2015)** have found that the inhibitory potential can be associated to synergistic compounds.

The high acetylcholinesterase inhibitory activity of the ethanolic extracts of *Aloysia triphylla* can also be due to its high amount of total phenolics. Previous reports demonstrated the potential of phenolic compounds to inhibit acetylcholineterase activity (**Ramassamy, 2006; Williams *et al.*, 2011; Tripathi and Mazumder, 2019; Grodzicki and Dziendzikowska, 2020**). Phenolic compounds have different intracellular targets, so they may constitute an efficient approach to reduce the incidence of Alzheimer Disease (**Ramassamy, 2006**). The optimized and the conventional *Aloysia triphylla* leaves extracts analyzed with LC-DAD-ESI-MS/MS, were revealed the presence of an important diversity of phenolic compounds who possess an important anti- acetylcholinesterase activity. These compounds were apigenin, verbascoside, cistanoside F, theveside, apigenin-7-diglucuronide, apigetrin, acacetin, kaempferol, astragalin, demethoxycurcumin, eukovoside and luteolin which was identified as a potent inhibitor of Aβ fibrils, strongly implicated in Alzheimer Disease (AD) pathology and the neurotoxicity observed with this disease (**Simunkova *et al.*, 2019**).

Many works were proved the presence of luteolin, verbascosie and apegenin in *Aloysia triphylla* extracts (**Quirantes-Piné *et al.*, 2009; Quirantes-Pine´ *et al.*, 2010; Pereira *et al.*, 2017; Sánchez-Marzo *et al.*, 2019**). These observations suggest that these extracts can be a source of potential multi-targets agents against Alzheimer deseases.

VI.3.1. Acetylcholinesterase sensitivity

For sensitivity tests, different concentrations of the two ethanolic extracts leaf extracts were tested to determine the extract concentration that inhibited 50% of the enzyme activity. The results expressed as IC_{50} values were calculated from the regression equations obtained from the activity of samples at different concentrations. The results were presented in **Figure 8 (Annexe 2)**.

These results showed that, the IC_{50} of MAE was better than the IC_{50} of conventional extract with a significant difference ($p > 0.05$), it was 4.34±0.043 mg/ mL and 4.75±0.021 mg/ mL, for microwave and conventional extract, respectively. This result is explained by the fact that the content of phenolic compounds was higher in the extract obtained by MAE compared to the conventional extract. Considering this, *Aloysia triphylla* leaves extracts stands out as a potent inhibitor of AChE enzyme. **Abuhamdah. (2018)** had shown that the methanol extract of *Aloysia triphylla* leaves, excerts an antiacetylcholinesterase inhibition of 78.03± 0.065 % for an extract concentration at 100 µg/mL. This value is higher than the activity of the conventional extract.

Conclusion

Conclusion

Polyphenols are phytochemical compounds that have acquired significant importance due to several health-related benefits in relation to lifestyle diseases and oxidative stress. Until now, the development of a standard method for optimal, efficient and rapid extraction of polyphenols from plant matrices is still a challenge due to the limitations imposed by the various conventional extraction methods.

Several works dealing with the different processes of green extraction of polyphenols from the leaves of *Aloysia triphylla*. These methods are; Supercritical Fluid Extraction (SFE), Ultrasonic Assisted Extraction (UAE), Microwave Extraction (MAE), Pressurized Liquid Extraction (PLE) and Pressurized Hot Water Extraction (PHWE). They are alternatives to conventional extraction methods like Soxhlet, percolation, and maceration, which presents some disadvantages that make its application uneconomic due to excessive consumption of time, energy and polluting solvents. Several journals published in the literature indicate that it is also recognized as a clean process technology with several environmental, economic and social benefits. The green extraction techniques used in this work to extract polyphenols from the leaves of *Aloysia triphylla* is microwave-assisted extraction, where this process has been optimized to extract maximum polyphenol content.

The recovery of natural bioactive compounds with antioxidant or other biological activities from plant matrices is a topic of growing interest in the biotechnology field. To our knowledge, this study, which focuses on optimizing the extraction of phenolic compounds from *Aloysia triphylla* leaves by microwave, the study of their biological activities and their characterization, is the first one carried out. The response surface methodology (RSM) had yielded satisfactory results for the optimization of extraction conditions.

The phytochemical characterization of the bioactive compounds of the two extracts by colorimetric methods showed quantitative differences. The colorimetric determination of total phenols, total flavonoids and condensed tannins indicates that the plant studied is an important source of polyphenols, and that microwave-assisted extraction is the most effective for polyphenols extraction than conventional method.

In this study, microwave extraction depends on several factors, namely extraction solvent (ethanol), irradiation time, ratio, microwave power and particle size. The particle size that resulted in a maximum extraction efficiency of 64.84±1.82 mg GAE/g DW was 125 µm. For extraction solvent, the best TPC yield was achieved by ethanol (54±1.18 mg Gallic acid equivalent /g DW) and methanol (57.69 ± 1.3461 mg GAE/g DW) with statistically equal values, ethanol was chosen because it is a non-toxic and more environmentally responsible solvent. 40 % ethanol allowed the best TPC extraction (67.87 ± 1.61 mg GAE/g DW). 188 seconds, and 40:1 mL/mg was the irradiation time and the liquid-to-solid ration that allows a better extraction rate respectively.

Under optimal extraction conditions, the polyphenol content of the leaves was 67.87 ± 1.61 mg GAE/g DW, which is double the conventional extraction ($P < 0.001$) content which was 34.55 ± 0.90 mg GAE/g DW. Based on these results, the best efficiency of microwave assisted extraction can be deduced.

R^2 coefficient and adjusted R^2 coefficient, are 0.9854 and 0.972, respectively, they were closely related. It demonstrates the good fit of the model to the experimental results. F-value (72.36) and *P*-value (<0.0001*) show that the model is extremely significant.

The identification of *Aloysia triphylla* extracts with LC-DAD-ESI-MS/MS in negative and positive ionization mode, allowed highlighting an important variety of phenolic compounds. Indeed, the compounds identified are;

- Phenolic acids (malic acid, succinic acid, caffeic acid and 3-O-methyl rosmarinic acid).
- Irridoids (shanzhiside, gardoside, loganic acid, theveside).
- Phenylethanoids (verbascoside, Cistanoside F, β-hydroxyverbascoside, eukovoside, Cistanoside C, Glycosylated Verbascoside, Derhamnosyl verbascoside,),
- Flvonoids such as homoisoflavone, (methylophiopogonone A), Flavones (Luteolin-7-diglucoronide, apigenin-7-diglucuronide, chrysoeriol-7-diglucoronide, astragalin, scutellarin, baicalin, acacetin-7-diglucoronide, acacetin-7-diglucoronide, apigenin, diomestin and genkwanin, genistein-C-glucosylglucoside, quercetin 3-O-glucoside, apigenin-7-O-glucoside, apigenin 4′-O-glucuronide, luteolin 7-O-glucoside, tricin-7-O-glucuronide, 5,6,7-trihydroxy-8-methoxy flavone-7-O-glucuronide, acacetin), Flavonol (quercetin-3-O-glucoside, nepetin,

luteolin, isorhamnetin, quercetin-7-O-glucuronide, Kaempferol-(2''-O-methyl)-4'-O-α-D-glucopyranoside, astragalin ,isorhamnetin-3-O-glucoside, kaempferide .

- Isorhamnetin, diosmetin, Isokaempferide and rhamnazine), demethoxycurcumin and Fatty acyl glycosides (12-hydroxyjasmonic acid glucoside).

Some of these phenolic compounds have been identified for the first time in the plant of *Aloysia triphylla*, namely; succinic acid, cistanoside C, methylophiopogonone A, astragalin, scutellarin, genkwanin, quercetin-3-O-glucoside, isorhamnetin, theveside and curcuminoide.

The varied contents of this plant in phenolic compounds, explains the different biological activities that it possesses, and its wide use in the phytotherapy field.

Aloysia triphylla leaf extracts were tested for several antioxidant activities such as DPPH, ABTS, FRAP, TAC, ORAC and HOSC. The results showed that these extracts were endowed with interesting antioxidant powers, with better results for the extract optimized by microwave.

These extracts were also tested for antibacterial activity. They were effective against the strains tested (*Escherichia coli, Staphylococcus aureus, Bacillus subtilis* and *Pseudomons aeruginosa*). According to the results, MAE extract has better antibacterial activity with low MBC levels (1.56 to 18.75 mg/mL), compared to the MBC of the conventional extract, with higher concentrations (9.37 to 25.00 mg/mL).

The last biological activity tested is the anti-acetylcholinesterase activity, the two extracts of *Aloysia triphylla* had shown an interesting enzymatic activity with a better result for the optimized extract. Indeed, IC_{50} of MAE was better than the IC_{50} of conventional extract with a significant difference (p >0.05), it was 0.434±0.043 mg/ mL and 0.475±0.021 mg/ mL, for microwave and conventional extract, respectively.

Based on the results obtained, the extracts studied exhibit very interesting antioxidant properties, which will probably be a good alternative in pharmacopoeia, to fight against the oxidative stress associated with several diseases.

Our results help to highlight the role of extracts of this plant, in the fight against certain bacterial pathogenic strains as a substitute for antibiotics with side effects, that could be harmful to human health.

Regarding the antiacetylcholinesterase activity, this study opens new perspectives to study the importance of this plant in the treatment of Alzheimer's disease, and to use it as a natural treatment, without side effects, unlike chemical drugs, which can pose a long-term health hazard.

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Annexes

Annexes 1

Table I: Solvents and chemicals used for practical work

Chemicals		Provider
Extraction	Acetone 99 % Ethanol 99 % Methanol 99 % Distilled water	Sigma Aldrich (BBBS Lab) Sigma Aldrich (BBBS Lab) Sigma Aldrich (BBBS Lab) BBBS Lab
Evaluation of total phenolic compounds and flavonoids	Folin-Ciocalteu NaH2PO4 $AlCl_3$, 6 H_2O	Sigma Co., St. Louis, MO. USA Sigma-Aldrich (St. Louis, Missouri, USA) Sigma Aldrish
Antioxidant activities	Gallic acid	Sigma-Aldrich (Portugal)
	Ethanol 99 %	Sigma-Aldrich (Portugal)
	Aluminum Chloride	Fluka Biochemika
	AAPH (2,2'-Azobis (2-amidinopropane)dihydrochloride	Sigma-Aldrich (St Quentin Fallavier, France)
	Trolox (6-hydroxy-2,5,7,8-tetramethyl chromane-2-carboxylic acid)	Sigma-Aldrich (St Quentin Fallavier, France)
	Disodium fluorescein (FL)	Sigma-Aldrich (S^t Quentin Fallavier, France)

	Potassium chloride (KCl)	Sigma-Aldrich (S^t Quentin Fallavier, France)
	Sodium chloride (NaCl)	Sigma-Aldrich (St Quentin Fallavier, France)
	Monopotassium phosphate (KH2PO4)	Sigma-Aldrich (St Quentin Fallavier, France)
	Fluorescein sodium	Vetec Química (São Paulo, Brazil).
	Sodium hydroxide	Sigma-Aldrich
	Ferricyanure de potassium (K3Fe(CN)6),	Biochem Chemopharma
	Acide tricholoroacetique (TCA)	Biochem Chemopharma
	chlorure ferrique (FeCl3),	Biochem Chemopharma
	molybdate d'ammonium ((NH4)2 MoO4)	Biochem Chemopharma
	Nitroprusside de sodium (Na2[Fe(CN)5NO]·2H2O)	Sigma-Aldrich
	DPPH (1,1-diphenyl-2-picrylhydrazyl)	Fluka Biochemika.
	ABTS (acide 2,2'-azinobis-3-éthylbenzothiazoline-6-sulfonique)	Fluka Biochemika.
Antibacterial activity	Mueller-Hinton medium	Sigma-Aldrich
	paper disks	Bio-Rad

	DMSO	Sigma-Aldrich
Acetylcholinesterase acticity	Ethanol	Sigma–Aldrich Química S. A. (Sintra, Portugal)
	5,5′-dithiobis [2-nitrobenzoic acid] (DTNB)	Sigma–Aldrich Química S. A. (Sintra, Portugal)
	Acetylcholinesterase enzyme	Sigma–Aldrich Química S. A. (Sintra, Portugal)
	acetylthiocholine iodide (AChI)	Sigma–Aldrich Química S. A. (Sintra, Portugal)
HPLC-LCMS	Acetonitrile	Fisher Scientific, Waltham/USA
	Formic acid (HCOOH) 99.5%	Merck®
	Acetonitrile (CH3CN) 99.5%	Fisher Scientific (Waltham/USA)
	Bi-distilled water/ ultra-pure water	Millipore-Direct Q3 UV system (Millipore, USA)
	Phosphoric acid	Panreac, Spain

Annexe 2

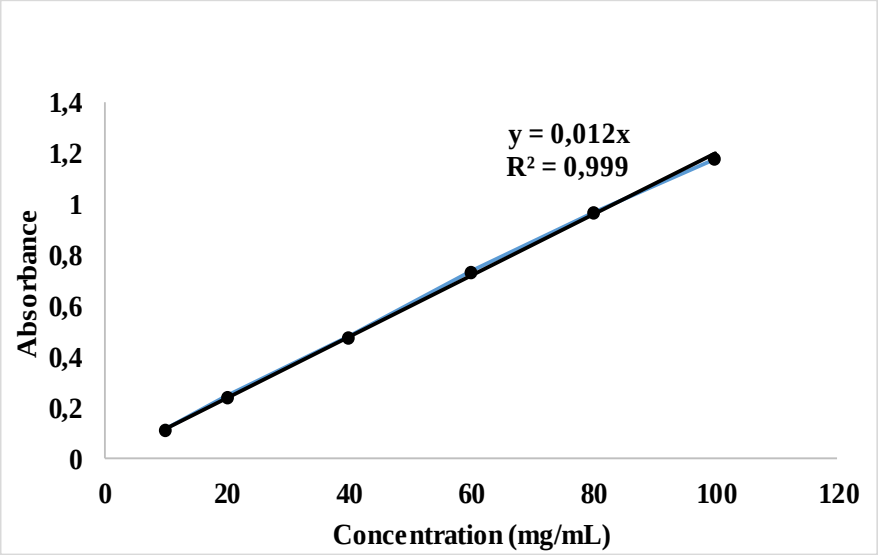

Figure 1: Calibration curve of Gallic acid

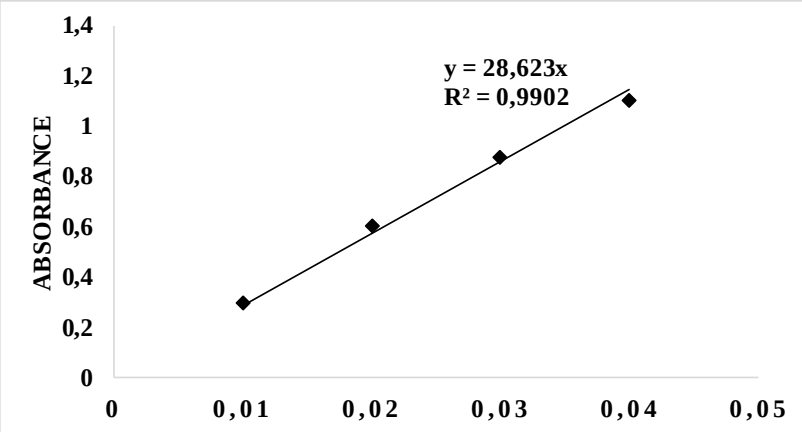

Figure 2: Calibration curve of catechin

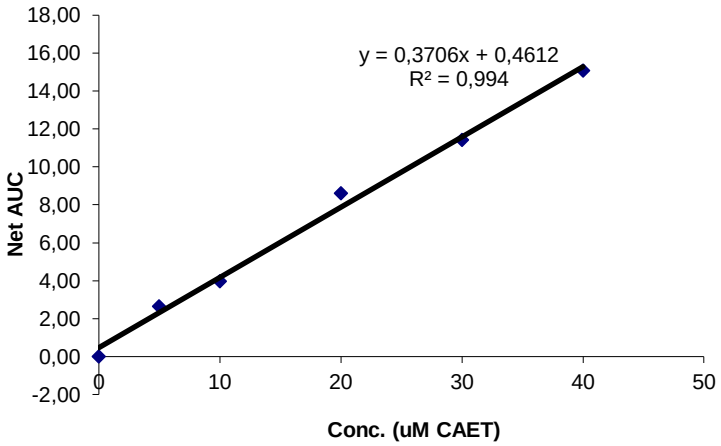

Figure 3 : Calibration curve of Trolox

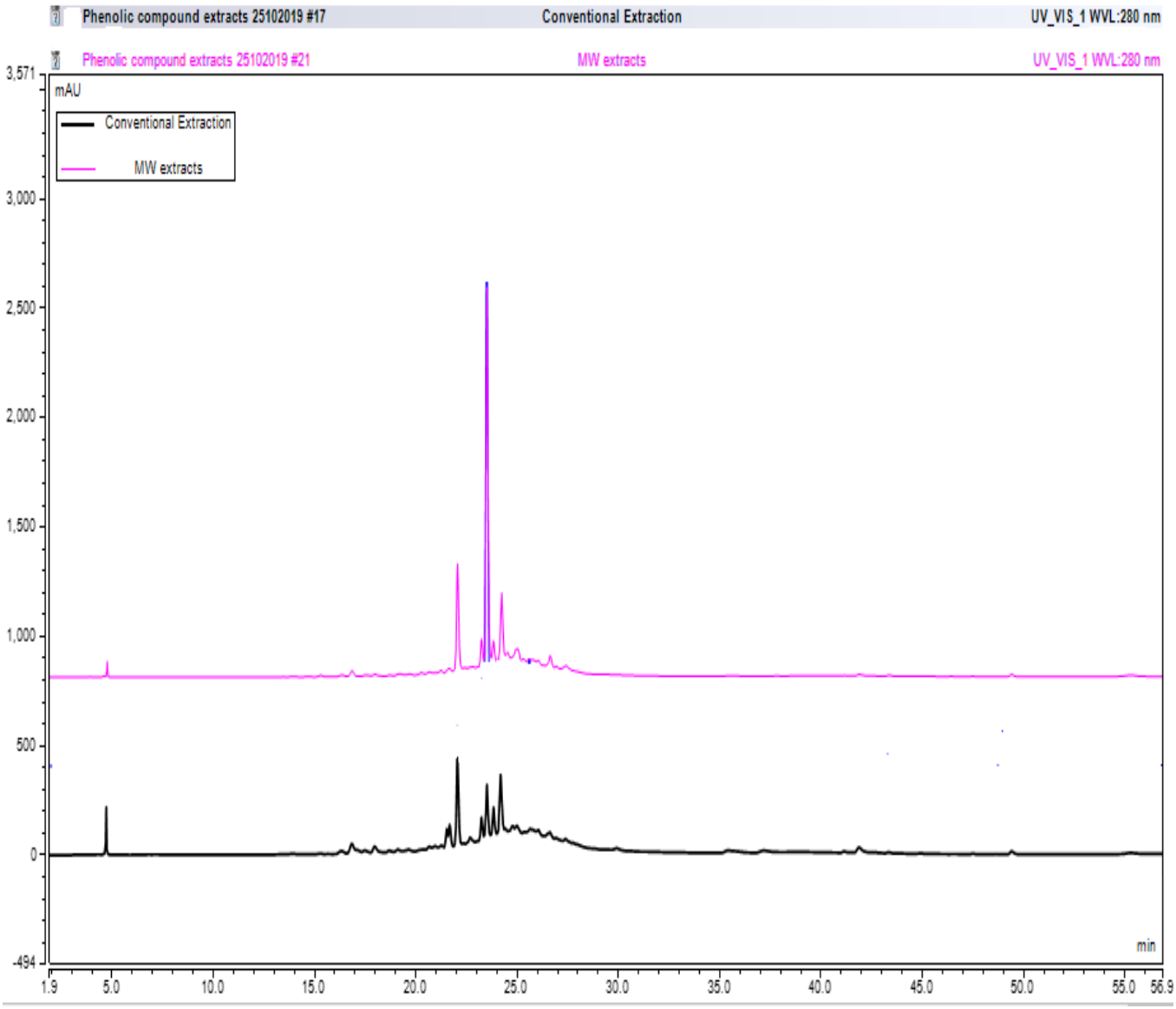

Figure 4: HPLC profile of *Aloysia triphylla* conventional and microwave extracts recorded at 280 nm

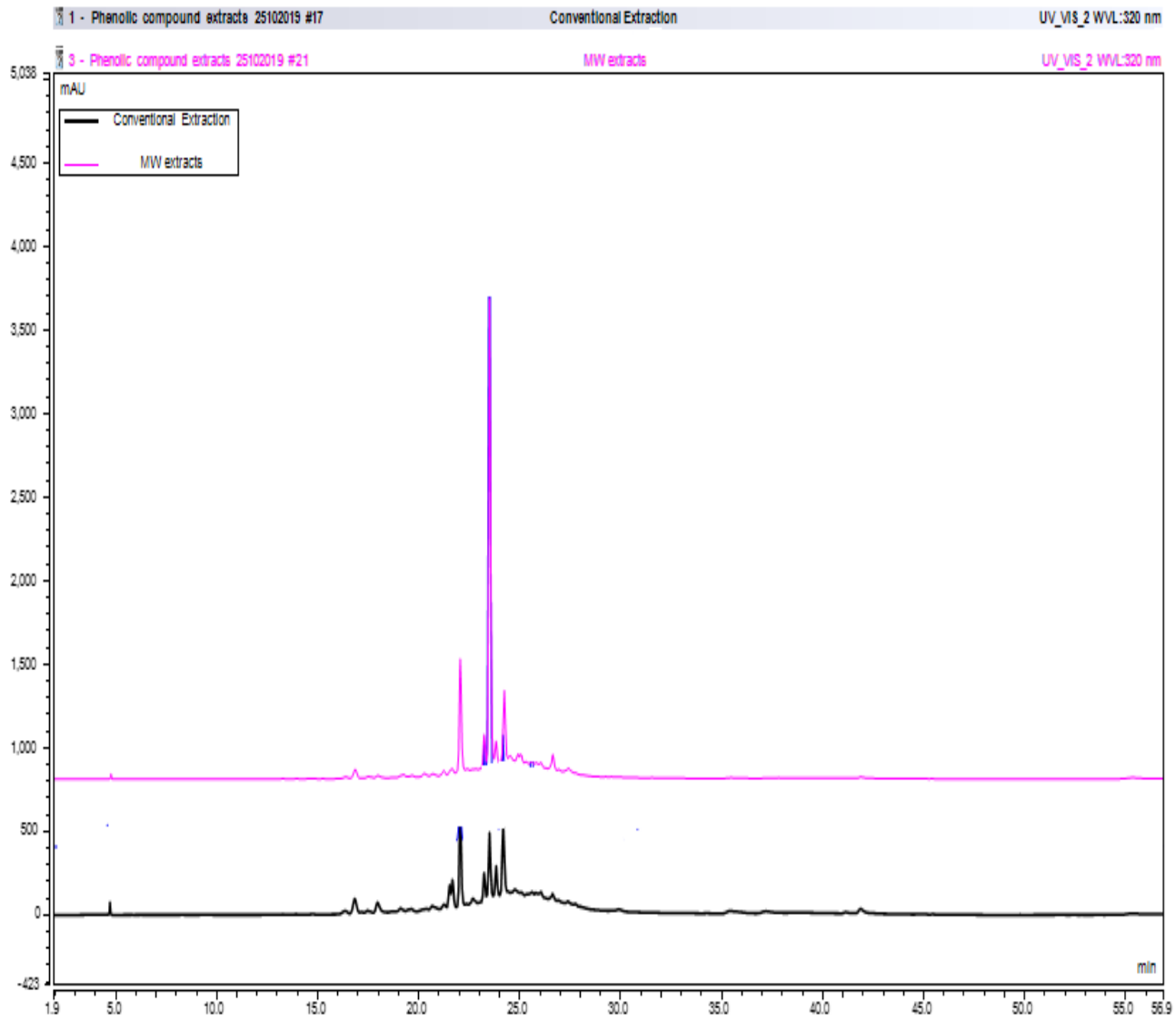

Figure 5: HPLC profile of *Aloysia triphylla* conventional and microwave extracts recorded at 320 nm

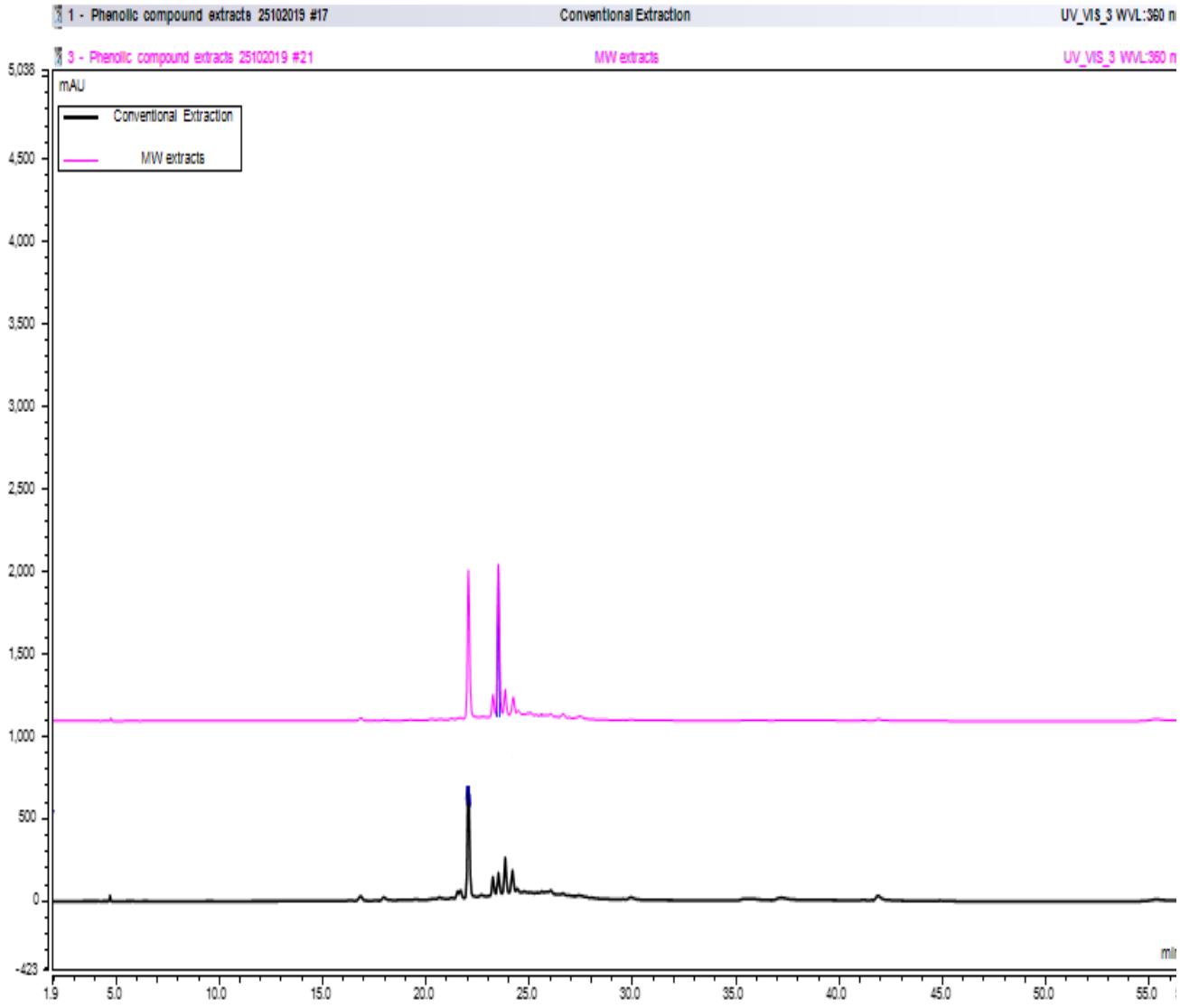

Figure 6: HPLC profile of *Aloysia triphylla* conventional and microwave extracts recorded at 360 nm

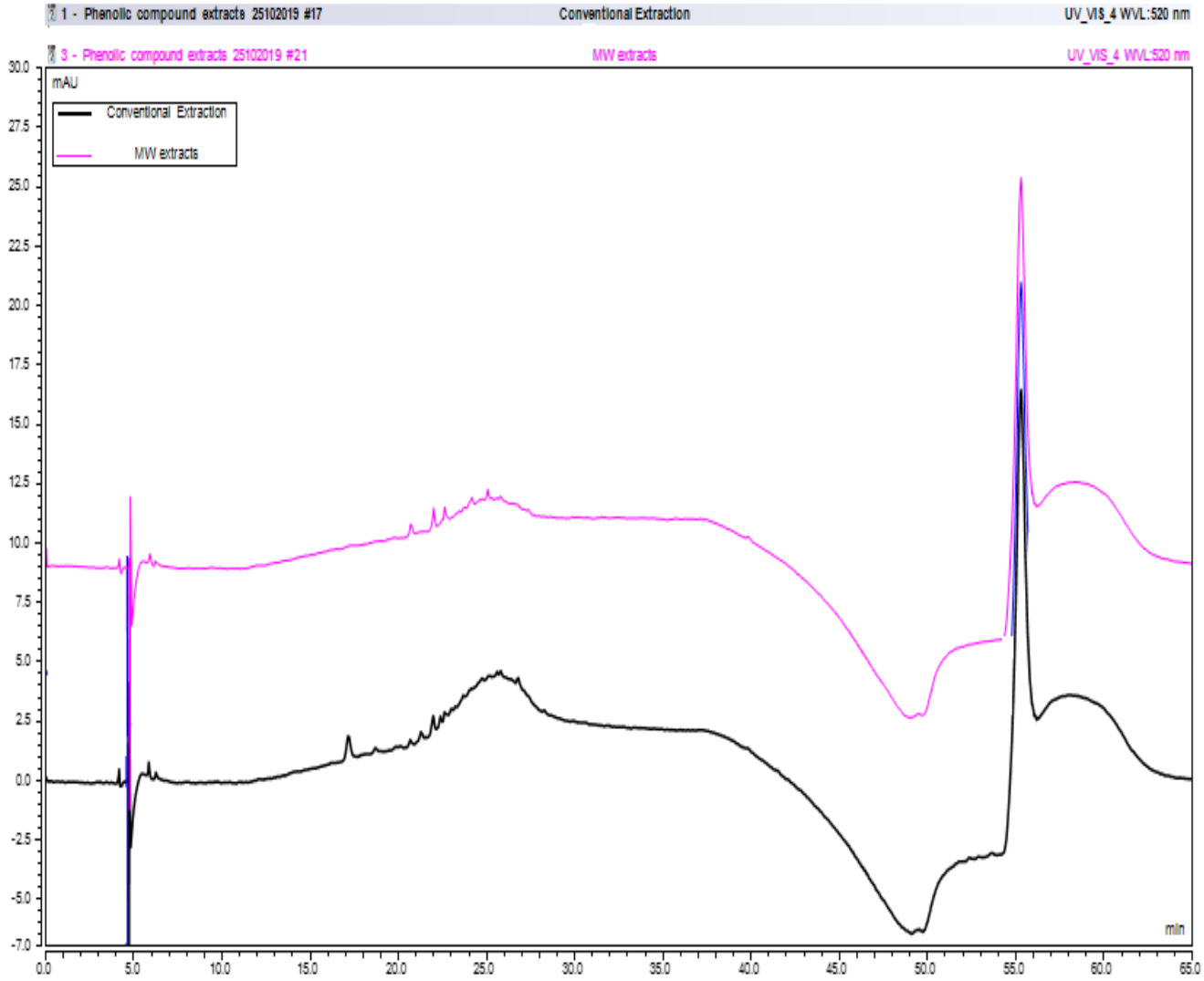

Figure7: HPLC profile of *Aloysia triphylla* conventional and microwave extracts recorded at 520 nm

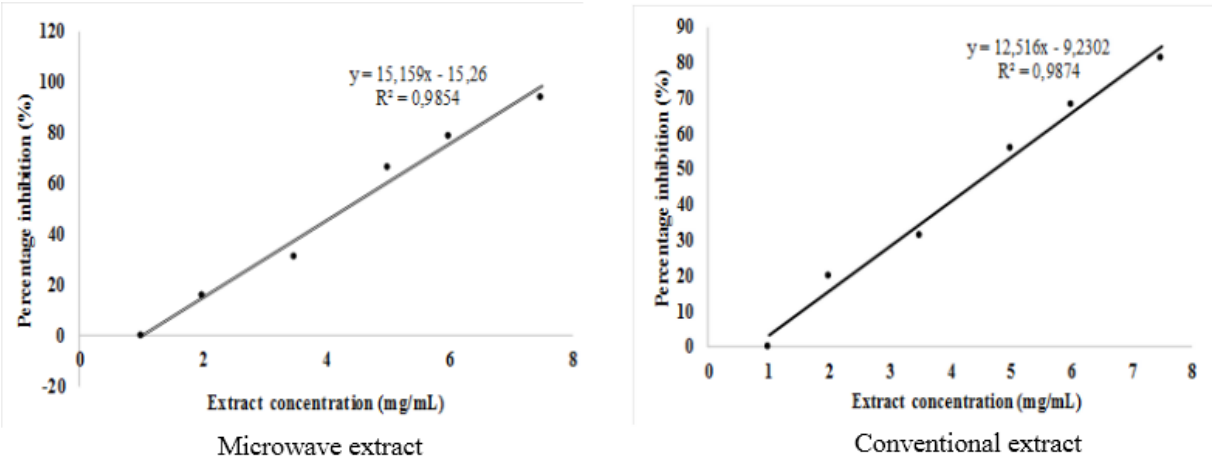

Figure 8: Microwave and conventional extracts sensitivity to acetylcholinesterase

Résumé

La présente étude vise à optimiser l'extraction des composés phénoliques par extraction assistée par micro-ondes (MAE) à l'aide de la méthodologie de surface de réponse (RSM), à partir de feuilles d'*Aloysia triphylla*, suivie par l'étude des différentes activités biologiques des extraits conventionnels et optimisés par microonde, à savoir les activités antioxidantes, antibactériennes et anti-acetylcholinesterase et enfin caractériser ces extraits en terme de composition en polyphénols. La méthode Cental Composite Design (CCD), une méthodologie de surface de réponse largement utilisée, a été appliquée pour étudier l'effet des variables des procédés sur l'extraction assistée par micro-ondes (MAE) pour la récupération des composés phénoliques totaux (TPC). Ces variables indépendantes, à savoir la taille des particules, la concentration en éthanol (%), la puissance du micro-ondes (W), le temps d'irradiation (min), le rapport solide-solvant (g/mL) pour l'EAM ont été étudiées. L'analyse statistique a révélé que les conditions optimales de l'EAM ont été obtenues avec 125 µm, 40 % d'éthanol comme solvant d'extraction, 1:40 g/mL de rapport solide-éthanol, 188 seconde et 600 W pour la taille des particules, le solvant d'extraction, le ratio, le temps d'irradiation et la puissance, respectivement. La récupération maximale prévue des TPC dans les conditions optimisées était de 67.87±1.61 mg GAE/g DW, ce résultat était très proche de la valeur expérimentale de 67.86±0.92 mg GAE/g DW, cela permet la validation du modèle de régression et l'affirmation du succès du RSM à optimiser les conditions d'extraction. Les résultats des TPCs, des activités antioxydantes, antibactériennes et anti-acetylcholiesterase ont confirmé l'efficacité des deux extraits avec un meilleur résultat pour l'extrait obtenu par microonde comparé à l'extrait conventionnel. La capacité antioxydante a été évaluée à l'aide de plusieurs méthodes, y compris DPPH, FRAP, ABTS, TAC, ORAC et HOSC, et l'activité antibactérienne a été évaluée contre deux souches GRAM- et deux souches GRAM+. L'analyse HPLC-DAD-ESI-MS/MS nous a permis d'identifier plusieurs composants bioactifs. 30 composés, dont 4 acides phénoliques, 15 flavonoïdes, 7 phénylpropanoïdes et 4 iridoides. 8 de ces composés ont été identifié pour la premiére fois dans cette plante.

Mot clés : feuilles d'*Aloysia triphylla*, composés phénoliques, optimisation, extraction assistée par micro-ondes, activité antioxydante, activité antibactérienne, activité anti-acétycholinestérase, HPLC-DAD-ESI-MS/MS.

Abstract

The purpose of this study is to optimize the extraction of phenolics by microwave-assisted extraction (MAE) using the Response Surface Methodology (RSM), from leaves of *Aloysia triphylla*, followed by the study of the different biological activities of conventional extracts and optimized by microwave, namely antioxidant, antibacterial and anti-acetylcholnesterase activities, and finally characterize these extracts in terms of composition in phenolic compounds. The Cental Composite Design method, a widely used response surface methodology, was applied to study the effect of process variables on microwave-assisted extraction (MAE) for the recovery of total phenolic compounds (TPC). Independent variables, including particle size (µm), ethanol concentration (%), microwave power (W), irradiation time (seconds), solid-solvent ratio (g/mL) for MAE were investigated. Statistical analysis revealed that the optimum MAE conditions were obtained with 125 µm, 40% ethanol as extraction solvent, 1:40 g/mL solid-to-ethanol ratio, 188 second and 600 W for particle size, extraction solvent, ratio, irradiation time and power, respectively. The maximum expected recovery of TPC under optimized conditions was 67.87 1.61 mg GAE/g DW, which was close to the experimental value of 67.86 0.92 mg GAE/g DW. This results allows the validation of the regression model and the success of the RSM to optimize the extraction conditions. The results of TPCs, antioxidant, antibacterial and anti-acetylcholiesterase activities confirmed the efficacy of both extracts with better results for the extract obtained by microwave compared to the conventional extract. Antioxidant capacity was evaluated using several methods including DPPH, FRAP, ABTS, TAC, ORAC and HOSC, and antibacterial activity was evaluated against two GRAM- and two GRAM+ strains. HPLC-DAD-ESI-MS/MS analysis allowed us to identify several bioactive components. 30 compounds, including 4 phenolic acids, 15 flavonoids, 7 phenylpropanoids and 4 Iridoids. 8 of these compounds were first identified in this plant.

Keywords: *Alloysia triphylla* leaves, phenolic compounds, optimization, microwave-assisted extraction, antioxidant activity, antibacterial activity, anti-acetylcholinesterase activity, HPLC-DAD-ESI-MS/MS.

ملخص

تهدف هذه الدراسة الى تحسين إستخلاص المركبات الفينولية من أوراق لويزة الليمون بطريقة المساعدة بالمكروويف و ذلك بمنهجية إستجابة السطح (RSM) . تمت بعد ذلك دراسة النشاطات البيولوجية المضادة للاكسدة، المضادة للبكتيريا والمضادة لانزيم كولين استيراز لمختلف المستخلصات العادية والمحسنة بالميكروويف. اتبعت هذه الدراسة بتمييز التركيبة الفينولية لهذه المستخلصات .استعملت طريقةCentral Coposite Design (CCD) لدراسة تأثير متغيرات المعالجة على الإستخلاص بطريقة المساعدة بالميكروويف لإستخراج مجمل المركبات الفينولية. تمت دراسة المتغيرات المستقلة المتمثلة في حجم الجزيئات (µm) ، تركيز الايثانول (%)، إستطاعة الميكروويف (W)، زمن التعريض للاشعاع (د)، و النسبة مذاب ـ مذيب (غ/مل) . أظهرت نتائج التحليل الإحصائي أن الظروف المثلى لطريقة EAM هي 125 µm لحجم الجزيئات، 40٪ إيثانول كمحلول إستخلاص، 1:40 (غ/مل) كنسبة مذاب ـ مذيب، 188 ثا كزمن التعريض للإشعاع و 600 واط إستطاعة الميكروويف. قدر الإستخراج الأقصى المتوقع ل TPCs في الظروف المحسنة ب 67.87±1.61 mg GAE/g DW و التي تبدو قريبة جدا من النتيجة التجريبية (67.86±0.92 mg GAE/g DW). تسمح هذه النتيجة بتصديق نموذج الإنحدار و تأكيد منهجية RSM في تحسين ظروف الإستخلاص. أظهرت نتائج TPCs ، و النشاط المضاد للأكسدة، للبكتيريا و لإنزيم كولين إستراز فعالية المستخلصين العادي و المحسن مع فعالية أكبر للمستخلص المحسن. تم تقدير النشاط المضاد للأكسدة بعدة طرق و هي DPPH, FRAP, ABTS, TAC, ORAC et HOSC . النشاط المضاد للبكتيريا تم تحديده ضد سلالة +GRAM و -GRAM . كما سمح تحليل HPLC-DAD-ESI-MS/MS بالكشف عن 30 مركب حيوي نشيط، منها 4 أحماض فينولية، 15 فلافونويد، 7 فنيل بروبانويد و 4 إريدرويد. 8 من بين هذه المركبات تم الكشف عنها مسبقا في هذه النبتة.

الكلمات المفتاحية: أوراق لويزة الليمون، المركبات الفينولية، تحسين، إستخلاص بمساعدة الميكروويف، نشاط مضاد للأكسدة، نشاط مضاد للبكتيريا، نشاط مضاد لإنزيم كولين إستراز، HPLC-DAD-ESI-MS/MS