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Experimental study of the biological and therapeutic effects of two local plants Milk thistle (*Silybum marianum (L.) Gaertner*) and Globularia (*Globularia Alypum.E*)

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ملخص

هذا العمل مكرس لاستقصاء وتثمين نباتين محليين: شوكة الجمل و تسلغا وكذلك المقارنة بينهما في بعض الأنشطة البيولوجية. يهدف هذا العمل إلى استكشاف التركيب الكيميائي النباتي لكلا النباتين، وتحديد ومقارنة أنشطتهم البيولوجية (مضادات الأكسدة ومضاد للبكتيريا). تم إجراء التقطير المائي ل نبتة التسلغا التي تم جمعها من منطقة آفلو بالأغواط في فبراير 2020 وتم استرداد الزيت العطري بعد عملية الإبانة باستخدام ثنائي إيثيل الأثير حيث تم استخلاص زيت عطري متطاير للغاية من نبتة التسلغا ،يتبخر في درجة حرارة الغرفة.

Résumé

Le présent travail est consacré à l'étude et à la valorisation de deux plantes locales: le chardon-Marie et le Globularia ainsi qu'à une comparaison entre elles dans certaines activités biologiques. Ce travail vise à explorer la composition phytochimique des deux plantes, à déterminer et à comparer leurs activités biologiques (antioxydantes et antibactériennes). L'hydro-distillation a été réalisée pour Globularia qui a été collectée à Aflou / Aghouat en février 2020 et l'huile essentielle a été récupérée après décantation à l'éther diéthylique. L'huile essentielle de Globularia s'est révélée très volatile et s'évapore à température ambiante.

Abstract

The present work is dedicated to the investigation and valorization of two local plants: Milk thistle and Globularia as well as a comparison between them in some biological activities. This work aims at exploring the phytochemical composition of both plants, determining, and comparing their biological activities (antioxidant and antibacterial). Hydro distillation was performed for Globularia that was collected in Aflou/Aghouat in February 2020 and essential oil was recovered after decantation with diethyl ether. The essential oil of Globularia was found very volatile and evaporates at room temperature.

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INTRODUCTION

Men has relied on nature, since antiquity and still, to cover his daily needs from nutrition and clothing to treating illnesses based on observations he made throughout ages giving birth to traditional medicine[1,2]. Traditional medicine or Folks medicine uses local plants and herbs and their infusions to treat and prevent diseases and their symptoms. According to the World Health Organization WHO, nearly 80% of population depend on traditional medicine to cure diseases[3]. Thus, developing and supporting traditional medicine is primordial for both health care and economic systems[3].

Plants are an inexhaustible source of bioactives that are exploited by Men. Plants' bioactives are called secondary metabolites. They are luxury products synthesized for different purposes mainly to defend against predators and to communicate with other plants[2]. Medicinal plants have ever since been a growing source of interest for scientific research as well as food and chemical and pharmaceutical for their diverse applications thanks to their antioxidant, antibacterial, stimulant and inhibiting properties[2].

Obtaining secondary metabolite can be a challenge as the wrong choice of extraction methods could alter or destroy bioactive compounds such as thermolabile substances. Hence, a beforehand knowledge of secondary metabolites properties and mastering extraction methods are primordial for an effective extraction. Extraction methods can range from conventional extraction such as solvent extraction to modern techniques like enzymatic assisted extraction EAE, that are more efficient methods and more environment friendly as they require very little to no use of solvents.

Oxidation and the generation of free radicals has been a major hazard to good health and been a key factor in the development of countless diseases such as cancer, hypertension and diabetes[1]. Antioxidants released by the body can be over balanced by oxidant agent creating oxidative stress. Therefore, the consumption of antioxidant rich dietary is key in preventing fatal illnesses. Moreover, bacterial infections are becoming increasingly challenging to master owing to the complexity of bacteria and the rapid development of resistance against the used antibiotic. Thus, the conception of new antibacterial agents is crucial to saving public health[1].

Regarding the immerging concerns raised about the current abusive utilization of chemicals and the dangerous side effects related to their administration, orienting to medicines based on plants (phytomedicines) and exploiting their antioxidant and antibacterial properties to combat modern public health problems, is an immediate necessity[4].

As a matter of consequences, milk thistle and globularia were investigated and valorized. Milk thistle and globularia are two abundant wild plants in Algeria famous in folks medicine for their liver protective and antibacterial properties and have been used since ages as infusions or powdered either sole or combined with honey. Milk thistle has recently become the center of interests in the western world for its anticancer properties. Yet, a lack of literature, especially in Algeria, in this matter is noticed. The present work consists of elaborating a study on the phytochemical composition and biological activities in both local plants. The general aim remains in the context of valorization of local plants and contributing to the conception of plant-based medicines.

Chapter I

Active substances in plants

I.1. Introduction:

Plants produce a wide range of bioactive molecules that serve many purposes. Those that are destined for biological functions and surviving are called primary metabolites. On the other hand, those compounds that are luxury products are called secondary metabolites. They serve as extra Nutritionals and may have adverse effects on other living organisms. Secondary metabolites have been used since antiquity in traditional medicine due to their benefits on human health. They are potent antioxidants, anticancer, antidiabetic, anti-inflammatory, stimulants... Their use has witnessed a dramatic increase in pharmaceutical, chemical, and food industries for the advantages they present. However, obtaining these compounds is a challenging process as they represent a small portion of plants raw material. Diverse extraction methods, from conventional methods such as solvent extraction to modern techniques like enzymatic assisted extraction EAE, have been used to improve extraction yield in terms of quality and quantity as well as environmental safety. This chapter discusses the main active substances categories, their role for plants and men as well as the different extraction methods used to obtain them.

I.2.Essential oils:

I.2.1- Definition:

Essential oils (EO) are complex mixtures of volatile, lipophilic, and liquid compounds, extracted from different parts of a plant by physical processes. These interesting natural products have the characteristic odor of the plant.

According to ISO norm (ISO/D159235) "An essential oil is a product made by distillation with either water or steam or by mechanical processing of citrus rinds or by dry distillation of natural materiel. Following the distillation, the essential oil is physically separated from the water phase."

Essential oils are highly concentrated substances and therefore rarely used neat[5,] they evaporate slowly when exposed to air at room temperature, and because of this, they are sometimes referred to as volatile oils[5].

EO's quality depends on many factors:

- Origin and parts of the plant used.
- Harvest way and extraction technique.
- Purification process.

I.2.2-Essential Oils composition:

The EOs are composed of terpenoids in large proportion, phenylpropanoids, and other compounds of diverse origin such as that resulting from the degradation of fatty acids.

I.2.2-a. Terpenoids:

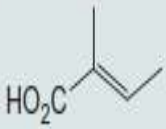
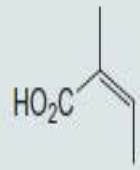
The Terpenoids represent the major part of essential oil compounds; they are defined as substances composed of isoprene (2-methylbutadiene) units [6]; they are synthesized by acetate via the mevalonic acid pathway.

The Terpenoids are classified according to the number of C_5 isoprene units that they contain; the classes are:

- **Hemiterpenes C_5:**

Hemiterpenes are the simplest type of terpenoids; they consist of a single unit of isoprene. There is evidence that these compounds may assist in plant defense by repelling herbivores or by attracting predators and parasites of herbivores[7].

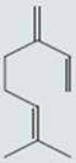
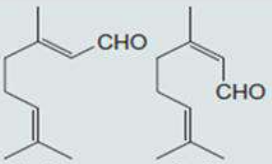
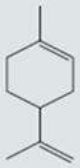
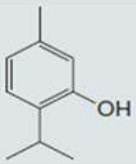
Table I.1 *The Chemical Structures, Resources, and Habitats of some Hemiterpenes* [8]

Name of Compounds	Structure	Resource	Habitat
Tiglic acid		*Schoenocaulon officinale* (Schltdl. & Cham.) A. Gray (Melanthiaceea) *Croton tiglium* L. (Euphorbiaceae)	Southern North America, Guatemala, Venezuela Africa, South America, Indian subcontinent
Angelic acid		*Archangelica officinalis* Hoffm. (Apiaceae)	Europe, the United States
		Peucedanum ostruthium (L.) Koch (Apiaceae)	Central and southern Europe
		Levisticum officinale Koch (Apiaceae)	Europe and southwestern Asia

- **Monoterpenes C_{10}:**

Monoterpenes are compounds made up of two units of isoprene, and of chemical formula $C_{10}H_{16}$, among the classes included in this group of terpenoids: iridoids and pyrethrins; they have anti-inflammatory pharmacological properties.

Table I.2 *The Chemical Structures, Resources, and Habitats of Monoterpenes*[8]

Name of Compounds	Structure	Resource	Habitat
Acyclic Monoterpenoids			
Myrcene		Varbena oil (*Lippia citriodora* Kunth., Verbenaceae)	South and central America, tropical Africa
		Bay laurel oil (*Laurus nobilis* L., Lauraceae)	Mediterranean region
Citral		Lemongrass oil (*Cymbopogon flexuosus* (Nees ex Steud.) J.F. Watson	Southern India
		and *Cymbopogon citratus* (DC. ex Nees) Stapf, Poaceae)	Malaysia
Monocyclic Monoterpenoids			
Limonene		Major constituent of several citrus essential oils (Rutaceae): orange (*Citrus sinensis* (L.) Osbeck), lemon (*Citrus limon* (L.) Burm. f.), mandarin (*Citrus reticulata* Blanco), lime (*Citrus aurantifolia* (Christm.) Swing), and grapefruit (*Citrus paradisi* Macfad)	Southeast Asia (southern China, India, Indonesia, Malay Archipelago)
Thymol		Thyme oil (*Thymus vulgaris* L., Lamiaceae)	Southern Europe from the western Mediterranean to southern Italy

- **Sesquiterpenes C_{15}:**

This class of terpenes has a crude formula $C_{15}H_{24}$ (three units of isoprene), these molecules are found in linear or cyclic form; they have several applications but are rarely found in EOs because of their low volatility.

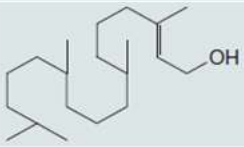
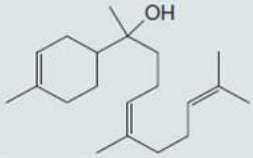
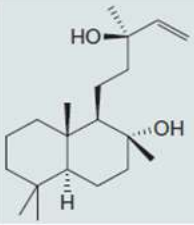
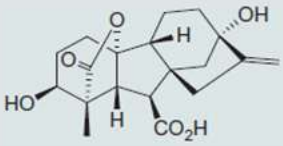
- **Diterpenes C_{20}:**

Diterpenes consist of a group of chemically heterogeneous compounds, their crude formula is $C_{20}H_{32}$ (four units of isoprene).Because of their higher boiling points, they are not

considered to be essential oils; instead, they are classically considered to be resins, the material that remains after steam distillation of a plant extract [7].

Diterpenes can be classified as linear, bicyclic, tricyclic, tetracyclic, pentacyclic, or macrocyclic Diterpenes depending on their skeletal core [8].

Table I.3: *The Chemical Structures, Resources, and Habitats of Diterpenes*[8]

Name of Compounds	Structure	Resource	Habitat
Phytol		Present as the ester attachementin the molecule of chlorophyll	
9-Geranyl-α-terpineol		*Helichrysum heterolasium* Hilliard (Asteraceae)	Southern Africa
Sclareol		Clary sage (*Salvia sclarea* L., Lamiaceae)	Native to the northern Mediterranean, along with some areas in north Africa and central Asia
Gibberellin A1		Belongs to gibberellins—plant hormones that regulate growth. First isolated from fungal strains (*Giberella fujikuroi*)	

- **SesterterpenesC$_{25}$:**

Sesterterpenes consist of 5 units of isoprene (25 carbon backbone). They exist in a wide variety of forms, including linear, monocyclic, bicyclic, tricyclic, tetracyclic, and macrocyclic frameworks [8]; as shown below in the **Table I.4**.

- **Triterpenes C$_{30}$:**

The triterpenes are made up of six isoprenes, they have relatively complex cyclic structures; the **TableI.5** shows some examples of triterpenes.

There are also tetraterpenes which consist of 8 units of isoprene ($C_{40}H_{64}$); and when the number of isoprene units exceeds 8 units, terpenes are called polyterpenes (C> 40).

Table I.4: *The Chemical Structures, Resources, and Habitats of Sesterterpenes* [8]

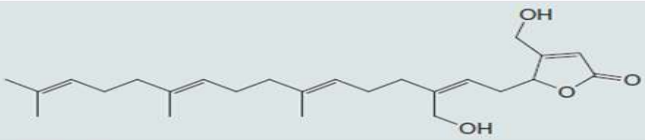
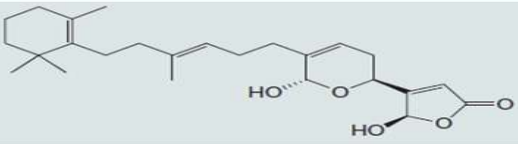
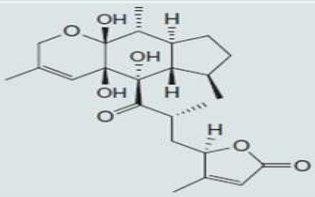
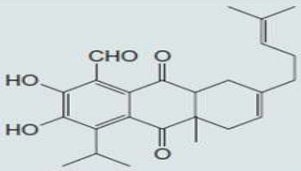
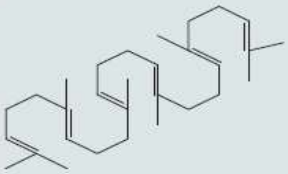
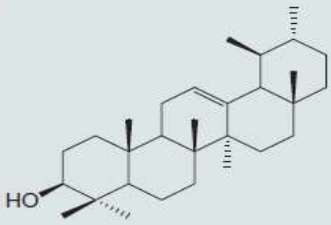
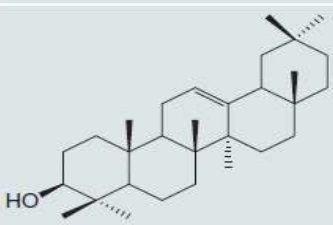
Name of Compounds	Structure	Resource	Habitat
Hippolide E		Marine sponge *Hippospongia lachne* de Laubenfels (Spongiidae)	Distributed around the world in the western central Atlantic
Manoalide		Marine sponge *Luffariella variabilis* Polejaeff (Thorectidae)	Distributed widely through the Indo-Pacific
Leucosceptrine		*Leucosceptrum canum* Sm., Lamiaceae	Native to south western China (Sichuan, Tibet, Yunnan)
Heliocide H1		Upland cotton (*Gossypium hirsutum* L., Malvaceae)	Native to Mexico, the West Indies, northern South America, and Central America

Table I.5: *The Chemical Structures, Resources, and Habitats of Triterpenes* [8]

Name of Compounds	Structure	Resource	Habitat
Squalene		Liver oil of shark (e.g., gray reef shark, *Carcharhinus amblyrhynchos*). Olive oil from the fruit of *Olea europaea* L., Oleaceae	Africa, the Mediterranean Basin from Portugal to the Levant, the Arabian Peninsula, and southern Asia as far east as China, as well as the Canary Islands, Mauritius and Reunion
α-Amyrin		Resins of *Bursera* and *Protium* species (Burseraceae)	Native (for many species endemic) to the Americas, from the southern United States south through to northern Argentina Native to the Neotropics, Madagascar, New Guinea, and southern Asia from Pakistan east to Vietnam
		Taraxacum officinale F. H. Wigg., Asteraceae	Temperate regions of the world
β-Amyrin		Lotus (*Nelumbo nucifera* Gaertn., Nelumbonaceae)	Native to Tropical Asia and Queensland, Australia
		Cuachalalate (*Amphipterygium adstringens* (Schltdl.) Standl., Anacardiaceae)	Central and southern Mexico

I.2.2-b.Phenylpropanoids:

The phenylpropanoids are a family of organic compounds with an aromatic ring and a three-carbon propene tail [9] (Zhang and Stephanopoulos, 2016); they are synthesized via shikimic acid pathway (biosynthesis).

I.2.3- essential oils properties:

The different properties of OEs are related to the species and their chemical composition.

I.2.3-a.Physicochemical properties:

Essential oils are characterized by several physicochemical properties:

- The EOs are known by their volatile, odorous, and flammable character.
- They are colorless or have a yellowish color in the liquid state and at ambient temperature.
- They have a low polarity and a low solubility in water however they are soluble in alcohol and most of the organic solvents.
- They have a lower density than water (0.8 to 1.08).

I.2.3-b. Biological properties:

Essential oils have various biological properties due to the variety of their chemical composition, they are using as:

- Antimicrobial;
- Antioxidant;
- Anti-inflammatory;
- Antiseptic……..

I.3. Lipids:

I.3.1- Definition:

Unlike the various families of basic molecules in the living world characterized by chemical structures, lipids are defined by their solubility not on the basis of their chemical structure[10].

Lipids are among the primary metabolites, they consist of a heterogeneous group of compounds characterized by their insolubility in water, on the other hand they are soluble in non-polar organic solvents such as: chloroform and alcohols.

Lipids play mainly important roles in plants, as signaling and energy storage compounds[11].

I.3.2- Classification:

Lipids mainly include fatty acids, simple lipids and complex lipids.

I.3.2-1. Fatty acids:

The fatty acids are carboxylic acids R-COOH; radical R is an aliphatic chain of hydrocarbon type of variable length which gives the molecule its hydrophobic character (fatty).

They are generally mono carboxylic, and characterized by a linear chain with an even number of carbons [12].

I.3.2-1.a. Saturated fatty acids:

A fatty acid consists of a hydrocarbon chain, more or less long, strongly apolar, and a polar carboxyl group. The general formula of fatty acids is $CH_3 - (CH_2)_n – COOH$ [12].

The most abundant fatty acids are: palmitic acid and stearic acid.

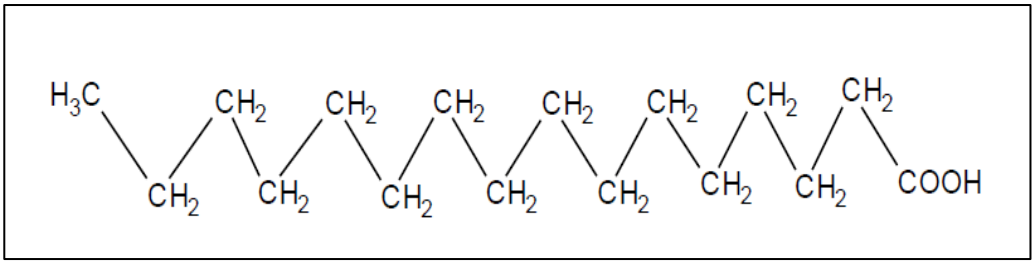


Fig I.1: *palmitic acid.*

I.3.2-1.b. Unsaturated fatty acids:

The structure of unsaturated fatty acids includes one or more double bonds; the presence of these double bonds gives them specific physicochemical properties [12].

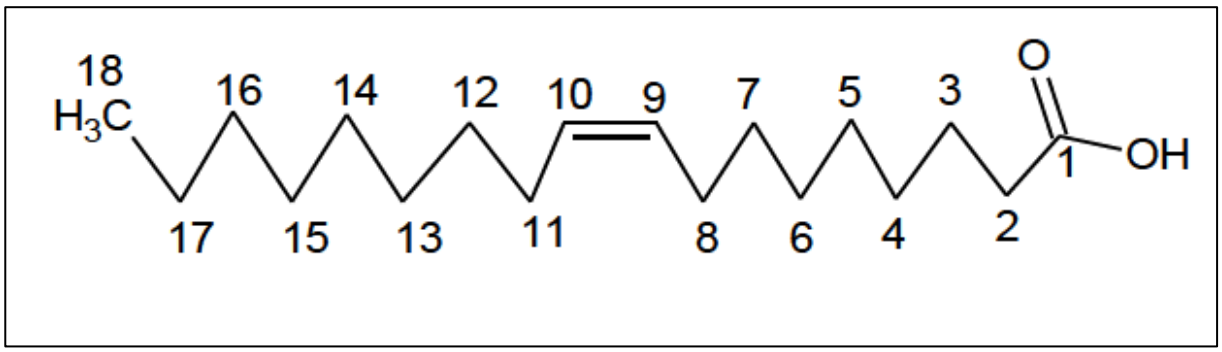


Fig I.2: *oleic acid.*

I.3.2-1.c. Atypical fatty acids:

Atypical fatty acids are characterized by an odd number of carbons; they are present in animal fats or in microbial lipids [12].

I.3.2-2. Simple lipids:

Simple lipids or homolipids are ternary bodies (C, H, O), they are fatty acid esters, depending on the alcohol we distinguish the following classes [12]:

- **Acylglycerols:** or glycerides are esters of glycerol,
- **Cerides:** long chain alcohol esters (fatty alcohol),
- **Sterids:** sterol esters (polycyclic alcohol).

I.3.2-3. Complex lipids:

Complex lipids are hetero lipids which contain phosphate, sulfate or carbohydrate groups. They are classified according to the molecule that fixes fatty acids [12].

I.3.2-3.a. Glycerophospholipids:

These are the most numerous and most represented lipids which are constructed from the skeleton of a glycerol mono ester [12].

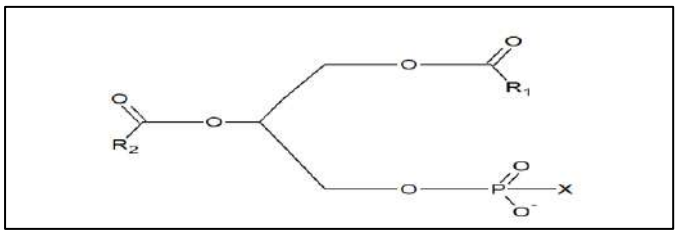


Fig I.3: *Glycerophospholipids.*

I.3.2-3.b. Glyceroglycolipids:

The alcohols of the C1 and C2 carbons of glycerol are esterified by fatty acids and the C3 carbon alcohol, unlike the glycerolipids, is not esterified, but it is linked to an ose by a glycosidic bond [12].

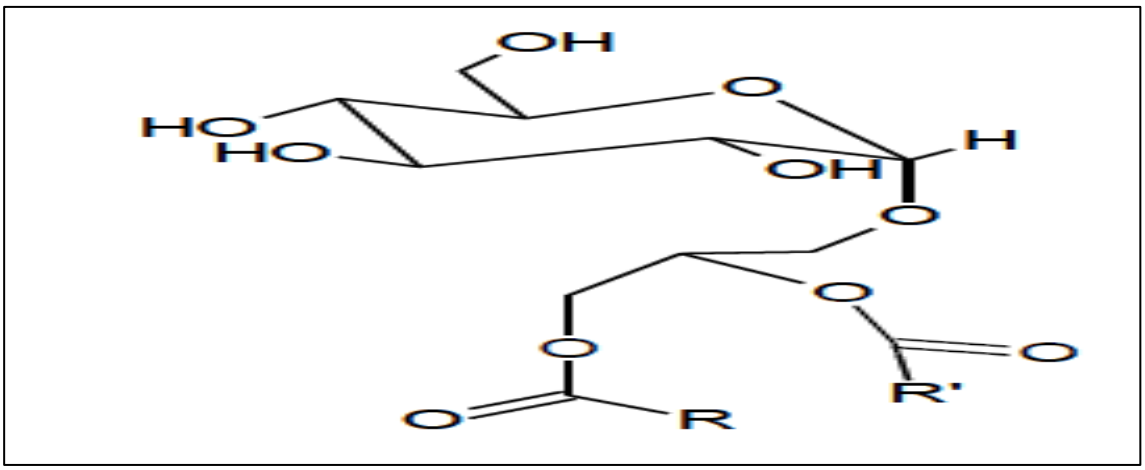


Fig I.4: *Glyceroglycolipids.*

I.3.2-3.c. Sphingolipids:

The skeleton from which these lipids are made is not glycerol but a long-chain carbon diol amine of the sphingoid type; the binding of a fatty acid to the amine group gives a ceramide which is the lipid precursor molecule of this group [12].

I.4. Alkaloids:

Alkaloids are natural compounds with low molecular weight[13] that are characterized by the presence of nitrogen atoms. They are mostly found in plants as secondary metabolites and some other animals and fungi[14].

They are derived from amino acids containing one or more heterocyclic nitrogen atoms[15]. Alkaloids have played a huge role in traditional medicine in various civilizations since antiquity due to their potential therapeutic properties. Toxic alkaloids were similarly used in poisonous arrows[16]. However, it was until 1804 that they were isolated and characterized by Friedrich Sertürner[17] and Derosne[13].

I.4.1- Classification:

Due to their diverse structures, finding a common classification was complicated[18]. Some studies considered a classification based on the ring systems: indolizidine- and quinolizidine-based systems and quinoline-, quinazoline-, and acridone-based systems[15]. Yet, this

classification mode causes confusion as some alkaloids can be categorized in more than one class[19]. Henceforth, a new classification was adopted[13]:

True alkaloids: The nitrogen atom, originating from the precursor amino acid, is part of the alkaloid heterocyclic ring.

Protoalkaloids: The nitrogen atom, originating from the precursor amino acid, is not part of the alkaloid heterocyclic ring.

Pseudoalkaloids: They are not originated from amino acids, including steroids-like, purine-like and terpene-like alkaloids.

Each class is divided to subclasses according to the precursor amino acid.

I.4.2- Therapeutic usefulness:

Alkaloids have a wide range of potential therapeutic activities. Morphine has narcotic effect, cocaine is a potent central nervous system stimulant, taxol as an effective anticancer chemical, colchicine as an anti-inflammatory[15], tubocurarine is a poisonous alkaloid used to relax muscles prior to surgeries[20], vinblastine, one of the antitumor alkaloids, and quinine being an effective anti-malarial[21].

I.4.3- Role for plants:

Despite the various advantages they present for Man, their role in the plant is not well known. Some studies suggest that they help the process of seed formation[21] and protect the plant against herbivores and unwanted plants[22] whereas some others propose they are waste products[21].

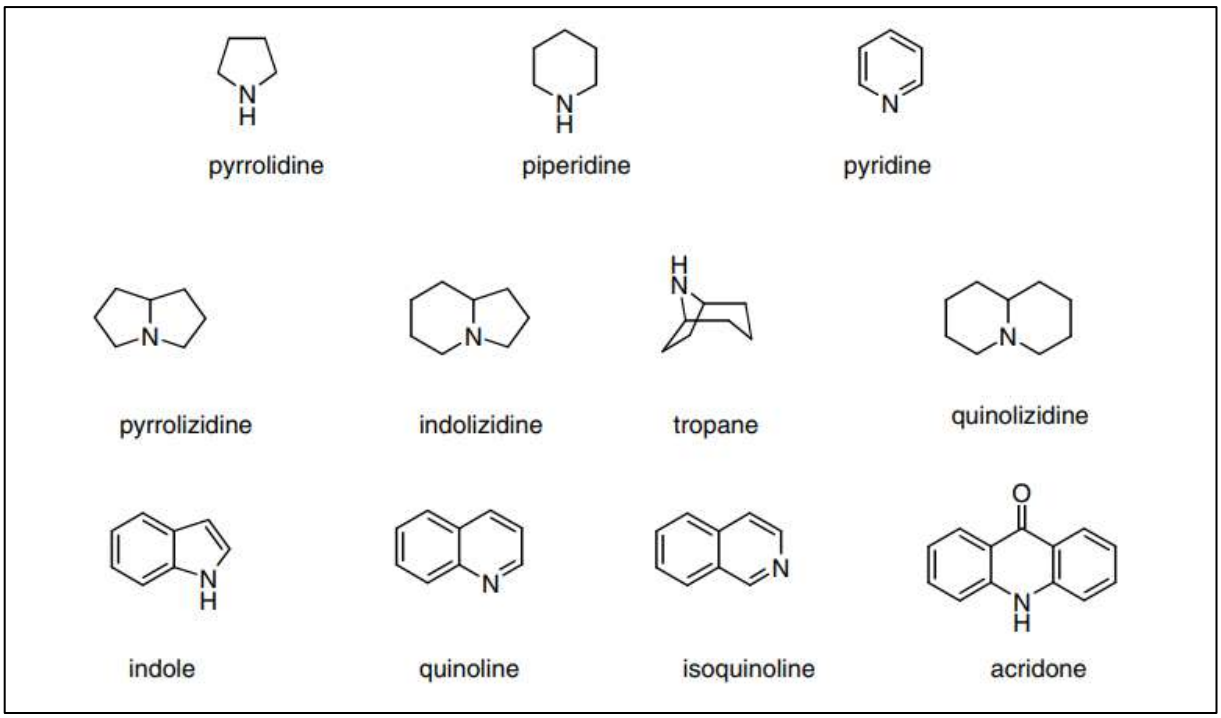


Fig I.5: *Alkaloids classes based on ring systems*[15]

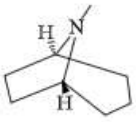
True alkaloids

Precursor: L-ornithine

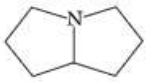
Pyrrolidine



Tropane

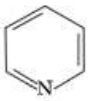


Pyrrolizidine



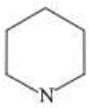
Precursor: nicotinic acid

Pyridine

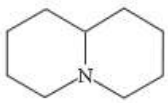


Precursor: L-lysine

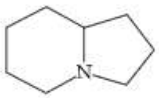
Piperidine



Quinolizidine

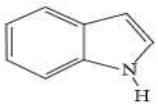


Indolizidine

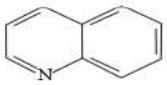


Precursor: L-tryptophan

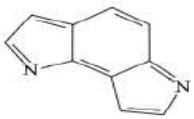
Indole



Quinoline

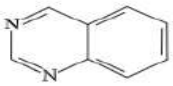


pyrroloindole

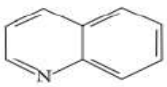


Precursor: anthranilic acid

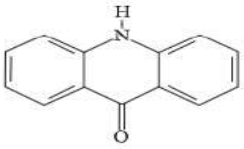
Quinazoline



Quinoline

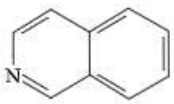


Acridone



Precursor: L-tyrosine or L-phenylalanine

Isoquinoline

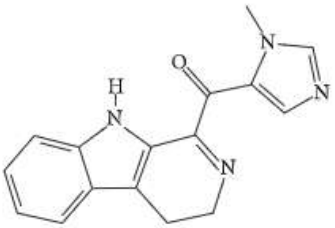


Precursor: L-histidine

Imidazole



Manzamine



(a)

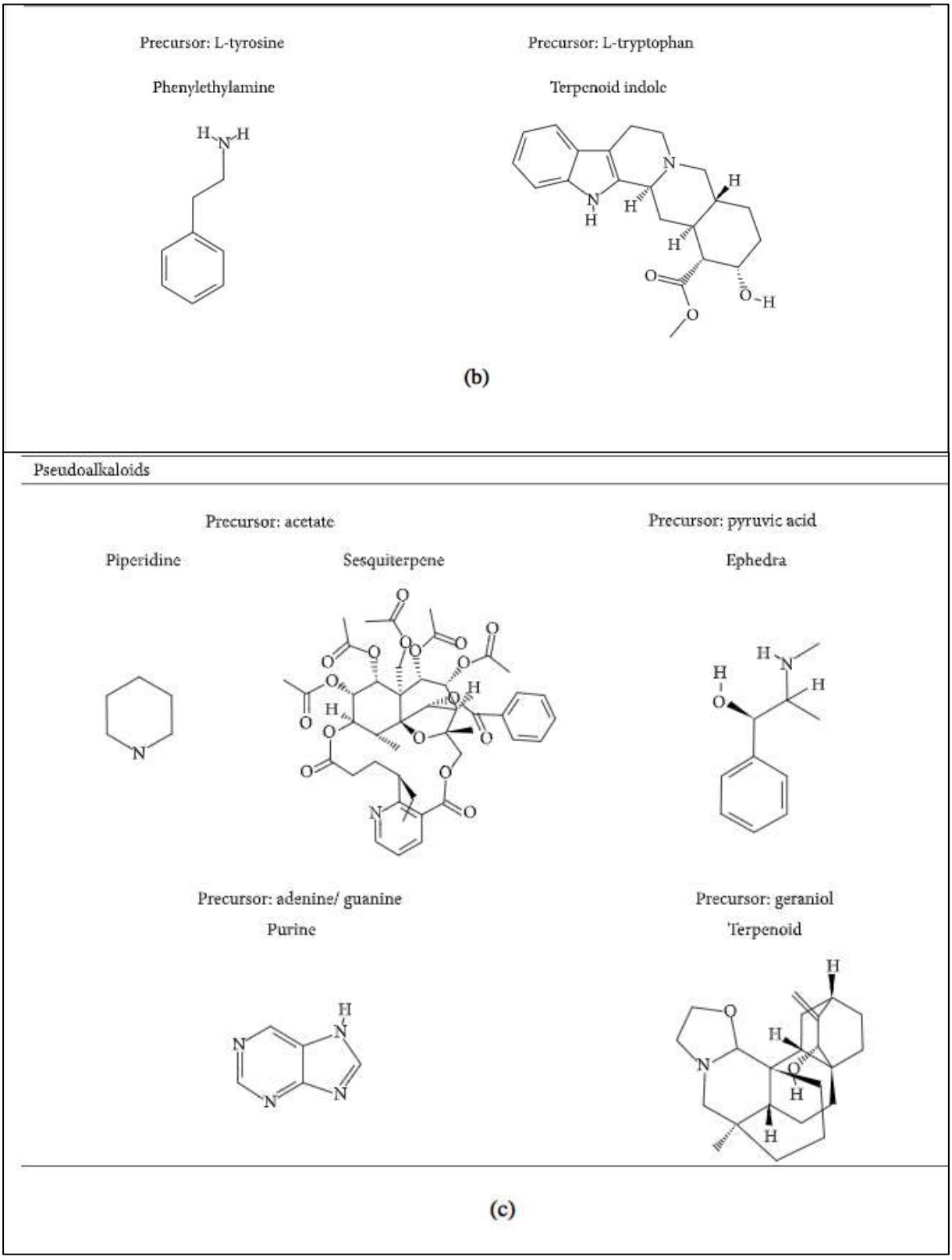


Fig I.6: *Main alkaloids groups: True Alkaloids (a), Protolakaloids (b) and Pseudoalkaloids (c), precursors, and skeleton structures.*[13]

I.4.4- Extraction methods:

There exist as many extraction methods as the diversity of alkaloids structures[19]. They are extracted exploiting their solubility in organic solvents and water for their salts[23]. Base or acidic extractions followed by purification are widely used to obtain alkaloids.

Base extraction consists of the use of alkaline solvents including 1,2-dichloroethane, chloroform, diethyl ether or benzene. Base Alkaloids are then converted to salts by the addition of weak acids and are washed with water to crystallize. The purification method is repeated until the desired purity is reached.

Acidic extraction, on the other hand, processes the finely grounded raw plant material with weak acidic solutions mentioning acetic acid in water, methanol or ethanol... followed by the addition of basic solutions to convert alkaloids to basic forms to allow their extraction with organic solvents. The same purification steps are followed[18].

Different alkaloids are separated based either on the difference of solubility or boiling temperature[23].

I.5. Phenolic compounds:

Phenolics are organic compounds containing one aromatic ring to which is attached one or more hydroxyl groups[15]. Phenolic compounds represent one of the most extensive groups of plants' secondary metabolites[24]. They are contained in a great share in the daily diet due to their countless benefits for human health and well-being. Henceforth, the consumption of phenolic-rich fruits and vegetables, such as berries and spinach, is increasingly recommended[24].

They are synthesized either through the shikimic acid or the acetic acid pathways. Phenylpropanoids are the result compounds of the shikimic acid pathway while simple phenols result from the acetic acid pathway acid. However, both pathways result in the formation of flavonoids, abundant phenolic compounds[25].

In addition to the presence of aromatic rings and hydroxyl substitutions, phenolic compounds are less likely to be found in the free form, rather, they are most frequently bound to other complex compounds as sugars to reduce their toxicity[25].

I.5.1- Classification:

Due to the wide variety of phenolic compounds ranging from simple to polymeric, there exist multiple classifications:

- Based on the carbon chain:

From simple phenols to complicated lignins, 16 different classes can be distinguished. The main classes are shown in table I.6 and figure I.7.

- Based on solubility:

Depending on the form phenolics are found in plans (either free from any bound to other compounds or attached to cell wall polysaccharides or proteins), their solubility varies. Therefore, two categories can be defined:

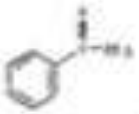
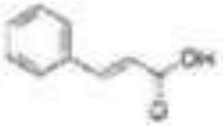
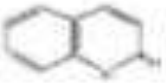
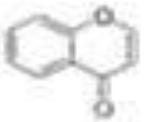
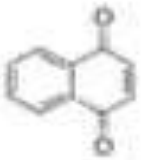
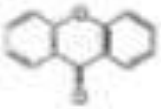
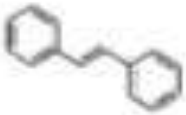
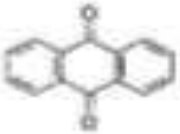
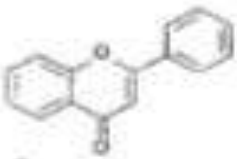
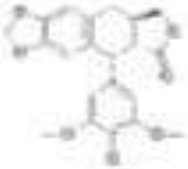
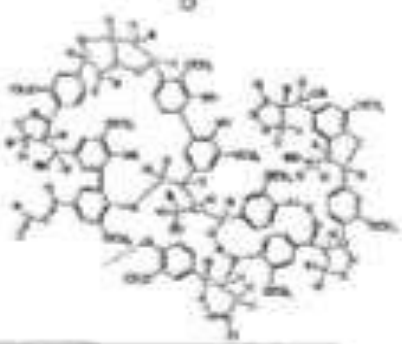
- Soluble phenolics: Free phenolics found in the soluble fraction of

the cell such as phenols, flavonoids, and tannins.

- Insoluble phenolics: Bound to other more complicated molecules to

form stable insoluble compounds. They majorly include condensed tannins and phenolic acids.

Table I.6: *Main classes of phenolic compounds based on carbon skeleton*[25]

Class	Basic skeleton	Basic structure
Simple phenols	C_6	
Benzoquinones	C_6	
Phenolic acids	C_6-C_1	
Acetophenones	C_6-C_2	
Phenylacetic acids	C_6-C_2	
Hydroxycinnamic acids	C_6-C_3	
Phenylpropenes	C_6-C_3	
Coumarins, isocoumarins	C_6-C_3	
Chromones	C_6-C_3	
Naphthoquinones	C_6-C_4	
Xanthones	C_6-C_1-C_6	
Stilbenes	C_6-C_2-C_6	
Anthraquinones	C_6-C_2-C_6	
Flavonoids	C_6-C_3-C_6	
Lignans and neolignans	$(C_6$-$C_3)_2$	
Lignins	$(C_6$-$C_3)_n$	

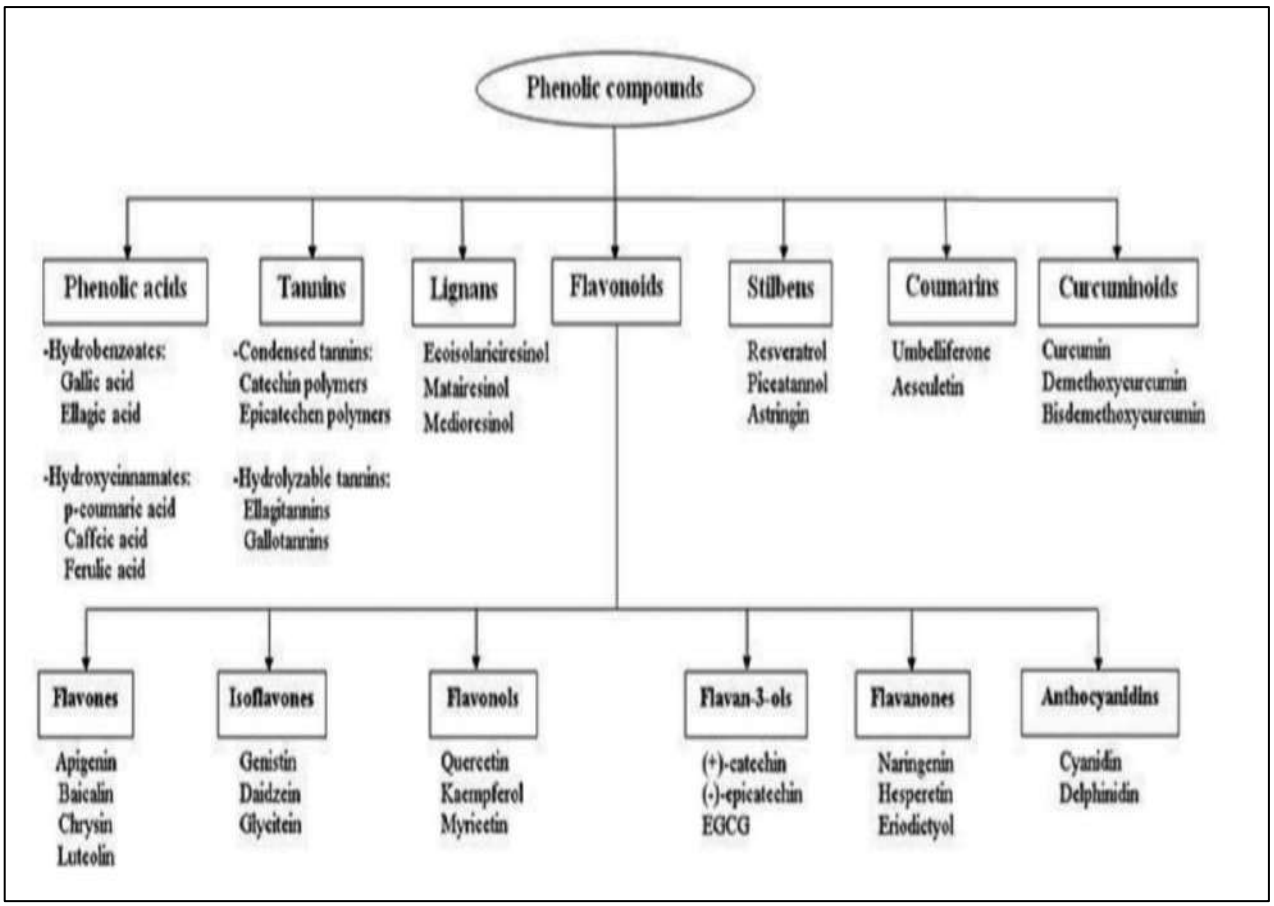


Fig I.7: *Classes of phenolic compounds*[26].

- Based on distribution:

Phenolic compounds are found in nature in different proportions. They thus can be divided to shortly distributed (simple phenols, pyrocatechol, hydroquinone, resorcinol ...), widely distributed (flavonoids and their derivatives, coumarins, and phenolic acids) and polymers (lignins and tannins)[25].

I.5.2- Main classes:

I.5.2-a. Simple phenols:

They are monomers constituted of various substitutions and represent the forming blocs of polymeric phenolic and acid compounds that make up the plant tissue. Some of them (p-hydroxybenzoic acid, protocatechuic acid, vanillic, syringic, salicylic, and gallic acids) can be

obtained by the hydrolysis of the plant tissue whereas some other free simple phenols do not require the destruction the cell wall polymers[15].

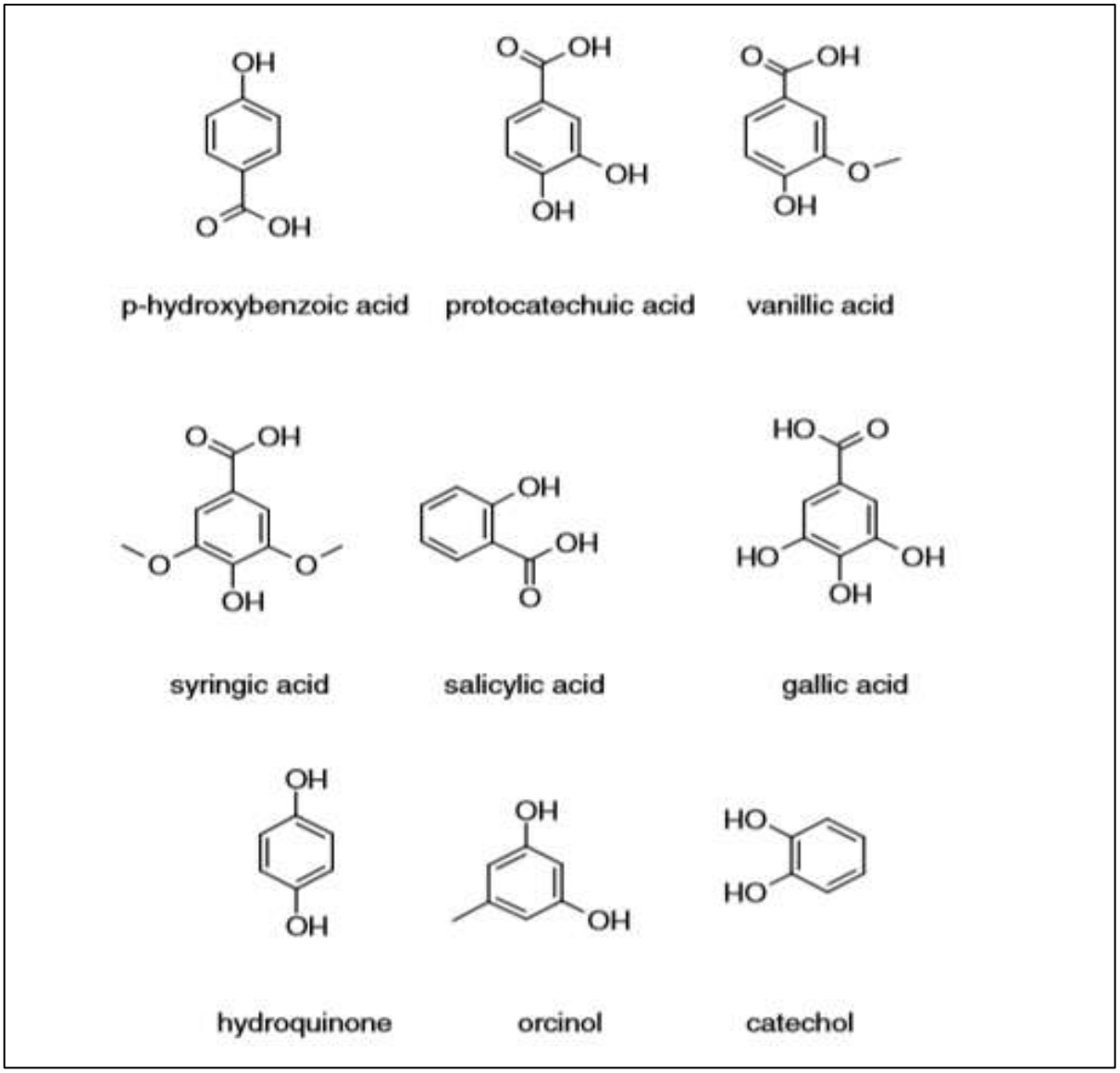
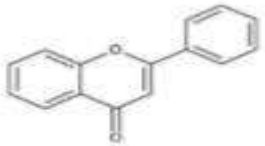
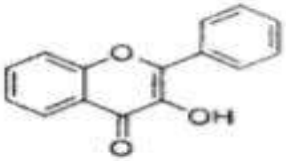
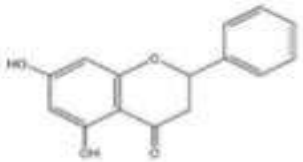
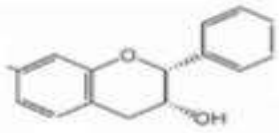
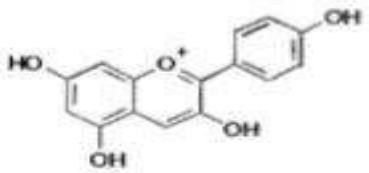
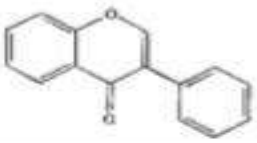


Fig I.8: *Simple phenols[15].*

I.5.2-b. Flavonoids:

They are compounds containing two aromatic rings attached by a bridge of triple carbon atoms (C6-C3-C6). The bridge often contains double carbon bonds and hetero-elements, which forms another mid-cycle. Therefore, flavonoids can be classified into 13 subgroups among which e flavonols, flavanols, flavones, isoflavones, anthocyanidins or anthocyanins, and flavanones[25].

Table I.7: *Main classes of flavonoids*[25].

Flavonoid	Basic structure
Flavones	
Flavonols	
Flavanones	
Flavanols	
Anthocyanidins	
Isoflavones	

I.5.2-c. Tannins:

They are compounds with a medium to high molecular weight that comprise two major classes: hydrolyzable and condensed tannins. Hydrolyzable tannins are glucose or a polyhydric alcohol centered compounds esterified with gallic acid or hexahydroxydiphenic acid to form gallotannin and ellagitannins, respectively. They are hydrolyzed by enzymatic, acidic or base treatment. On the other hand, condensed tannins, also known as non-hydrolyzable tannins or recently proanthocyanidins, are polymers of catechin and leucoanthocyanidin. Their antioxidant activity is dependant on their degree of polymerization. High molecular weight tannins have been demonstrated to be fifteen to thirty times more active than simple phenols[25].

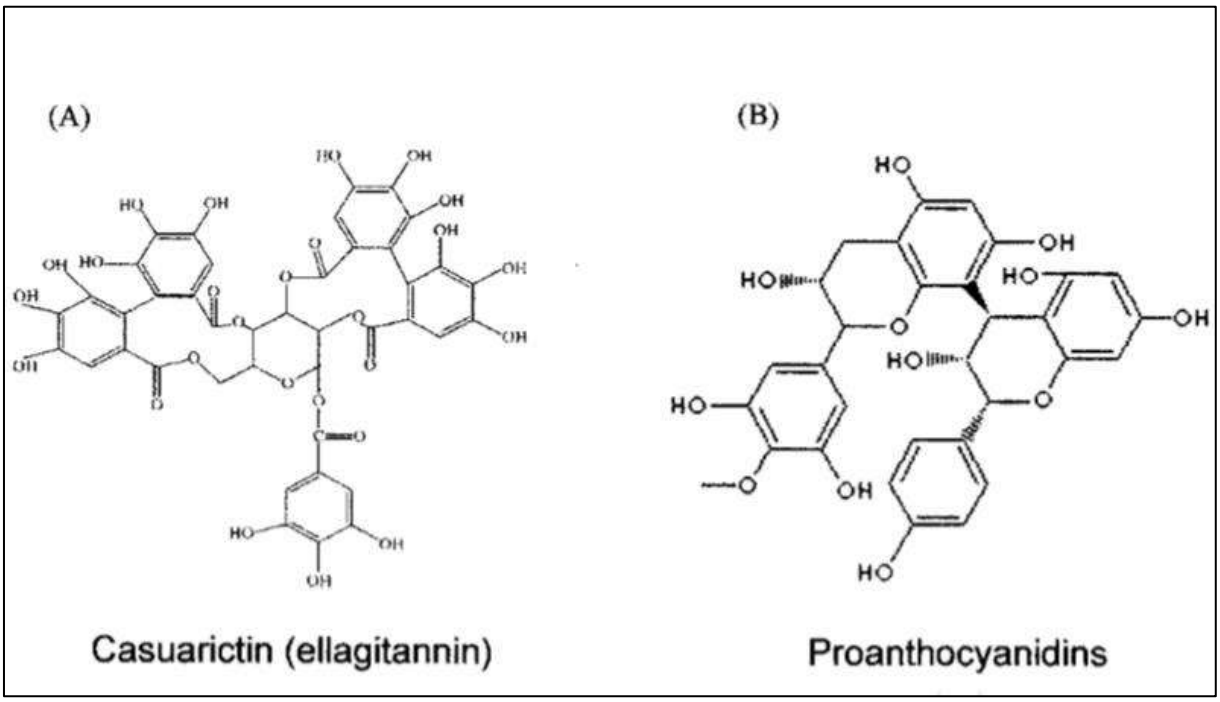


Fig I.9: *Chemical structures of (A) hydrolysable tannins and (B) condensed tannins*[25].

I.5.2-d. Phenolic acids:

As their name implies, phenolic acids are characterized by the presence of a phenolic ring and a carboxylic group. They comprise two categories:

- *Benzoic acids:*

They are the simplest phenolic acids in nature. They contain seven carbon atoms C6-C1 with hydroxyl or methoxyl roots.

- *Cinnamic acids:*

They contain nine carbon atoms C6-C3 with hydroxyl or methoxyl roots.

Their nomenclature varies according to the nature of roots attached to the main ring. Figures I.10 and I.11 illustrate their nomenclature and their structures[25].

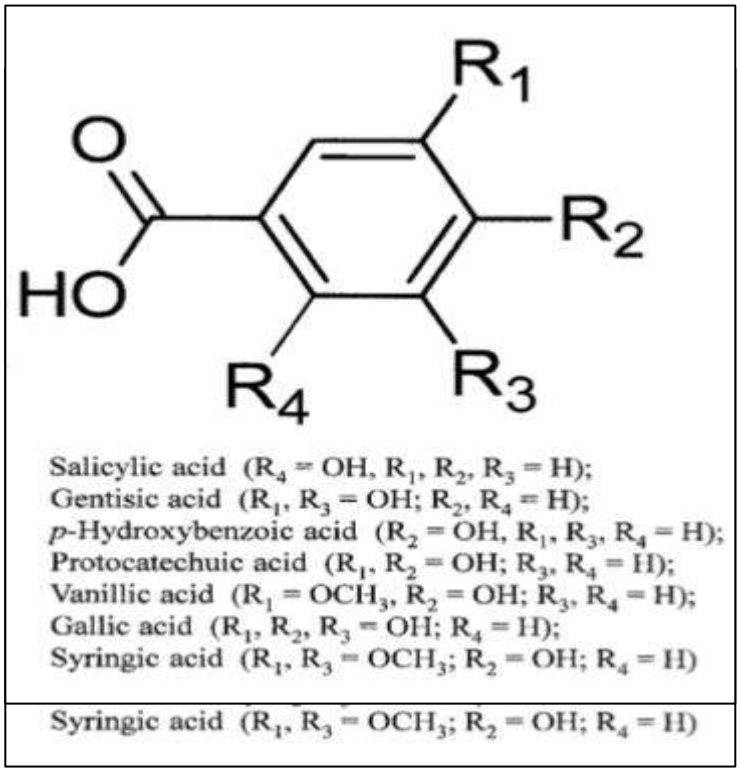


Fig I.10: *The general formulas and names of the main benzoic acids*[25].

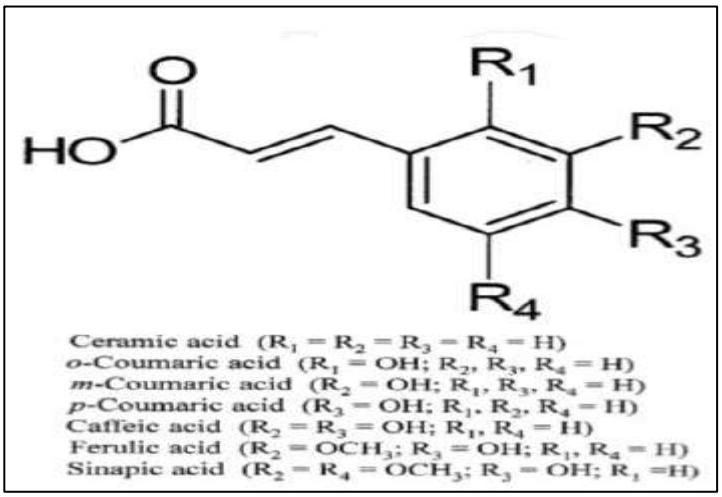


Fig I.11: *The general formulas and names of the main cinnamic acids*[25].

I.5.3- Therapeutic usefulness:

Phenolic compounds hold a variety of potential therapeutic properties ranging from antioxidant activity, anticancer[26], bacteriostatic, liver-protecting, anti-infection, cholesterol-lowering, immunity enhancement properties[27], cardioprotective and vasodilatory influences[24]. They have increasingly been part of the human diet for centuries for their benefits through the consumption of fruits and vegetables[25,28]

I.5.4- Role for plants:

Phenolics stand of great importance to plants. Not only are they responsible for the protection of the plants against exterior hazards[24], but they are also crucial to physiology and cellular metabolism. They play a key role in sensorial traits such as the plants' color and aroma, germination of seeds and reproduction[25].

I.6. Extraction methods:

I.6.1- Hydrodistillation:

Principle of the method:

Hydrodistillation is a simple extraction technique which consists in putting in a flask proportional quantity of distilled water and the plant, then heating until boiling; the rising vapor condenses using a refrigerant to recover the distillate.

Mounting:

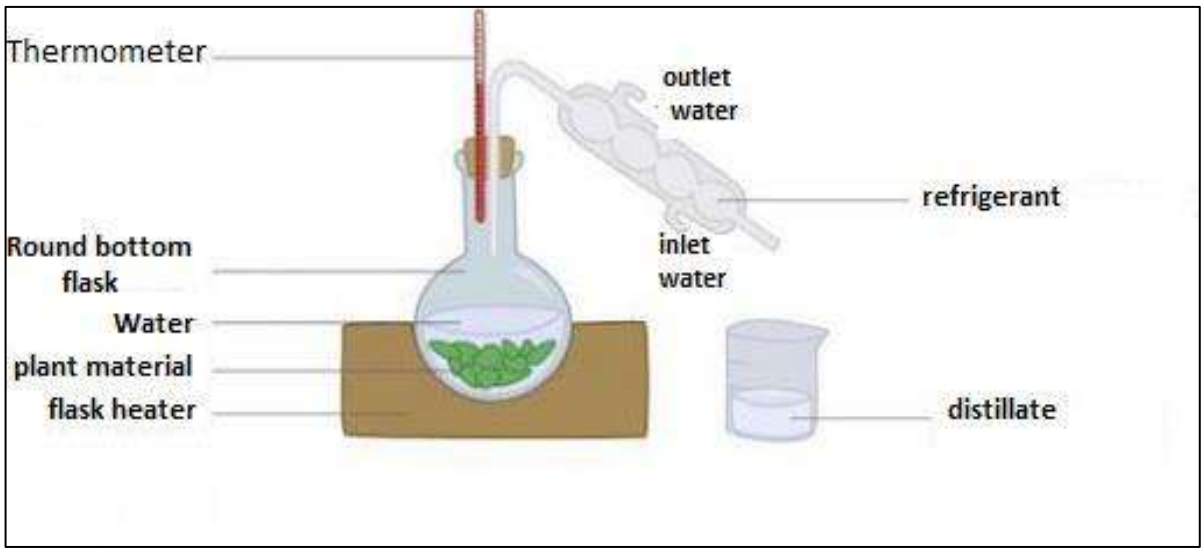


Fig I.12: *Installation of Hydrodistillation*

I.6.2- Solvent extraction:

This method consists in bringing the plant material into contact with the appropriate solvent; this protocol is carried out cold or hot. Among the most used solvents for the extraction of natural products from plant elements: petroleum ether, methanol, ethanol, and hexane.

One of the glassware used for this extraction technique is soxhlet:

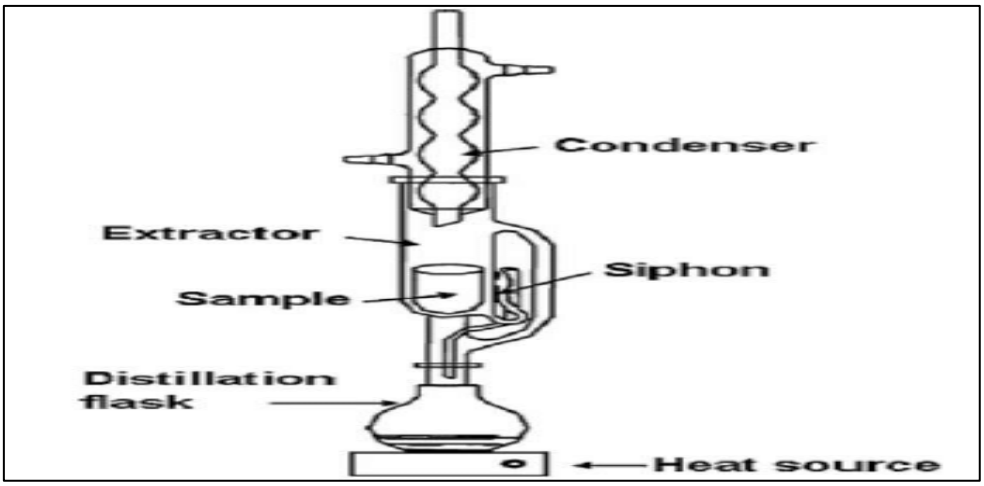


Fig I.13: *Soxhlet*

I.6.3- Enzyme Assisted Extraction:

Enzyme Assisted Extraction is an extraction technique that consists of the destruction of the source material cell wall using specific enzymes to liberate bio-active compounds[29]. Not only does this method augment the extraction yield compared to other conventional techniques, but it also is considered environmentally friendly as it does not require the use of toxic solvents[30,31].

The most used enzymes are cellulases, hemicellulases, pectinases, and other hydrolytic enzymes[31]. These have the potential of catalyzing the hydrolysis of plant cell wall components such as polysaccharides and proteins. Table I.8 shows some of the useful plants metabolites and enzymes used for their extraction[30].

Table I.8: *List of bioactive compounds of industrial importance obtained by enzyme-assisted extraction from plants*[30]

Product type	Product	Source	Enzyme used	Maximum yield (%)
Oils and carotenoids	Oil	Grape seed	Cellulase, protease, xylase and pectinase	17.5
	Carotenoids	Marigold flower	Viscozyme, Pectinex, neutrase, corolase and HT-proteolytic	97
	Volatile oil	Mandarin peel	Xylan-degrading enzymes	15
	Carotene	Carrot pomace	Pectinex Ultra SP-L	0.0064
	Lycopene	Tomato	Pancreatin	2.5-fold
		Tomato	Cellulase and pectinase	206
	Capsaicin	Chilli	Cellulase, hemicellulase and pectinase	n.d.[a]
	Colourant	Pitaya	Pectinolytic, hemicellulolytic and cellulolytic enzymes	83.5
	Anthocyanin	Grape skin	Pectinex BE3-L	n.d.[a]
Glycosides	Sugar	Grapefruit peel waste	Cellulase and pectinase	0.6377
	Oligosaccharide	Rice bran	Cellulase	39.9
	Inulin	Jerusalem artichoke	Inulinase	n.d.[a]
	Starch	Cassava	Pectinase enzyme	45.6
	Pectin	Pumpkin	Xylase, cellulose, β-glucosidase, endopolygalacturonase and pectinesterase	14.0
Others	Vanillin	Vanilla green pods	β-glucosidase and pectinase	14–21
	Flavonoid (naringin)	Kinnow peel	Recombinant rhamnosidase	n.d.[a]
	Phenolics	Citrus peel	Celluzyme MX	65.5
	Proteins	Lentils and white beans	Glucoamylases	50.3
	Polyphenols	Grape pomace	Pectinolytic	98.1
	Catechins	Tea beverage	Pepsin	80
	Lignans	Flax	Cellulase and glycosidase	40.75 mg/g
	Soluble fibre	Carrot pomace	Cellulase-rich crude preparation	77.3

[a]Abbreviation: n.d., not defined.

Plant cell walls:

Due to their high insolubility and complex structure, plant cell walls are the major barrier to extracting bioactive compounds. Cellulose, the main component of cell walls, is a carbohydrate polymer that is characterized by low solubility and hydrogen-bonded crystalline

fibers, which render its degradation greatly arduous. Components of the cell wall intervene in the process of extraction. Therefore, the nature of the cell wall matrix, the nature of the desired compounds, and their location are key factors controlling the extraction yield[29].

Principle and mechanism of action:

Enzymatic assisted extraction is mainly based on the selectivity and ability of enzymes to intrude the matrix of the cell wall through interaction with the cell wall complex components. Thus, the release of bio-actives in the bulk solution is enhanced. Enzymes bind to their specific substrates by conformational complementarity forming the enzyme-substrate complex and therefore, allowing the hydrolysis to occur. This process is a function of various parameters such as temperature, hydrogen potential, enzyme concentration, the particle size of the substrate, and time of extraction that directly influence the efficiency of EAE. Optimizing these factors implies ensuring a high yield extraction in terms of quality and quantity[29].

Optimum operating conditions:

The choice of enzymes is the first parameter to study. It is dependent on the chemical structure of the targeted compounds, the structural complexity of the cell wall, and the nature of the raw material. A combination of different enzymes is possible. Optimum temperature and pH are then selected based on the enzymes chosen[31].

Particle size is also a determinant parameter. Small particles were observed to have a better contact between enzymes and substrate and thus lead to a better release of bio-actives.

A prior understanding of the composition of the raw material, the structure of the cell wall, and the nature of the destinated compounds are necessary for the determination of the optimum operating conditions as it facilitates the selection of enzymes and optimizing related parameters[31].

Table I.9: *Different enzymes and their optimum operating conditions*[31]

Enzyme used	Bioactive extracted	Source material	Conditions used
Cellulase	Polysaccharides	Garlic	Temperature 45 °C, pH 5.0, time 80 min
α-Amylase and glucoamylase	Oleoresin	Turmeric	–
Cellulase, papain, and pectinase	Polysaccharides	Alfalfa	Temperature 52.7 °C, pH 3.87, time 2.73 h
Cellulase, pectinase, and protease	Seed oil	Pumpkin	Temperature 44 °C, time 66 min
Alginate lyase	Fucoxanthin and lipids	*Undaria pinnatifida*	Temperature 37 °C, pH 6.2
α-Amylase	Polysaccharides	*Panax ginseng*	–
Pectinase and cellulase	Carotenoids	Tomato waste	–
Lipase and phospholipase	Proteins	Olive pulp and stone	Temperature 30–40 °C, pH 7.0, time 15 min
Papain, protease, and trypsin	Fatty acids	*Strongylocentrotus nudus*	Temperature 40–55 °C, pH 7.8–8.5, time 180 min

Advantages of EAE:

Enzyme Aided Extraction is an advantageous technique and has served as an objective for countless recent studies as it remarkably improves the extraction yield, does not alternate the bio-actives properties, and selectively removes the unwanted components of raw material. Moreover, it does not require the use of toxic organic solvents nor does it harm the environment[30-32].

I.6.4- Steam distillation:

Steam distillation is practically the oldest and most famous way of essential oils' extraction[33]. Steam distillation is a separation process for temperature sensitive materials like oils which are insoluble in water and may decompose at their boiling point[34]. It consists of spraying with a certain amount of moisture on the plant material placed on the grid in a similar system to water distillation and allow steam transfer of the essential oils to occur[33]. The plant raw material is placed on a performed plate and the steam, generated by a separate boiler, volatilizes and carries essential oils as it passes through the plant material[33]. The vapors are then condensed in water cooling system only to be collected at the end of the cooler in a separate appropriate collector. Steam distillation is advantageous as it allows the vaporization of essential oils without reaching their boiling point[34]. Moreover, the boiler being kept in a separate chamber from the plant chamber keeps the ambient temperature at which the material to be distilled is located is kept below 100° C and hence it diminishes and prevents alterations due to heat effect. However, steam distillation is avoided for the reason that it is time and energy consuming and that, in case of high vapor flow rates, degradation of volatile compounds that can occur[33].

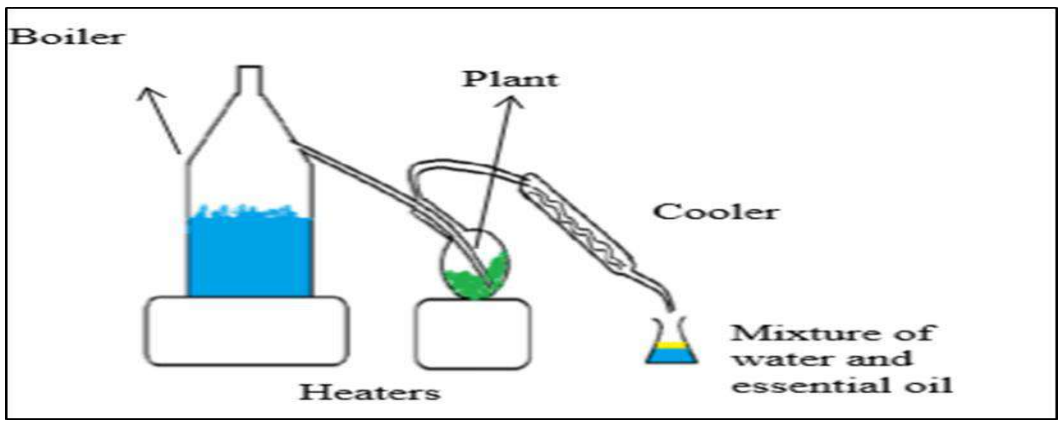


Fig I.14: *Steam distillation*

I.6.5- Microwave assisted extraction:

Microwave assisted extraction MAE is modern technique used in extraction. The first patent for MAE was in 1995for the extraction of a natural product using microwaves by Pare[35].

The target for heating in dried plant material is the minute microscopic traces of moisture that occurs in plant cells. The heating up of this moisture inside the plant cell due to microwave effect, results in evaporation and generates tremendous pressure on the cell wall. The cell wall is pushed from inside due to the pressure and the cell wall ruptures. Thus, the exudation of active constituents from the ruptured cells occurs, hence increasing the yield of phytoconstituents[36]. MAE allows the boiling point to be reached earlier then supported water distillation as shown in **figure I.15**. It has also been determined that the amount and quality of essential oil obtained by microwave-assisted water distillation in 30 minutes is equivalent to the amount and quality of essential oil obtained in 4 hours and thirty minutes by water distillation[33]. Not only does MAE save time and energy, but it also is considered environment friendly as it uses very little or no solvents[35,36].

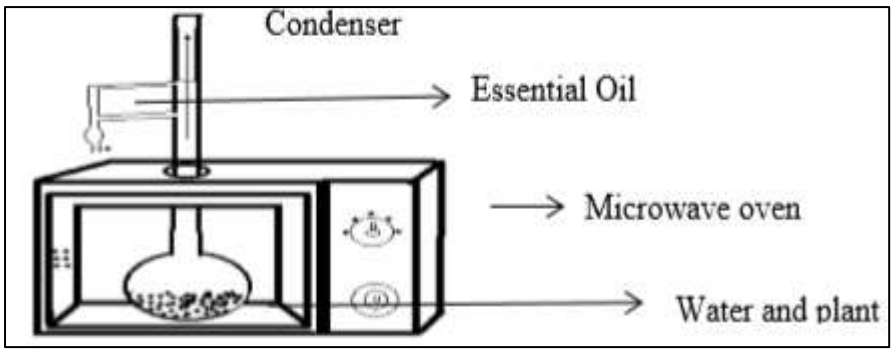


Fig I.15: *Microwave assisted extraction*

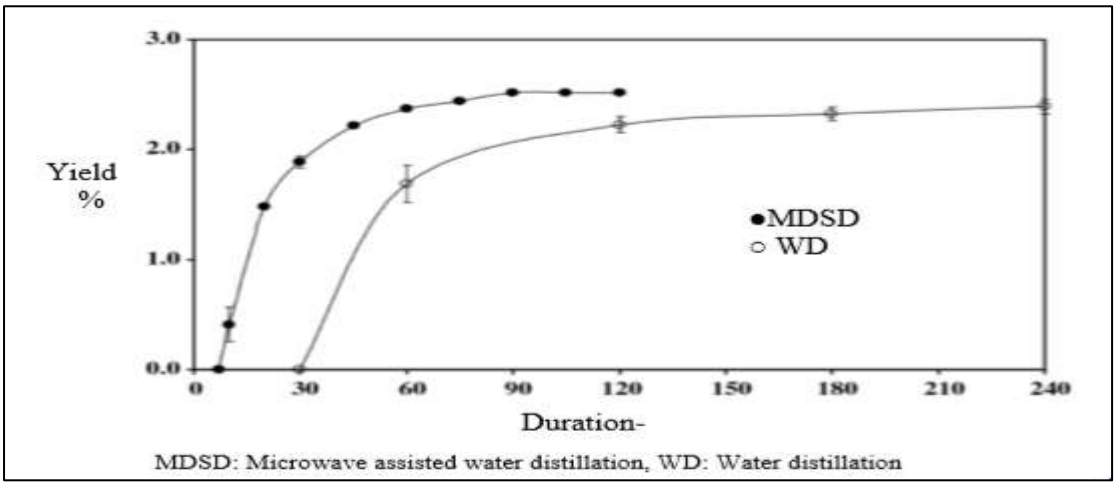


Fig I.16: *Comparing water distillation and microwave assisted water distillation of obtaining thyme essential oil*

I.7. Conclusion:

The rich composition of plants (EOs, primary and secondary metabolites) offers several advantages in the phytotherapy field, therefore the use for different therapeutic purposes.

The extraction method of bioactive molecules depends on several factors and also depends on the plant properties.

In the next chapter, we will present the two plants used in our practical part.

Chapter II

PLANTS' PRESENTATION

II.1. Introduction:

Numerous plants and herbs have been used since antiquity in folk medicine for their therapeutic effects among which many grow naturally and are abundant. Some of them have been the aim of countless scientific studies in the purpose of validating their effects and even using them in medicine as drugs and, consequently, have been increasingly used in pharmaceutical, chemical and food industries. Milk thistle and Globularia are widely used plants both in traditional[37] and modern medicine and industry for their potential benefits for Men[38]. This chapter discusses their monograph, distribution, chemical composition and therapeutic usefulness.

II.2. Milk thistle:

II.2.1- Identity:

Preferred Scientific Name: *Silybum marianum (L.) Gaertn*[39].

Preferred Common Name: variegated thistle[39].

Other Scientific Names:

Carduus Mariae Crantz.

Carduus Marianus L.

Carthamus maculatum (Scop.) Lam.

Cirsium maculatum Scop.

Mariana lactea Hill

Mariana Mariana (L.) Hill

Silybum maculatum (Scop.) Moench,

Silybum Mariae (Crantz.) Gray[39].

International Common Names:

English: blessed milk thistle; blessed milk thistle; Holy thistle; Lady's thistle; St. Mary's thistle

Arabic: Shawk El Djamel شوك الجمل

Spanish: Cardo asnal; Cardo Blanco; Cardo lechero; Cardo Mariano; Cardo Santo; Poma

French: Chardon argente; Chardon Marie; Chardon Notre Dame[39]

Local Common Names:

English: blessed thistle; bull thistle; cabbage thistle; Gundegai thistle; Gundy; holy thistle; lady's thistle; Marian thistle; Mary's thistle; Marythistle; milk thistle; spotted thistle; St. Mary's

Arabic: shawk el-gamel, shawk el hmir

French: Chardon argente; Chardon Marie; lait de Nore Dame; Silybe de Marie

Germany: Gemeine Mariendistel; Mariendistel[39].

EPPO code: SLYMA (*Silybum marianum*)[39].

II.2.2- Taxonomic Tree:

Domain: *Eukaryota.*

Kingdom: *Plantae.*

Phylum: *Spermatophyta.*

Subphylum: *Angiospermae.*

Class: *Dicotyledonae.*

Order: *Asterales.*

Family: *Asteraceae.*

Species: *Silybum marianum*[39].

II.2.3- Description:

Annual or biennial. Stems branched above[40], ridged, with sparse mealy hairs at least above, 0.5 - 2 m tall, not winged. Radical leaves glabrous or glabrescent, spiny but with weak spines, with terminal lobe barely longer than the others. Bracts of the involucre almost all terminated in a strong brownish spine (this one not exceeding 5 cm long and generally overturned behind), glabrous or sparse[41]. Seeds are the only part utilized[38].



Fig II.1: *Milk thistle open flower and large rosette*[27]



Fig II.2: *Milk Thistle mature[39] and dried flowers*



Fig II.3 *Milk thistle seeds*

.

II.2.4- Distribution:

S. Marianum is native to the Middle East, the Arabian Peninsula, the Indian subcontinent, North Africa, and parts of Europe. It has been introduced to Japan, sub-Saharan Africa, and the Americas, parts of Europe, Australia, and New Zealand[39].

In Algeria, Milk thistle is native and wildly grows in open spaces. It flowers in April and is ripe by mid-June.

II.2.5- History:

Milk thistle is so-called because of the white markings (variegation) on the leaves, which have been consumed as a vegetable. According to folk tradition, its characteristic violet flowers and white-veined leaves came from the Virgin Mary's milk. Roasted seeds have been used as a coffee substitute. The mature untreated seeds of milk thistle have been used for 2000 years in traditional medicine to treat melancholy, headache, digestive and liver complaints, detoxification, and promote lactation[38]. The Greek physician and botanist Dioscorides (40–90 AD) was the first to describe milk thistle's healing properties. Later, in 1597, John Gerard noted that milk thistle was "the best remedy against melancholy diseases"[37].

More recently, a small randomized study from Iran showed similar improvements in patients with obsessive-compulsive disorder who received either fluoxetine or extract derived from leaves of the Milk thistle plant[37].

In the 1960s the ingredients of milk thistle were investigated. The mixture found was named silymarin. The research concentrated primarily on the use of milk thistle in the treatment of liver disorders and protection from liver injury. In the 1990s reports based on preclinical models suggested preventative, and therapeutic, potential in cancer warranted further research[38].

II.2.6- Chemical composition:

The main effects of milk thistle are thought to stem from the flavonolignans combined with other ingredients. A crude extract of dried seeds of milk thistle contains 65 – 80% of a flavonolignan complex termed silymarin[38]. Silymarin itself is a complex of at least seven flavonolignans (silybin A and B, isosilybin A and B, silychristin, isosilychristin, and

silydianin) and the flavonoid taxifolin[38,40]. Silybin A and B, isosilybin A, and B are isomers. Silybin, synonymous with silibin and silybinin, is the processed form of silymarin and contains the isomers silybin A and B in equal amounts. Silybin is considered the main active ingredient of the milk thistle. The seeds also contain fatty acids such as linoleic acid[38].

II.2.7- Silybin chemical structure and properties:

Silybin is a flavonolignan, a polyphenol, an aromatic ether, a benzodioxine, and a secondary alpha-hydroxy ketone. It contains oxygen, carbon, and hydrogen atoms and has the formula $C_{25}H_{22}O_{10}$, a molecular weight of 482.441g/mol. It is a combination of two equimolar diastereomers, silybin A and silybin B[42]. Silybin has five rings among which three are benzenes. It is made up of two units: the first one is based on a flavonol group in flavonoids called taxifolin. The second unit is conyferil alcohol (a phenylpropanoid). The two units are bridged together with an oxeran. The presence of alcohol groups is key for the majority of the compound's activity. It comprises five hydroxyl groups: C-3 OH,C-5 OH, C-7 OH, C20 OH, and C-23 OH that have various properties and characters: phenolic, carboxylic esterification, or oxidation[40].

Silybin is a mixture of two isomers: Silybin A and B. These latter are considered trans diastereoisomers with a difference in reference positions C-10 and C-11 in the 1,4-benzodioxane ring. Silybin A can be separated from silybin B popularly and simply using high-pressure liquid chromatography HPLC by the difference in retention time Tr. More visibly, Silybin A forms yellowish flat crystals with melting point 162– 163○C, whereas silybin B gives yellow grain crystals with a melting point of 158–160○ C[40].

Table II.1: *Chemical properties of silymarin*

Property Name	Property Value
Molecular Weight	482.4 g/mol
XLogP3-AA	2.4
Hydrogen Bond Donor Count	5
Hydrogen Bond Acceptor Count	10
Rotatable Bond Count	4
Exact Mass	482.121297 g/mol
Monoisotopic Mass	482.121297 g/mol
Topological Polar Surface Area	155 Å²
Heavy Atom Count	35
Formal Charge	0
Complexity	750
Isotope Atom Count	0
Defined Atom Stereocenter Count	4
Undefined Atom Stereocenter Count	0
Defined Bond Stereocenter Count	0
Undefined Bond Stereocenter Count	0
Covalently-Bonded Unit Count	1
Compound Is Canonicalized	Yes

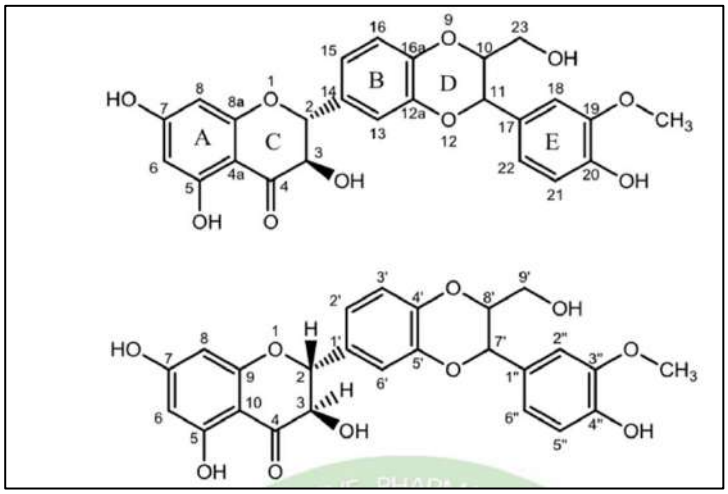


Fig II.4: *The chemical structure of Silybin* [28]

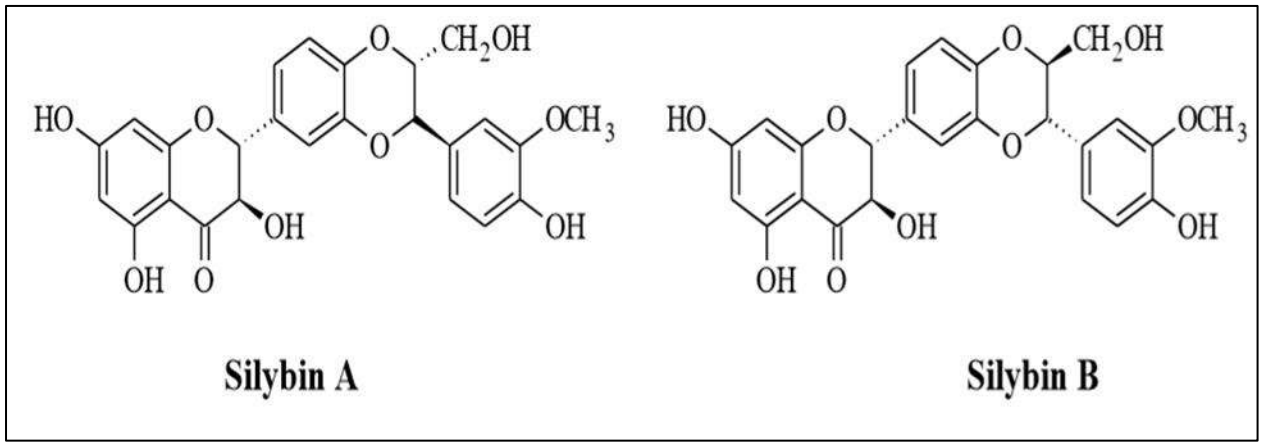


Fig II.5: *Chemical structures of silybin A and B*[40]

II.2.8- Therapeutic usefulness:

Milk thistle has a wide range of uses, from fighting hangover to potentially life-saving for patients who have ingested poisonous mushrooms. A review of more than 2000 patients exposed to amanita mushrooms in Europe and North America suggested that intravenous silybinin was the most effective therapy available against this toxin[37]. Milk thistle is also reported to be an effective antiviral especially for the treatment of Hepatitis C. Mechanisms of antiviral activity against hepatitis C include inhibition of a viral polymerase critical for

replication. Interestingly, a case report of a patient co-infected with both hepatitis C and HIV showed clearance of both hepatitis C and HIV after 2 weeks of intravenous silybinin[37]. According to the European Scientific Cooperative on Phytotherapy (ESCOP), milk thistle has the following indications: toxic liver damage; supportive treatment in patients with chronic inflammatory liver conditions, and hepatic cirrhosis. Orally, milk thistle is used for liver disorders including toxic liver damage caused by chemicals, Amanita phalloides mushroom poisoning, jaundice, chronic inflammatory liver disease, hepatic cirrhosis, chronic hepatitis, gallbladder complaints, hangover, and indigestion[37,38]. It has also been used in prostate cancer, pleurisy, malaria, depression, uterine complaints, allergic rhinitis, stimulating breast milk, and menstrual flow[37]. Intravenously, milk thistle is used as a supportive treatment for Amanita phalloides (death cap) mushroom poisoning. In cancer, the claims are that milk thistle can be protective by inhibiting tumor development, and supportive in ameliorating adverse treatment effects as well as enhancing chemotherapeutic effect. These claims rest on presumed and acknowledged effects of milk thistle extracts in pre-clinical trials and case reports. In Germany, where the government regulates herbal medicine use, milk thistle has been listed in the Commission E monograph for the treatment of dyspepsia, cirrhosis, and liver damage due to toxins[37,38].

II.2.9- Mechanisms of action:

The precise mechanism of action is unclear with silymarin considered a multi-functional, multi-target drug. Several mechanisms are thought to contribute to the therapeutic effect on liver disease. Silymarin reduces hepatocyte membrane permeability to toxins. Antifibrotic action has been demonstrated by silybin in an in vitro model of human hepatic fibrogenesis. Silymarin demonstrates antioxidant and anti-inflammatory effects in several animal cell models. Silybinin is thought to have anti-cancer properties in different tumor types via e.g. apoptotic, tumor growth modulating, anti-carcinogenic, anti-inflammatory, anti-metastatic, and anti-angiogenic mechanisms. The presumed modes of action are varied and include enhancement of pro-apoptotic molecules (e.g. caspases), enhancement of growth inhibitory proteins, presumed interaction with tumor necrosis factor (TNF), and inhibition of cell proliferation via a number of pathways including inhibition of various protein kinases (e.g. mitogen-activated protein kinase MAPK), and inhibition of anti-apoptotic signaling. In animal experiments and in vitro studies, anti-carcinogenic effects were seen for skin cancer, breast cancer, lung cancer, colon cancer, urinary bladder cancer, prostate cancer, ovarian cancer,

leukemia, and cervical cancer. In in-vitro studies, a synergistic action with different chemotherapeutic agents has been seen[38].

II.2.10- Safety and counter-indications:

Milk thistle is considered generally safe. Orally, milk thistle is usually well-tolerated. The rate of adverse events of milk thistle was low in clinical trials[38]. Related side effects are mild: diarrhea, gastrointestinal upset, intestinal symptoms, headache, and dizziness. It can be counter-indicated when taking other drugs as it might interact with metabolic enzymes such as cytochrome P450, leading to interactions with other drugs[37]. Patients with oestrogen-responsive breast cancer are warned when using milk thistle as it is believed to have an oestrogen-like action for it has been reported that tumor growth was seen in an animal experiment and in an experiment with an oestrogen responsive breast cancer cell line (MCF-7) when adding silymarin[38]. Yet, the more commonly used milk thistle seed extracts are not known to have oestrogenic effects in vivo. There is insufficient information to assess safety in pregnancy and lactation. Patients who are hypersensitive to plants in the Asteraceae family must be cautious before consuming milk thistle[38].

II.3. Globularia:

II.3.1- Identity:

Scientific name: *Globularia* [43].

Vernacular names: Globulaire (in French), Globulaire turbith, Sene de Provence, Zerga, Tasselgha, Chelra, Tide n'tnet, and Zouitna [43].

II.3.2- Taxonomic tree:

Reign: *Plantae* [44].

Sub-reign: *Trachebeonta* [44].

Division: *Magnoliopsida* [44].

Class: *dicotyledons* [45].

Sub-class: *Asteridae* [44].

Order: *Scrophulariales* [44].

Family: *Globulariaceae* [44].

Genus: *Globularia* [44].

II.3.3- Globulariaceae family:

The Globulariaceae family is among dicotyledons plants, it in principle includes 10 genera and 300 species which are distributed in Europe, Western Asia, Africa, and Madagascar [46].

The most known genera are Selago (180 species), Habenstretia (40 species), Walafrida (40 species), and Globularia (25 species); but the approved classification includes only 30 species divided into 2 genera [46] (Globularia and *Poskea* [47]).

II.3.4- Description of *Globularia*:

This botanical genus occurs in the form of low shrubs, subshrubs, or perennials with woody base. Leaves are coriaceous, simple, lanceolate to suborbicular or spathulate, sometimes emarginated, or with few teeth in the upper part [47].

Inflorescence: capitulum with bracts and an involucre at the end of the stem or on short lateral branches with the end of the stem remaining vegetative. *Globularia* has zygomorphic flowers, calyx with five acute lobes as long as or longer than the tube, corolla bilabiate; the larger upper lip with three short teeth or deeply laciniate, the lower lip consisting of two linear lobes 2/3 as long as the upper lip or much shorter, sometimes completely lacking or only one lobe present. The fruit included in the persisting calyx [47].

Globularia has several species as mentioned above, the only species that exists in Algeria is "*Globularia alypum*" with its two subspecies (*Globularia Arabica* and *Globularia eu alypum)* [45].



Fig II.6: *two different Globularia species*

Globularia alypum:

- Plant: sub-shrub of 30 - 60 cm;
- Stems: very branching in bushes usually erect;
- Leaves: all sparse on the persistent, oblong or obovate, leathery branches attenuated in short whole petiole or bi-tridentate mucronate uninervated;
- Flowers: a beautiful fragrant blue with sub-sessile terminal and lateral heads 15 – 20 mm wide;
- Leaflets of the involucre: oval ciliate at the edge. Spiky receptacle and sequins;
- Calyx: long-bearded with almost equal deep divisions;
- Corolla: Upper lip almost null, the lower lip has 3 oval-acute lobes [17].



Fig II.7: *Globularia flowers and leaves*

II.3.5- Ecology and geographic distribution of *Globularia*:

Most *Globularia* species are located in Cape Verde and the Canary Islands, the Atlas, the Baleares, Italy, Greece, and Turkey [47].

Globularia alypum species is a Mediterranean species of dry and arid land, and which is found even in the rock gardens of the Saharan Atlas and in the Ahaggar [43].

II.3.6- Chemical composition:

Studies on the chemical composition of *Globularia* prove several secondary metabolites responsible for therapeutic activities.

The main natural products of this plant are as follows:

- **Phenol acids:** p-hydroxybenzoic acid, vanillic acid, syringic acid, caffeic acid, 3-resorcylic acid, sinapic acid, p-coumaric acid, and ferulic acid [43,44]
- **Flavonoids:** 4 ', 7-dihydroxyflavone, apigenin-7-glucoside, quercetol, luteolin-7-glucoside, bayine and rutoside [44].
- **Iridoids:** Globularin (globularoside) the most known [43], catalpol, Globularimine, globularinine, globularicisin, globularidine, globulanoside [44].
- **A glycoside aryl:** Syringin, liriodendrine [44].
- **Anthocyanins:** Cyanidine and paéonidine [44].
- **Phenylethanoids** [44].
- **Protocatechuic acid**[45].

Iridoids, particularly globularin, are the active compounds of *Globularia* [43].

➢ ***Globurarin* chemical structure and properties:**

It is an iridoid, derived from monoterpenes characterized by a cyclopentapyran skeleton[45], its chemical formula is $C_{24}H_{28}O_{11}$ [48].

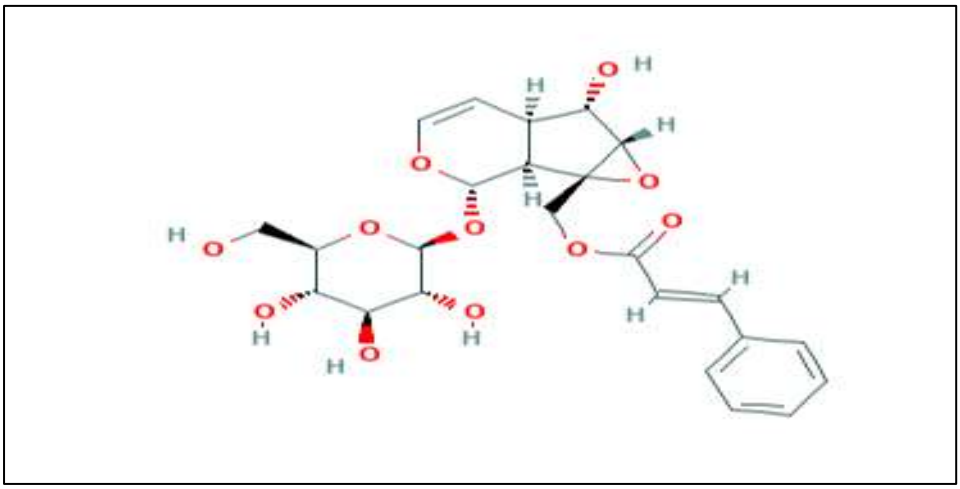


Fig II.8: *Globularin chemical structure* [48]

Chemical and Physical Properties:

The different physical and chemical properties of *Globularin* are illustrated in the figure below:

Table II.2: *Chemical and Physical Properties of Globularin* [48]

Molecular Weight	492.5 g/mol
XLogP3-AA	-0.6
Hydrogen Bond Donor Count	5
Hydrogen Bond Acceptor Count	11
Rotatable Bond Count	8
Exact Mass	492.163162 g/mol
Monoisotopic Mass	492.163162 g/mol
Topological Polar Surface Area	168 Å²
Heavy Atom Count	35
Formal Charge	0
Complexity	826
Isotope Atom Count	0
Defined Atom Stereocenter Count	11
Undefined Atom Stereocenter Count	0
Defined Bond Stereocenter Count	1
Undefined Bond Stereocenter Count	0
Covalently-Bonded Unit Count	1
Compound Is Canonicalized	Yes

II.3.7- Therapeutic usefulness:

Globularia leaves are considered a potential source of natural antioxidants; it is widely used for the treatment of digestive disorders and skin diseases [44].

In traditional medicine, this plant is used as a hypoglycemic, laxative and purgative agent[44]; It is also known for its anti-hypertensive, anti-diabetic, and anti-inflammatory properties ; Other studies have identified anti-leukemic and anticancer activity [43].

II.3.8- Safety and contraindications:

The risk may arise from the use of *Globularia* in "long-term" treatments; therefore, it is recommended to use it for limited treatment of three weeks with rest periods [43].

In large doses, it causes oliguria, diarrhea, colic, dizziness, headache, chills, limb pain, hypothermia, and slow pulse [43].

II.4. Conclusion:

The botanical presentation of *Milk thistle* and *Globularia* has demonstrated the morphological specificity with which the species of each plant can be determined.

The phytochemical study according to the literature has shown that the two plants have several main natural products, in particular, flavonolignans for *Milk thistle* and iridoids for *Globularia*.

The usefulness of these plants is known in the therapeutic field where they were used in traditional medicine to treat many diseases; Ongoing studies have proven the efficacy of the two plants for the therapeutic purposes for which they were used by proving many biological activities, some of these biological activities will be covered in the next chapter.

Chapter III

PLANT' BIOLOGICAL ACTIVITIES & LITERATURE REVIEW

III.1. Introduction:

The main reason behind the fame of plants is their biological activities. They are due to the presence of secondary metabolites such as phenols that possess antioxidant, anti-inflammatory, antibacterial … Antioxidant activity is considered a cure for many illnesses cause by oxidation such as cancer and Alzheimer. Antibacterial activity combated bacterial related diseases such as infections. Milk thistle and globularia have been reported to be potent antioxidants and antibacterial. This chapter details antioxidant and antibacterial activities, their principles, the main assessment methods used and milk thistle's and globularia's literature reviews in that matter.

III.2. Antioxidant activity:

III.2.1- Definition :

Anti-oxidation is the process of retarding or inhibiting oxidation from taking place[23]. Oxidation is the loss of electrons from atoms, molecules or ions. As a result, oxidized compounds become unstable as they are left with unpaired valence electrons[49]. When it happens inside human bodies, oxidation generates compounds called free radicals. Free radicals can either be generated physiologically: endogenous (resulting from respiration and metabolism) or pathologically: exogenous (resulting from environmental factors such as UV-light exposure, smoking, heavy metal consumption, stress, lack of sleep …)[23]. The free radicals may be either Oxygen derived (ROS) or Nitrogen derived (RNS). The most common reactive oxygen species include superoxide anion (O_2), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO) and reactive hydroxyl radicals (OH). The nitrogen derived free radicals are nitric oxide (NO), peroxy nitrite anion (ONOO), Nitrogen dioxide (NO_2) and Dinitrogen trioxide (N_2O_3)[50,51].

III.2.2- Free radicals production:

- **Physiological production:**

Free radicals are normally produced by the body through the respiration cycle. Cytochrome C Oxidase (or complex IV) transfers electrons to oxygen which leads to the formation of superoxide anion (O_2), hydrogen peroxide (H_2O_2) and reactive hydroxyl radicals (OH)[49].

- **Pathological production:**

Free radicals can be generated during an inflammation by macrophages through the activation of NADPH Oxidase by lysosomal enzymes resulting in the release of two electrons which are captured by nearby oxygen molecules to form superoxide radicals. Another enzyme, then, called superoxide dismutase interferes to transform superoxide to hydrogen peroxide, which is eventually a less harmful product. This process is called the respiratory burst. Macrophages also have an enzyme called Nitric oxide synthase that produces Nitric oxide. Nitric oxide has a double role: directly attacks the pathogens that the macrophages have gobbled, and reacts with superoxide to form peroxy nitrite (ONOO) free radical that also damages the pathogens by breaking down their cell membranes and damaging their proteins. Another way free radicals are generated are when radiations hit water molecule in tissues, exciting the outer electron and thus forming hydroxyl radicals. Metals build up in the body can also be a way to produce free radicals. For instance, hemochromatosis (the absorption of high amount of iron) undergoes the Fenton Reaction: Iron2+ ions are oxidized by hydrogen peroxide forming iron3+ and a hydroxyl radical $OH^{\cdot}$ and hydroxide ion OH^{-}. The excess amount of hydrogen peroxide can reduce Fe^{3+} to Fe^{2+} in an endless loop releasing peroxide radical and a proton. The accumulation of free radicals created through the Fenton Reaction ($OH^{\cdot}$ and OH^{-}**)** cause cell death and thus tissue fibrosis in different organs. Finally, free radicals can also be generated though the metabolism of certain drugs and chemicals by the liver. Acetaminophen metabolism, for example, produces free radicals which damage the liver[49].

III.2.3- Antioxidants and oxidative stress:

As a matter of defense, organisms generate compounds that are susceptible of suppressing the formation of pro oxidants or opposing their actions. They are called antioxidants[52]. Antioxidants are either enzymatic (such as Glutathione peroxidase) or non-enzymatic products (such as uric acid)[50,51]. Antioxidants can act as chain breakers, scavenging chain initiating radicals like hydroxyl, alkoxyl, or peroxyl, quenching singlet oxygen, decomposing hydro peroxides, and chelating pro oxidative metal ions. Chain breaking antioxidants able to scavenge radical species are called primary antioxidants. Secondary antioxidants are singlet oxygen quenchers, peroxide decomposers that yield non radical species, oxidative enzyme (e.g., lipoxygenase) inhibitors, UV radiation absorbers, or compounds that act by metal chelating[50].

Vitamins A, C and E donate electrons to neutralize free radicals without themselves being oxidized[50].

Glutathione neutralizes hydrogen peroxide by giving electrons. Two glutathione molecules need to be in the reduced state to donate two electrons to hydrogen peroxide to form two water molecules. Glutathione molecules become oxidized and need to be reduced again. This insured by an enzyme named Glutathione reductase that uses NADPH as electron donor. After losing its electron, NAPH becomes $NADP^+$ and gets back its electron after the intervention of an enzyme called Glucose 6 Phosphate Dehydrogenase (G6PD) that reduces $NADP^+$ back to NADPH by oxidizing glucose 6 phosphate which a metabolite of glucose[49,50].

Another antioxidant mechanism is carrying (chelating) metals[51] by proteins so as to prevent them from generating free radicals, such as Transferrin that binds to iron and Ceruloplasmin that binds to copper and carry them in the blood[49].

Finally, scavenging enzymes[50] is another way of defense that transforms free radicals to harmless products. For instance, superoxide dismutase transforms superoxide into hydrogen peroxide that is transformed into water by catalse in peroxisomes and glutathione peroxidase in cytoplasm[49]. The presence of pro oxidants and these endogenous antioxidants create a balance that keeps cells healthy. However, this balance can be shifted towards the pro-oxidant when production of oxygen species is increased or when levels of antioxidants are diminished. This state is called ‘oxidative stress’ and can result in serious cell damage if the stress is massive or prolonged[23]. Oxidative stress can also damage DNA proteins resulting in DNA cuts or mutations which increase cancer possibilities[52].

In order to equilibrate pro oxidants: antioxidants proportions, exogenous antioxidants are necessary. Herbal antioxidants, represented by carotenoids, tocopherols, vitamin D, phenolic acids, flavonoids, or ascorbic acid, as well as high-molecular weight metabolites such as tannins, have henceforth been successfully employed as rejuvenators, for several centuries in traditional medicine. Epidemiological studies confirm that the incidence of oxidative stress-related conditions is lowered by the consumption of fruits and vegetables rich in compounds possessing high antioxidant activity. Foods containing antioxidants and antioxidant nutrients play an important role in prevention[50,52].

III.2.4- Antioxidant capacity assessment methods:

There exist various analytical methods[50] that serve the measurement of plants' antioxidant capacity some of which will be detailed.

- **The DPPH method:**

DPPH• (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical, due to the delocalization of the spare electron on the whole molecule. Thus, DPPH• does not dimerize, as happens with most free radicals. The delocalization on the DPPH• molecule determines the occurrence of a purple color, with an absorption band with a maximum around 520nm. When DPPH• reacts with a hydrogen donor, the reduced (molecular) form (DPPH) is generated, accompanied by the disappearance of the violet color. Therefore, the absorbance diminution depends linearly on the antioxidant concentration[50]. Trolox, ascorbic acid or gallic can be used as standard antioxidant.

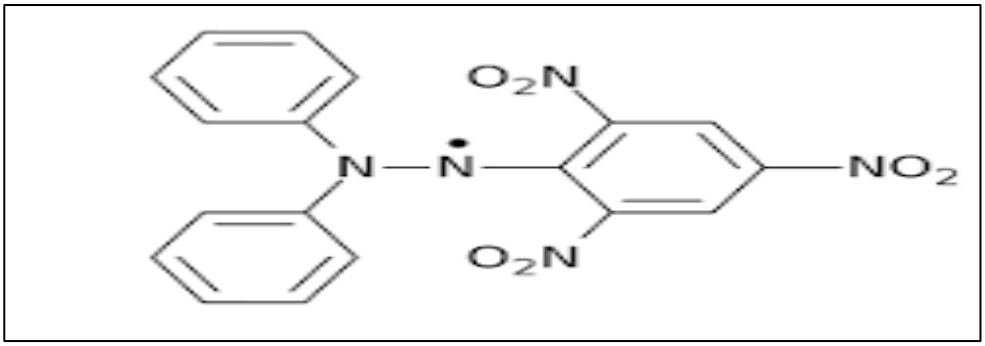


Fig III.1 *DPPH chemical structure*

- **The ORAC (oxygen radical absorption capacity) assay:**

The method measures the antioxidant scavenging activity against the peroxyl radical, induced by 2,2'-azobis-(2-amidino-propane) dihydrochloride (AAPH), at 37°c. Fluorescein was used as the fluorescent probe. The loss of fluorescence was an indicator of the extent of the decomposition, from its reaction with the peroxyl radical[50].

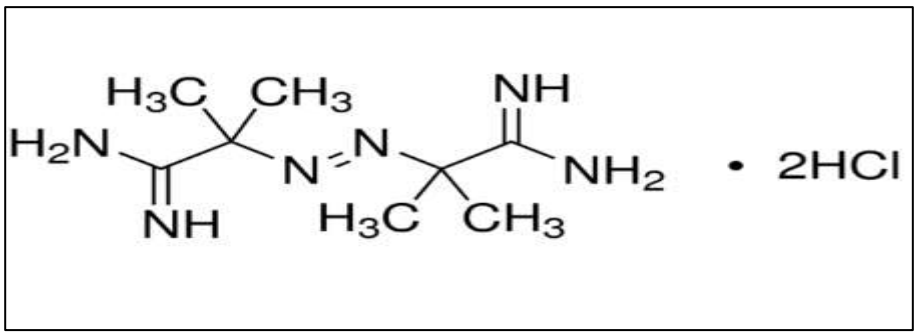


Fig III.2 *AAPH chemical structure*

The Table III.1 resumes other assessment methods.

Table III.1 *Antioxidant assessment methods*[50]

Antioxidant capacity assay	Principle of the method	End-product determination
Spectrometry		
DPPH	Antioxidant reaction with an organic radical	Colorimetry
ABTS	Antioxidant reaction with an organic cation radical	Colorimetry
FRAP	Antioxidant reaction with a Fe(III) complex	Colorimetry
PFRAP	Potassium ferricyanide reduction by antioxidants and subsequent reaction of potassium ferrocyanide with Fe3+	Colorimetry
CUPRAC	Cu (II) reduction to Cu (I) by antioxidants	Colorimetry
ORAC	Antioxidant reaction with peroxyl radicals, induced by AAPH (2,2'-azobis-2-amidino-propane)	Loss of fluorescence of fluorescein
HORAC	Antioxidant capacity to quench OH radicals generated by a Co(II) based Fenton-like system	Loss of fluorescence of fluorescein
TRAP	Antioxidant capacity to scavenge luminol-derived radicals, generated from AAPH decomposition	Chemiluminescence quenching
Fluorimetry	Emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength	Recording of fluorescence excitation/ emission spectra
Electrochemical Techniques		
Cyclic voltammetry	The potential of a working electrode is linearly varied from an initial value to a final value and back, and the respective current intensity is recorded	Measurement of the intensity of the cathodic/ anodic peak
Amperometry	The potential of the working electrode is set at a fixed value with respect to a reference electrode	Measurement of the intensity of the current generated by the oxidation/reduction of an electroactive analyte
Biamperometry	The reaction of the analyte (antioxidant) with the oxidized form of a reversible indicating redox couple	Measurement of the current flowing between two identical working electrodes, at a small potential difference and immersed in a solution containing the analysed sample and a reversible redox couple
Chromatography		
Gas chromatography	Separation of the compounds in a mixture is based on the repartition between a liquid stationary phase and a gas mobile phase	Flame ionisation or thermal conductivity detection
High performance liquid chromatography	Separation of the compounds in a mixture is based on the repartition between a solid stationary phase and a liquid mobile phase with different polarities, at high flow rate and pressure of the mobile phase	UV-VIS (e.g. diode array) detection, fluorescence, mass spectrometry or electrochemical detection

III.2.5- Milk thistle antioxidant activity: Literature review

The antioxidant activity of Silybum marianum seed extract against DNA oxidation, protein damage and lipid peroxidation was assessed by *Aynur Serçe et al*[53]. and demonstrated that milk thistle seeds have good DPPH free radical scavenging activity and can prevent lipid peroxidation. Therefore, Silybum marianum can be used as potentially rich source of antioxidants and food preservatives. The results obtained show that ethanol extract of seeds can be used as a dietary supplement and it can protect DNA from damage. The inhibitory activity of seed extract against hydroxyl radical-induced DNA, protein and lipid damage may be mainly responsible for the cancer chemoprevention and hepatoprotection effects. The presence of silymarin could account for the increased antioxidant activity and enhanced DNA, protein and lipid damage protection capacity of the extract. Protection against the DNA, protein and lipid oxidation is due to phenolic and flavonoid compounds that scavenge the free radicals. These findings also imply that the presence of antioxidants can decrease the occurrence of degenerative disorders in which free-radical damage may be involved.

Anca Hermenean et al[54]. assessed Antioxidant and hepatoprotective activity of milk thistle (Silybum marianum L. Gaertn.) seed oil, using biochemical markers, pathological histology and electron microscopy, showed that Silybum marianum seed oil (SMSO) pre-treatment could prevent CCl_4-induced oxidative stress and liver injury. These findings suggest that the SMSO was effective in counteracting the damaging effect of CCl_4 in murine hepatocytes and indicate that the preventive effects of SMSO against CCl_4-induced liver damage are related to its antioxidant properties, which are likely due to the presence of tocopherol and ascorbic acid 6-hexadecanoate. It is well known that chemical agents inducing hepatic injury can lead to significant increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity.

One of the most sensitive indicators of liver injury after CCl_4 administration is the release of intracellular enzymes in the circulation, such as aminotransferases. It was stated that elevated activities of these enzymes are indicative of cellular leakage and loss of the functional integrity of liver cell membrane. The elevation of plasma enzymes concentration is generally regarded as one of the sensitive markers of hepatic damage. In this cited study study, CCl_4 was found to cause significant increases in plasma AST, ALT and gamma glutamyl transferase (GGT) levels compared to control. Pre-treatment with 10 g/kg of SMSO effectively protected the animals against CCl_4-induced hepatic injury, as evidenced by decreased plasma AST (by 42.45%), ALT (by 50%) and GGT (by 32%) compared to CCl_4 group. The antioxidant effect of all these natural products (tocopherol and ascorbic acid 6

hexadecanoate, sterols, sterylglycosides and squalene), which are present in SMSO, could diminish the membrane peroxidation as well as the AST, ALT and GGT leakage in plasma.

III.2.6- Globularia antioxidant activity: Literature review

N.-E. Es-Safi et al.[55] studies on hydroalcoholic extracts and isolated compounds of *globularia alypum* by DPPH method showed that *GA* has significant antioxidant activity, the results demonstrated that the flavonoids have a potent antioxidant activity, and the less antioxidant activity was noted for the iridoids.

Daycem Khlifi et al.[56] determined the antioxidant potential of *GA* by DPPH and ABTS assays, the results demonstrated that the strongest antioxidant is obtained for the methanolic extract (IC_{50} = 15.58 mg/L).

In *Hnia Chograni et a.l*[57] Studies, leaves and flowers from Tunisian *GA* were assessed for their phenolic and flavonoid contents and antioxidant activity; the results demonstrated that *GA* represents a considerable source of potential antioxidant .

III.3. Antibacterial activity:

III.3.1- Antibacterial agents:

An antibacterial agent is a substance that has the power to kill bacteria or prevent its multiplication; Therefore the antibacterial action can be bactericidal which destroys microorganisms, or else can be bacteriostatic which only inhibits the growth of microorganisms without killing them[44].

- **Minimum Inhibitory Concentration (MIC):** lower concentration of antibiotic which inhibits any visible culture of a bacterial strain after 18 hours of culture at 37 ° C. This value characterizes the bacteriostatic effect of antibiotic[58].
- **Minimum Bactericidal Concentration (MBC):** lower concentration of antibiotic leaving 0.01% or less of survivors of the initial inoculum after 18 hours of culture at 37 ° C. This value characterizes the bactericidal effect of an antibiotic[58].

III.3.2-antibacterial activity tests:

The sensitivity of a bacterial strain to an antibacterial can be determined by the antibiogram, this technique also allows the determination of the minimum inhibitory concentration MIC; the two main methods included in the antibiogram are:

- Dilution method.
- Dissemination method.

a. Dilution method:

The dilution method consists in incubating the bacterial inoculum in a liquid medium or in a solid medium, then bringing it into contact with increasing concentrations of antibacterial[59].

For the liquid medium, the incubation is carried out in a series of tubes (macrodilution method) or in a series of wells (microdilution method); the tube which contains the lowest concentration of antibacterial and no culture growth is visible corresponds to the MIC [59].

For the solid medium, the bacterial inoculum is inoculated in an agar medium poured into a petri dish with increasing concentrations of the antibacterial agent, the MIC is unbalanced by the medium which contains the lowest concentration and which corresponds to inhibition growth of incubated strains[59].

b. Dissemination method:

The diffusion method or the standard antibiogram consists in placing on an agar medium previously sown with the strain to be studied blotting paper discs soaked in the antibacterial agent to be tested[59].

As soon as the discs are disposed, the antibacterial agent begins to distribute uniformly around the disc; the absence of growth around the disc corresponds to the inhibition of the strain, the circular zones formed are known by the zones of inhibition, which have different diameters according to the sensitivity of the strain tested[59].

- **Determination of strain sensitivity:**

The strain to be tested can be of a resistant (R), intermediate (I) or sensitive (S) category. To know the category of the strain, critical values for the diameters of the zones of inhibition and for the concentrations were defined [59].

The diffusion method uses the relationship between the MIC and the diameter of inhibition so that the reading is relatively direct[59].

Table III.2: *relationship of strain sensitivity with MIC and inhibition diameter*[59]

Categories	**MIC (mg/L)**	**Diameter (mm)**
Sensitive	MIC≤ c	Diameter ≥ D
Resistant	MIC > C	Diameter < d
Intermediate	c < MIC ≤C	d ≤ Diameter < D

- Sensitive (S): corresponds to the MIC lower or equal to that of the lower critical concentration c, and a diameter of inhibition greater than or equal to the critical diameter D.
- Resistant (R): the MIC is greater than the high critical concentration C, and the diameter of inhibition is less than the critical diameter d.
- Intermediate sensitivity (I): the MIC and the corresponding inhibition diameter is between the two critical concentrations and the two critical diameters.

III.3.3- Milk thistle antibacterial activity: Literature review

According to *Dayanne Rakelly de Oliveira et al.*[60], silymarin demonstrated antimicrobial activity of little significance against the bacterial strains tested, with MIC (minimum inhibitory concentration) values of 512 µg/ml. Meanwhile, silibinin showed significant activity against Escherichia coli with a MIC of 64 µg/ml. Findings have pointed to a synergistic drug-modifying effect when silymarin and silibinin were combined with antibiotics, especially aminoglycosides, against the different bacterial strains evaluated, where silibinin had an antagonistic effect when combined with imipenem and gentamicin against P. Aeruginosa. Accordingly, phenolic compounds, for example, flavonoids and lignans, have demonstrated their therapeutic potential as antimicrobial agents, where they are considered responsible for this activity. The synergistic effect of flavonoids combined with commonly utilized antibiotics is well supported in the literature, emerging as an important complementary treatment modality in research. It is believed, therefore, that phenolic compounds possess the capacity to form complexes with extracellular soluble proteins that bind to bacterial cell wall. Studies have shown that many natural compounds alter the permeability of the cell membrane, favoring the penetration of antibiotics. The interaction with bacterial enzymes can also be related to the synergistic mechanism of natural products with antibiotics, which can be obtained from an extract or from the combination of extracts, synthetic products, antibiotics, and other natural products.

With respect to the antibacterial action of flavonoids, studies have demonstrated a significant inhibitory effect on DNA topoisomerase activity by the formation of complexes that alter enzyme binding. In this perspective, the antibacterial activity of these compounds could also be related to the presence of hydroxyl phenolic groups that interfere with the bacterial synthetic processes by enzyme inhibition.

Oshaghi Mojgan et al.[61] investigated the antibacterial activity of silymarin against enteric bacterial pathogen strains. For evaluation of antibacterial activity, different concentrations of silymarin (ranging from 2-128 µg/ml) were tested against twelve enteric bacterial pathogens by microdilution broth method. The results of study showed that the minimal inhibitory concentration (MIC) of silymarin against all of tested strains was >128 µg/ml. It appears that silymarin is not beneficial drug for treatment of gram negative enteric bacterial pathogens. Inhibitory effects of silymarin against different selected gram negative strains were performed at various concentrations from 2, 4, 8, 16, 32, 64, 128 µg/ml. The minimal inhibitory concentration of silymarin against all of the 12 gram negative bacteria was >128 µg/ml. Turbidity of Shigella strains were lower than other tested strains at 64 and 128 µg/ml concentrations, demonstrating decrease of growth and the number of bacteria. Results also reported that silymarin is not suitable candidate for treatment of gram negative enteric bacterial pathogens.

III.3.4- *Globularia* antibacterial activity: Literature review

The antibacterial activity was evaluated by *Bouchebrinewalid et al.* using the agar diffusion technique, the results demonstrate that the methanolic extracts of *Globularia alypum* have no antibacterial effect via the four strains tested in this study (*Salmonella spp*, *Pseudomonas aeroginosa* ATCC 27853, *Escherichiacoli* and *Staphyloccoccus aureus* ATCC 25923)[44].

According to *Messaoud Ramdani et al.*[62] The EO of *GA* showed a significant effect against gram-negative bacteria.

Boussoualim Naouel et al[63]. study on the antibacterial effect of *GA* extracts has shown that the atibacterial activity exhaled by ethyl acetate extract and Chloroform extract is more important than that obtained with the other extracts; it is noted that: the Ethyl acetate extract and raw extract are more active on the *Staphylococcus aureus* strain, and Chloroform extract on the *Acinetobacter baumannii*strain.

III.4. Conclusion:

In this chapter, we mentioned the biological activities to be tested on the two plants (the antioxidant activity and the antibacterial activity) with a brief explanation of their principles.

According to literature reviews studies of the biological activities the two plants are considered as a potential source of antioxidants; and for the antibacterial activity, the results are different according to the strains and the extracts tested.

In the next chapter, we will detail the protocols that have been followed to test these different activities and studies.

EXPERIMENTAL PART

IV.1. Introduction:

The practical part corresponding to the extraction of plants' essential oils, lipids and aqueous and alcoholic extracts, the phytochemical screening, indexes and antioxidant activity should have taken place at the laboratory of the technologic hall while the antibacterial activity should have been conducted at SAIDAL Medea.

IV.2. Methodology:

The present work was going to be conducted according to the following methodology[2]:

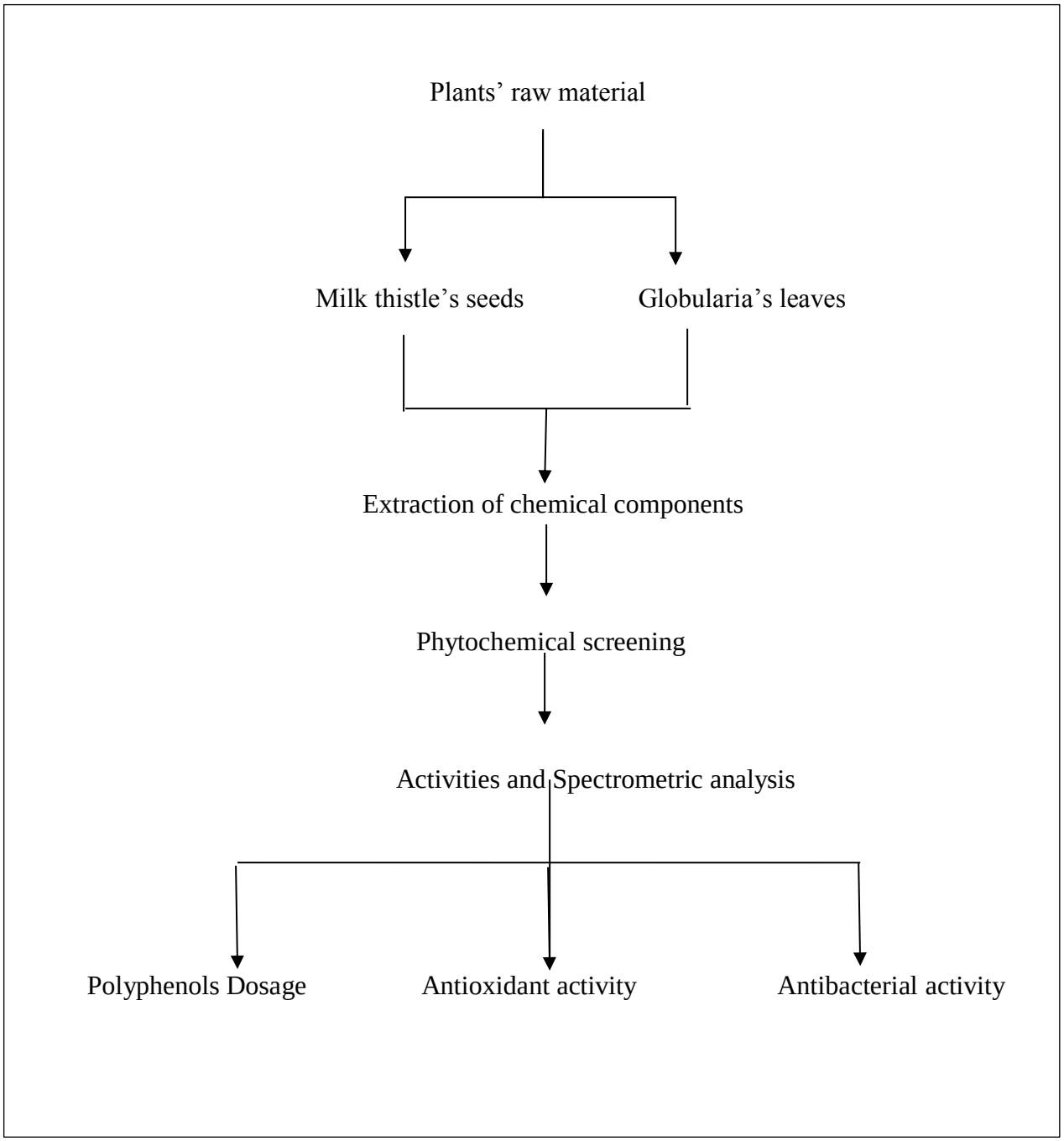


Fig IV.1 *Methodology scheme*

IV.3. Samples preparation:

- **Milk thistle:** Milk thistle dry, white and brown flowers were harvested in May 2019/2020 from the region of Ouzera/ Medea by hand and seeds were recovered. Seeds were grounded into fine powder using a shuffled coffee grinder. The obtained powder was kept in a dry well-sealed container in the dark.

- **Globularia Alypum:** The leaves of Globularia were collected from the mountains of the Aflou region / Laghouat; the leaves were dried and ground into a fine powder, the powder was kept away from moisture.



Fig IV.2: *Globularia leaves and powder*

IV.4. Milk thistle extraction:

- **Isolation of oil from the seed:**

The milk thistle seeds ought to be grounded and the moisture content is determined by drying to a constant mass at 105°C. The ground seeds (m = 50 g) of milk thistle are defatted in Soxhlet apparatus with 80 mL of hexane for 3 h. After filtration and evaporation of the solvent, a mass of pale yellow oil is obtained[64].

- **Isolation of silymarin from the seed:**

The defatted seed powder is transferred into a flask fitted with a condenser and 190 mL of acetone is added and stirred for t = 72 h at room temperature. After filtration and

concentration of the silymarine fraction under vacuum, the yellow residue is dissolved in methanol (100 mL) and partitioned with hexane (3 × 200 mL). Methanol extract is partitioned with diisopropyl ether, and evaporated. The remaining solid is dried to obtain silymarin[64].

IV.5. Extraction of *Globularia Alypum* essential oil:

Globularia essential oil was extracted by hydrodistillation technique; 60 g of globularia leaves powder were mixed in 600 ml of distilled water, the distillate obtained has a monophasic aspect.

Two assemblies were used for the hydrodistillation, the first comprises a serpentine condenser, and the second with a straight condenser. The distillation yield obtained for hydrodistillation by the right refrigerant is better than that obtained for the serpentine refrigerant (for a volume of 400 ml of distillate we find that the first assembly take a duration of approximately 24 hours and the duration obtained by the 2nd assembly is approximately 3 to 4 hours).

As soon as the distillation is finished, the distillate is poured into a separation funnel to carry out the salting-out step; for every 100 ml of distillate, 6 g of NaCl has been added, then the solution is stirred with degassing from time to time.

To separate the aqueous phase from the oily phase, 20 mL of diethyl ether for 100mL of the estract is added and the mixture is left to stand; after a certain time, the aqueous extract and the oily phase which contains the essential oil and the added solvent are recovered.



Fig IV.3: *separation of the oily phase by decantation*

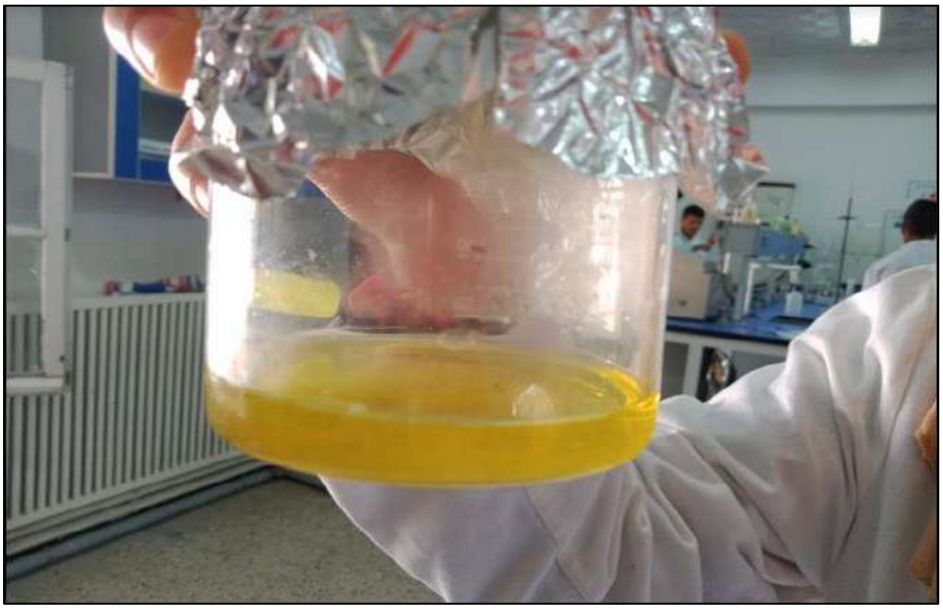


Fig IV.4: *the oily phase recovered*

To separate the essential oil from the solvent, the oily phase was placed in the rotary evaporator at a temperature of 35 ° C to 40 ° C until the solvent has completely evaporated.



Fig IV.5: *separation of essential oil in the rotary evaporator*

The essential oil obtained is very volatile which requires stabilizing it in neutral oil such as paraffin in order to use it in a pharmaceutical formulation (an ointment or a cream).

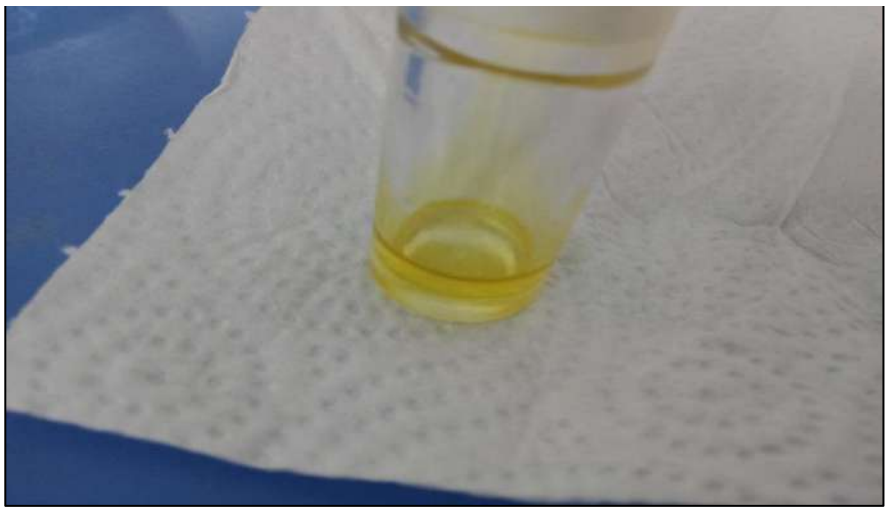


Fig IV.6: *The recovered essential oil*

- **Extraction yield calculation:**

Extraction yield is the ratio of the dried mass of the desired extracts and the dried mass of the used plants' parts (seeds, leaves,)[2]. It is calculated according to the following formula:

$$\text{Yield (\%)} = 100We/Wt$$

where We and Wt stand for the weight of completely dried extract and parts of plant which is undertaken of extraction, respectively[65].

IV.6. Extracts preparation:

In order to prepare the extracts of both plants, two solvents were tested: ethanol, methanol for Globularia and Milk thistle. 10g of the sample powder was weighed and mixed with 80mL of the solvents (each solvent in a separate flask) and 20mL of distilled water. The mixtures were left on medium agitation for 24 hours under room temperature and protected from light. The contents were then filtered and the filtrate was kept in the dark at 4°C while the cake was recycled into a second and a third extraction with the same solvents proportions and under the same conditions. Recycling the cake has the purpose of extracting all the solvents-soluble plant components. The third extraction solutions have a less intense green color compared to the first extraction as shown in figure IV.7.

IV.7. Phytochemical screening:

Phytochemical screening allows the confirmation of the existence or absence of secondary metabolites families in plants using indicators that change colors when a certain family is present[2].

Plants infusions are prepared by bringing a mixture of 5g of each plant powder and 100 mL water to boil for 15min and then filtering them to end up with an infusion volume of 100mL and a concentration of 5%[2].

- Phenols and tannins are indicated by the appearance of greenish blue or black color when few drops of ferric chloride ($FeCl_3$) are added to the infusions.
- Flavonoids are detected by the addition of few drops of sodium hydroxide (NaOH) and the appearance of an intense pink color.
- Alkaloids are revealed by an ethanolic plants solution of 0.1% to which is added 10mL of water containing 0.015g of iodine (I_2) and 0.16g of potassium iodide (KI). A dark brownish red color should appear if alkaloids are to be present[2].

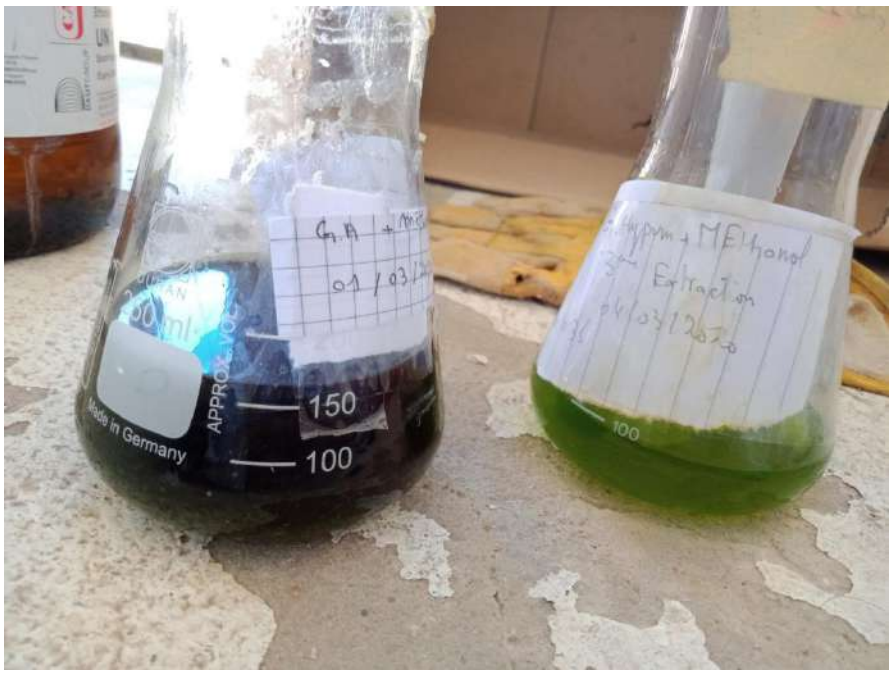


Fig IV.7: *Globularia Alypum methanol extracts: 1st and 3rd extractions (dark and light green respectively)*

IV.8. physicochemical constants:

The physico-chemical constants determine the nature of the oils and their degree of purity, they are hence referred to as oils' constants.

Acid number:

The acid number of a fatty substance is the quantity of potash in mg necessary to neutralize its free acidity. The free acid content of fatty substances increases over time[2].

The acid number of essential oils amounts to neutralizing the free acids of these by potassium hydroxide (KOH) by applying the following protocol:

- In an Erlenmeyer flask, a mass of 0.4 g of EO was introduced with 40 ml of ethanol (96%), 40 ml of cyclohexane and 2 drops of phenolphthalein were successively poured.
- The solution is stirred with a magnetic stirrer until dissolved.
- Then it is dosed with a 0.01 M alcoholic potassium hydroxide solution until a persistent pink color appears.

The calculation of the value of the acid index is as follows:

$$I_A = (m_p / m_o) \text{ with: } \quad m_p = cvM$$

m_o: mass of oil.

m_p: mass of potash.

c: concentration of KOH.

v: volume of KOH at the equivalent point.

M: molar mass of KOH.

Saponification index:

Saponification index is the mass of potash in mg necessary to neutralize the free fatty acids and to saponify the fatty acids combined in one gram of fatty substance[2].

The procedure includes the following steps:

- Prepare a solution of 200 mg of EO and 20 ml of a solvent composed of 10 ml of ethanol and 10 ml of cyclohexane, then shake to dissolve the fatty substance.

- In a beaker, 10 ml of the solution prepared above is introduced with 25 ml of alcoholic potash of concentration (0.5 M). Then the beaker is put in a boiling water bath for 45 to 60 minutes.
- 2 to 3 drops of phenolphthalein are added to the beaker, before dosing the solution with hydrochloric acid at a concentration of 0.025 M, stirring until the color of the phenolphthalein disappears. Carry out a blank test under the same conditions.

$$I_s = ((V_b - V_S) \times C_{HCl} \times M_{KOH}) / m_o$$

V_b: blank volume;

V_s: sample volume;

C_{HCl}: concentration of HCl.

M_{KOH}: molar mass of KOH;

m_o: mass of oil.

Esterification index:

Esterification index is the quantity in mg of KOH necessary to saponify 1g of oil devoid of fatty acid. The calculation formula is:

$$I_E = I_S - I_A$$

I_E: esterification index;

I_S: saponification index;

I_A: acid number.

Refractive index:

The Refractive index is the ratio of the velocity of light of a specified wavelength in the air to its velocity in the examined substance[66].

The refractive index is measured using a direct reading refractometer:

- After having cleaned the refractometer and direct it towards the light, 2 or 3 drops of compound to be analyzed are placed in the middle of the prism.

- When looking through the eyepiece, the adjustment is adjusted by turning the refractive index adjustment knobs to bring the dark and lighted areas to the center of the reticle.
- And finally the value of the refractive index is taken by direct reading.

IV.9. Determination of polyphenols:

The polyphenols were determined spectrophotometrically by the Folin-Ciocalteu method, this method consists in quantifying the total concentration of hydroxyl groups present in the extract, using the Folin Ciocalteu reagent which is a yellow acid, consisting of a mixture phosphotungstic acid (H3PW12O40) and phosphomolybdic acid (H3PMo12O40). It is reduced, during the oxidation of phenols, to a mixture of blue oxides of tungsten and molybdenum [2].

The coloring produced, whose maximum absorption at 765 nm, is proportional to the amount of polyphenols present in the plant extracts.

The concentration of polyphenols is determined by referring to a calibration curve which was carried out in parallel under the same operating conditions using Gallic acid at different concentrations.

The concentration of polyphenols is determined by referring to a calibration curve which was carried out in parallel under the same operating conditions using Gallic acid at different concentrations.

$$C_{ph} = K*A$$

C_{ph}: concentration of phenols.

A: the absorbance corresponding to the concentration of polyphenols.

K: a constant for a given tank thickness, a given wavelength and a given solution, this constant is obtained by calculating the tangent of the calibration curve of Gallic acid.

The protocol followed to quantify polyphenols is as follows [2]:

- In a test tube, 200 µL of extract are put with the appropriate dilutions, and then 1 ml of Folin's reagent (10 times diluted) is added.
- The mixture is stirred and incubated for 4 minutes, and then 800 µl of the Na_2CO_3 solution (at 7.5%) are added.

- After 30 min, the Absorbance is measured using a spectrophotometer at 765 nm.

IV.10. Antioxidant activity:

The antioxidant activity was supposed to be carried out for both plants using the following DPPH essay.

- **DPPH essay:**

A 3mg of DPPH in 100ml ethanolic solution is to be prepared and 1mL of this solution is added to 50µl of the solution of all extracts in ethanol at different concentration (50,100,200,400 & 800µg/mL). The mixtures are shaken vigorously and allowed to stand at room temperature for 30 minutes. Gallic acid (or ascorbic acid) is used as the reference. The control solution is prepared by adding 1mL of DPPH ethanolic solution to 50µl of ethanol. Then the absorbance is then measured at 517 nm using a UV-VIS spectrophotometer Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPPH radical is calculated by using the following formula.

$$DPPH\ scavenging\ effect\ (\%\ inhibition) = ((A_0 - A_1)/A_0) * 100$$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all the extract samples and reference. All the tests should be performed in triplicates and the results are averaged[2,67].

To calculate IC_{50} (Concentration that reduces 50% of DPPH), a curve of the inhibition percentage in function of extracts concentrations need to be drawn. The equation of the linear curve (y=ax+b) is then used to calculate IC_{50} value. The smaller value is, the more powerful the antioxidant is[2].

IV.11. Antibacterial activity:

The antibacterial activity was supposed to be evaluated at the level of the Saidal Complex, the method proposed for this evaluation is the diffusion disc method, and the antibacterial effect of the extracts will compare with other antibiotics supplied by the pharmaceutical manufacturing company SAIDAL - Médéa (injectable solution of the antibiotic Gentamine (GENT) and Amoxicillin (AMOXY)).

Table IV.1: *The strains used in the test for antibacterial activity*[2].

Strain	Code	Kind	Gram staining
Pseudomonas aeruginosa	ATCC 9027	Bacteria	-
Candidas albicans	ATCC 10231	Yeast	
Eschirichia coli	ATCC 8739	Bacteria	-
Bacillus subtilis	ATCC 6633	Bacteria	+
Salmonella typhimuium	ATCC 14028	Bacteria	-
Staphylococcus epidermidis	ATCC 12228	Bacteria	+
Staphylococcus aureus	ATCC 6538	Bacteria	+
Saccharomyces cerevisaie	ATCC 9763	Yeast	

- **Preparation of culture medium:**

40 g of agar powder is introduced into 1L of distilled water; the solution is placed on a heat source until it boils. The solution is then poured into sterile bottles, then these are placed in a sterilizer for 1 hour and a half [2].

Antibacterial test:

- Pour 1 ml of the bacterial suspension into petri dishes near a flame.
- Add a quantity of nutrient agar equivalent to 11 ml, then shake carefully until the mixture becomes homogeneous.
- Leave the petri dishes open in front of the flame until solidification.

- Make sure the agar surface is well dried.
- Using sterile forceps, place the discs (four discs per box) impregnated with plant extract on the surface of the agar.
- Place the petri dishes in the oven, at the optimal temperature for growth of the germ to be studied (35 ° C) for 16 to 24 hours. The petri dishes must be placed with the lid down.
- After incubation, the results are read by measuring the diameters of the inhibition zones (the clear zone around the disc) obtained for each strain in (mm). The petri dish with the largest diameter corresponds to the most resistant strain.

Determination of MIC by agar diffusion method:

- The extracts having shown positive antibacterial activity are selected to determine the minimum inhibitory concentrations (MIC) by the diffusion method on agar.
- Successive dilutions of arithmetic progression of reason 2 made it possible to prepare a range of dilutions (1/2, 1/4, 1/8 and 1/16).
- The minimum inhibitory concentration is that corresponding to the medium which contains the lowest concentration among the prepared dilutions and which inhibits the growth of the incubated strain.

Difficulties and aspirations

From the very first beginning of the process of this thesis elaboration to the last final step, we have received help countless times from supervisors, academics and colleagues, which were it not for, this work would have probably never seen light. However, there were some difficulties and obstacles that slowed down the advancement of the thesis realization. Difficulties were mainly related to lack of chemical solvents and substances and consumable glassware and the insufficiency of the allocated time to laboratory entry. Yet, the strongest and the most unfortunate condition was the pandemic spread of the Coronavirus COVID19 which consequently led to the total shut of the laboratories after only two weeks of discrete work and thus conducting to the incompletion of all goals and aims set. Still, it was primordial to ensure the delivery of an authentic work.

We aspire that future studies tackle other extraction methods such as enzymatic assisted extraction. Other biological activities could interestingly be tested such as anticancer activity. We also hope that further studies examine the possible synergy between milk thistle and globularia in terms of biological activities.

CONCLUSION

This work represents a phytochemical and biological study of Milk thistle and Globularia plants, which are known by their use in traditional medicine, whose biological effects have been tested for hydro-alcoholic extracts and essential oil of the two plants.

Medicinal plants represent an important source of therapeutic substances because of their composition varying from natural products, mainly essential oils, and secondary metabolites.

The monographic and chemical presentation of Globularia and milk thistle approves the botanical and chemical specificity for each plant, which explains the dissimilarity biological effects of each.

According to literature review studies of the biological activities, the two plants are considered as a potential source of antioxidants; Antibacterial activity studies have shown that Milk thistle has modest activity against gram-negative bacteria; however, Globularia has a significant antibacterial effect against this kind of bacteria compared to gram-positive bacteria.

The practical part has not been fully achieved because of the current state of health (epidemic of Coronavirus), but according to the extraction part made on Globularia, it has been found that the essential oil of this plant has very high volatility.

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