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By :

Mrs KADDOUR Faiza

Title :

Chemical and biological study of *Plumbago europaea*: Development of a new alcohol-free mouthwash against oral candidiasis

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Publicly defended on 06/06/2023 in Tlemcen before a jury composed of

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Spécialité : Chimie des produits naturels analyses et application

Par :

Mme KADDOUR Faiza

Sur le thème

Etude chimique et biologique de *Plumbago europaea* : Développement d'un nouveau bain de bouche sans alcool contre les candidoses buccales

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Soutenue publiquement le 06/06/2023 à Tlemcen devant le jury composé de :

Mr ARRAR Zoheir	Professeur	Université de Tlemcen	Président
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DEDICATIONS:

To the soul of my father (may Allah be merciful to him) who taught me the meaning of life, teaching me to work hard to achieve my goals.

To my beloved Mother who is always there for me, for her endless love, support, and encouragement.

To my husband, who has been a source of strength, support, patience, and motivation for me throughout this entire experience. I am truly blessed to have you as my partner in this life.

To my angels Ziad Ibrahim, Besma Nihel and Mohammed Yanis who have made me stronger, better and more fulfilled than I could have ever imagined.

To my brother and Sisters.

To everyone who has supported me, encouraged me, and believed in me.

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List of abbreviations:

AD: Antimicrobial drugs

BACs: Bioactive Compounds

DPPH: 2,2-Diphenyl-1-Picryl-Hydrazyl

EA: egg albumin

EE: Ethanolic extract

Eos: Essential oils

FICs : Fractional inhibitory concentrations

FICIs : fractional inhibitory concentration indices

FRs: Free radicals

FRAP: Ferric-reducing antioxidant power

GC: Gas Chromatography

HAT: Hydrogen atom transfer

HE: Hydrosol extract

HPLC: High-performance liquid chromatography

IR: Infrared

LBDD : Ligand-based drug design

LLE: Liquid-liquid extraction.

MDR: Multi-drug resistant

MICs: Minimum inhibitory concentrations

NCEs : New Chemical Entities

NMR : Nuclear Magnetic Resonance

NPs: Natural products

OC: Oral candidiasis

OCRF: Oral Cavity Residence Factor

PBS: Phosphate buffered saline

P. europea: Plumbago europaea

SBDD: Structure-based drug design

SMs: Secondary metabolites

UV: Ultraviolet

WHO: World Health Organization

Introduction

Humans have had a long and delicate relationship with nature. Humans have been probing and studying a wide range of natural phenomena from the beginning of recorded history, regardless of place, time, or culture. The oldest records of human activity frequently reflect spectacular and inventive answers to these natural events, and human life is inextricably linked to the rich tapestry of life that nature provides.

Many would agree, however, that humanity's interaction with natural resources has not only reaffirmed its importance over time, but has also led to a higher collective understanding about how to more effectively tap into these huge arrays of resources to advance human civilization.

It may be claimed that every knowledge system in existence today is linked to or has drawn inspiration from natural products, which are referred to collectively as natural products in the broadest sense. Modern knowledge systems in industry, commerce, and health care have been and continue to be inextricably linked to the natural world. Nature has served as a vital platform in promoting breakthroughs in the realm of healthcare and medically-related scientific advances.

Medicinal plants have been utilized for thousands of years as traditional cures for many human diseases throughout the world. In rural regions of developing countries, they still constitute the main source of medicines. The World Health Organization (WHO) estimates that 80% of the population in developing countries depends on traditional medicine for their primary medical needs and that about 85% of traditional medicine is based on plant extracts. This implies that around 3.5 to 4 billion people throughout the world depend on plants as drugs resources[1]. Using plants for therapeutic reasons is considered the most important use of the world's biodiversity. More plant species are used as medicines than as food. This fact indicates that natural products play an important role in the development of novel drugs.

Plants consist of many active compounds such as alkaloids, steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids, which found in their specific parts such as leaves, flowers, bark, seeds, fruits, root, etc. The therapeutic effects of plant materials are usually the result of the combination of these by-products. Natural products derived from

medicinal plants have been shown to be an abundant source of bio actives, many of which have been used as the basis for the development of new lead-based chemicals for pharmaceuticals [2]. From aspirin to Taxol, the modern pharmaceutical industry itself still relies heavily on the diversity of secondary metabolites from plants to find new molecules with biological properties previously unknown. It is also necessary to remember those such effective remedies as quinine, the leader in antimalarial drugs, morphine, a major analgesic, rye ergot, with anti-migraine properties, or curare, with muscle relaxant properties, are of plant origin. Some other relevant examples are: galegine, from *Galega officinalis L*., which was the model for the synthesis of metformin and other antidiabetic drugs; papaverine *from Papaver somniferum L*., which formed the basis for verapamil used in the treatment of hypertension [3]. This source appears inexhaustible, given that only a small percentage of the world's 440,000 plant species have been studied phytochemically and pharmacologically, and that each species can have thousands of different constituents, increasing the possibility of discovering new bioactive compounds [4].

Infectious diseases and microbial pathologies are the sources of millions of deaths every year. They continue to be the leading causes of death in the world's low-income countries, as well as the third most common cause of death across the world [5]. Unfortunately, the treatment of these infections has been hampered by the emergence of antimicrobial resistance, which is considered one of the most serious threats to the world healthcare system this century. This situation is further aggravated by the appearance and emergence of multi-resistant strains. Antibiotic research and development has slowed to the point that there are no new, more effective antibiotics on the market now [6]. As a result, we will need to look for new antimicrobial drug sources elsewhere, and plant-based material is an obvious choice.

The mouth, like all other parts of the body, is colonized by a commensal microbial flora that acts as a barrier against disease invasion. This flora is not pathogenic, although it can become so under certain circumstances. Most mouth problems are infectious diseases that arise when the oral ecosystem is disrupted. There is a well-established link between oral disease and oral microbiota. A total of more than 750 species of bacteria that live in the oral cavity have been linked to dental problems. The prevalence of people suffering from microbial etiology is the world's highest among all the dental diseases. With the increase and spread of antimicrobial resistance, oral diseases have become a serious public health concern around the world. Therefore, finding safe, efficient, and cost-effective prevention and treatment approaches as well as products for oral diseases has become critical. Numerous studies have been conducted

about the use of medicinal plant extracts as substitutes for synthetic pharmaceuticals against certain microbiological pathogens in the treatment of mouth diseases [7, 8].

When searching for new bioactive compounds from natural products, several issues need to be considered, including the choice of plant material and primary screening tests, which are essential to ensure the selection of molecules or extracts with relevant biological activity. In terms of plant material selection, it is therefore preferable not to choose plants solely on the basis of chance, but to narrow them down according to different criteria. The most widely used is that of their use in traditional or folk medicine, which appreciates the knowledge and experience gained by people all around the world. Following that, the plant material must go through an appropriate extraction process, which is a method for separating medicinally active compounds from natural products via various methods and techniques. The extracts obtained are then subjected to physicochemical analysis (chromatographic and spectroscopic) to determine their chemical compositions as well as biological assays in order to evaluate their biological activity.

In a country like Algeria, where flora is abundant, valuing the medicinal plant sector has become critical. Several scientific research laboratories have been established in order to develop this sector in order to use it in a variety of fields, including pharmaceutical, agricultural, and cosmetic manufacturing. The current research is part of the Laboratory of Natural and Bioactive Substances (LASNABIO) research program, which is focused on valuing the flora of the Tlemcen region in order to discover new active compounds. Our research aims to promote *Plumbago europaea,* a medicinal plant used in traditional local medicine that has yet to be published internationally. In this work, for the first time, a chemical analysis of a root hydrosol extract is presented.

The following objectives have been fixed:

- Determination of the chemical composition of essential oil and hydrosol extract from roots of *Plumbago europaea.*
- Investigation of the *in-vitro* antioxidant and antimicrobial activities of these essential oil and hydrosol extracts.
- *In-vitro* evaluation of the antimicrobial effect of a hydrosol extract combined with Gentamicin and Amphotericin B on a large panel of microorganisms to check whether there is a synergistic effect between the hydrosol extract and the antimicrobials chosen in order to minimize their effective dose and side effects.

- Isolation and purification of phyto-compounds derived from the ethanolic extract prepared from *Plumbago europaea* roots.
- Evaluation of the *in-vitro* antioxidant, antifungal, hemolytic and anti-inflammatory activities.
- Preparation of anti-candida mouthwash containing the ethanolic extract of *Plumbago europaea* roots as active ingredient.
- Conducting a stability study of the prepared mouthwash.

In the first part, we will go over a literature review describing the essential concepts to the understanding of our work. The second part describes the experimental work, which will be divided into three chapters. The first is devoted to the study of essential oils and hydrosol extracts, while the second is devoted to the study of ethanolic extracts. Finally, the last chapter is reserved for the development and formulation of an antifungal mouthwash and also conducting a study to determine its efficacy and stability.

PART I
Bibliographic part

I. Bibliographic part

I.1 Natural products:

Human societies have used natural products for thousands of years. Biologically active compounds obtained from natural sources, such as plants, animals and microorganisms, are defined as natural products [9]. The degree of characterization of the term natural product is both limited and controversial. Therefore, a common definition accepted by all concerned disciplines will still be an evolving goal, but one that will likely evolve as researchers discover the vast array of compounds expected to be found in this domain [10].

There are four main sources of natural products:

1. Whole organisms that have not undergone any processing or treatment other than simple preservation processes such as plants, animals or microorganisms.
2. Parts of organisms such as leaves, roots or flowers of plants or isolated animal organs.
3. Extracts of organisms or parts of organisms.
4. Isolated pure compounds from plants (alkaloids, steroids, flavonoids, carbohydrates glycosides etc.) animals or microorganisms.

The great diversity of natural chemicals is caused by several factors, most notably the enormous biodiversity of marine and terrestrial organisms, which produce different chemical structures with a wide range of biological properties [11]. In addition, chemical diversity is also the result of millions of years of evolution, which have altered biosynthesis pathways in response to a variety of biotic and abiotic stresses induced by natural events (for example, viruses and environmental changes), or unnatural (such as, chemicals or radiation) [12].

These molecules have undergone natural selection and screening over thousands of years to enhance specificity and cover a very broad range of functions, depending on the origin, habitat and specific activity of the source organism. Because of these intrinsic properties, natural products (NPs) have been used as medicines for thousands of years and remain the most important source of new potential therapeutic compounds today [11].

The prevalence of communicable and non-communicable diseases and the challenge of discovering drug candidates that can effectively treat these diseases with minimal or no side effects is enormous. Despite the successful development of drugs to treat and manage diseases such as HIV/AIDS, hypertension, malaria, diabetes and cancer, these diseases continue to afflict diverse populations around the world and result in significant mortality. There is a great need for innovative drug discovery strategies that differ from the pharmaceutical industry's current

"blockbuster" Research and Development (R&D) strategies. Currently, a viable approach is to look for answers in "nature," as it has historically been applicable to drug research. Anticancer drugs such as Taxol, Vinblastine and antimalarial drugs such as Quinine and Artemisinin have all been discovered from natural products and are efficient in treating these diseases. With global public health challenges, research and development (R&D) on natural products potentially plays a key role in the discovery of innovative new drugs [13].

I.1.1 Natural products and drug discovery:

Medicines and natural products (NPs) have been closely linked for thousands of years through the use of traditional medicines and natural poisons. Natural products have played an extremely important role in the treatment and prevention of human diseases worldwide. Clinical, pharmacological and chemical studies of traditional medicines, mainly derived from plants, were the basis for most early drugs such as aspirin, morphine digitoxin, quinine and pilocarpine [14].

The importance of natural products for the treatment and prevention of human diseases can be assessed as follows: the introduction of new chemically diverse units and their use as templates for semi- and total-synthetic modifications; the various diseases treated by these diverse compounds; and the rate of their use in disease treatment [15] .

The goal of all drug discovery processes is to find the most promising lead compounds that can be used as therapeutics agents and help to treat infections and multiples diseases such as cancer, hypertension, neurological disorders, and metabolic diseases. In the early stages of the drug design process, scientists must use different methods to isolate and purify lead compounds from natural sources, depending on the structural diversity, stability, and quantity of the desired compound. Various screening techniques are used to screen and select lead compounds for specific targets. At this stage of the drug design process, many lead compounds are not selective enough for their target molecules. In order to improve their selectivity, the scientists modified the structures of the lead compounds according to the expected structure-activity relationship. If the performed modifications enhance selectivity, promising molecules will enter *in-vitro* and *in-vivo* studies. If the results are positive, scientists must conduct safety tests on the selected compounds to determine the mechanisms of drug absorption, distribution, metabolism and excretion, which is termed the pharmacokinetics process. If all results and optimizations are positive, lead compounds can be potential drug candidates [16, 17].

What is now indisputable is that natural products have been and will be an important source of innovative drug therapies. The large share of natural products in drug discovery is attributed to their structural diversity and the complexity of their carbon skeleton. Because secondary metabolites are developed from natural sources in living systems, they are often considered "drug-like and more biocompatible" than fully synthetic molecules, which makes them good candidates for subsequent drug development [18, 19]. However, therapeutics formed from natural sources can avoid side effects because they produce physiological and pharmacological effects in living cells. Furthermore, natural products have a broader distribution of molecular properties, including lower molecular weights, partition coefficients, and structural diversity [20]. In addition, natural products interact more strongly with biomolecules such as proteins and enzymes. Moreover, natural products have fewer heavy metals and greater molecular stiffness than synthetic compounds and combinatorial libraries [16].

The search for natural products as a source of new human therapies peaked in the western pharmaceutical industry between 1970 and 1980, leading to the pharmaceutical field being heavily influenced by non-synthetic molecules. Of the 877 small molecule new chemical entities introduced between 1981 and 2002, approximately half (49%) were natural products, semi-synthetic natural product analogs, or synthetic compounds based on natural product pharmacophores [18]. Of the 1562 new chemical entities (NCEs) launched between 1981-2014, only 420, or 27%, are of synthetic origin. More specifically, it is reported that over 20 new drugs launched on the market between 2000 and 2005, are derived from terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates [21, 22].

The statistics described above have led researchers to renew their interest in the study of NPs. This new trend, probably destined to develop and lead a substantial amount of NPs to reach the market in the near future, is also supported by the objective truth that the plant kingdom includes a very large number of species, each is capable of producing a wide range of bioactive compounds based on different chemical scaffolding.

I.1.2 Medicinal plants:

Medicinal plants have been a valuable source of therapy for thousands of years, and even today many medicines are natural products derived from plants or their derivatives [23]. The oldest written evidence of medicinal plants being used in medicine is found on a Sumerian clay tablet in Nagpur, dating back nearly 5,000 years. [24]. Throughout this time, medicinal plants have been used only on an empirical basis without any mechanistic knowledge of their pharmacological activity or active ingredients. The rational discovery of drugs from plants began in the early 19th century, when the German pharmacist Friedrich Sertürner successfully isolated an analgesic and hypnotic drug from opium, which he called morphium (morphine).

This led to the study of other medicinal herbs, and in the following decades of the 19th century, many biologically active natural substances, especially alkaloids (e.g. quinine, caffeine, nicotine, codeine, atropine, colchicine, cocaine, capsaicin) were isolated from natural sources [23, 25]. According to the World Health Organization (WHO), even today, due to poverty and lack of access to modern medicine, about 80% of the population in many third world countries still use traditional medicine (such as medicinal plants) for primary care. About 25% of prescription drugs in industrialized countries also contain active ingredients that are still extracted or derived from higher plants, a situation that has persisted in recent decades [26].

According to the report of the International Union for Conservation of Nature (IUCN) in 2015, Approximately 310,000 plant species have been described to date [11]. Of the overall plant species known to date, only 60,000 species, or about 20%, have already been screened and they have supplied 135 known drugs [27, 28]. Therefore, by making a quick projection, these statistics suggest that screening the remaining plant species could detect approximately 700 new drug candidates. Furthermore, most of the 60,000 plants already screened have been studied for their effects on a limited number of disease targets, and there is still a chance of finding additional or new effects on neglected diseases targets [29]. Moreover, they may be useful as molecular probes to find disease-relevant targets [30].

The vital importance of botanical products for humanity is mainly due to their phyto-compounds, which are active constituents with therapeutic properties. Phyto is the Greek word for plant, and phytochemicals are naturally occurring compounds in plants. In order to protect themselves from natural dangers such as predatory insects, pollution, and diseases, plants produce phytochemicals. [31]. Compounds produced by plants are divided into primary metabolites and secondary metabolites. Primary plant metabolites are essential for normal plant

growth and basic metabolism, while most secondary metabolites are non-essential but can play key roles in other functions. They are bioactive molecules of plants with therapeutic, preventive, toxicological and immune stimulating effects [32].

Several approaches are used to select the raw materials for the discovery of new pharmacologically active plant substances. In random screening methods, plant extracts, enriched fractions or isolated phytochemicals are randomly selected according to their availability. The ethno-pharmacological approach is a classical knowledge-based approach in which the traditional medicinal use of plants serves as the basis for the selection of test materials and pharmacological trials. The ecological approach used to choose medicinal plants starting materials is based on observing the interaction between an organism and its environment, resulting in biologically active natural products [23, 33-36].

Computational approaches are another very powerful knowledge-based method that facilitates selection of plant materials or natural products with a high probability of biological activity. Computational methods commonly used in drug discovery can be divided into structure-based drug design (SBDD), ligand-based drug design (LBDD), and sequence-based methods (SBDD) such as molecular docking and de novo drug design rely on knowledge of the target macromolecular structure, mainly obtained from crystal structure and NMR data [37].

I.1.3 Secondary metabolites:

The plant kingdom provides hundreds of compounds with low molecular weight. The research community has classified these compounds into three major groups based on their purported functions: primary metabolites, that are directly required for plant growth; secondary metabolites, that mediate plant-environment interactions; and hormones, that regulate the processes and metabolism of organisms [38].

The plant primary metabolites are the compounds of nucleic acids, proteins, carbohydrates, fats and lipids. They are related to structure, physiology and genetics and play a crucial role in plant development. These products of primary metabolism are mainly obtained from glycolysis, the TCA cycle, or the shikimate pathway and typically serve as precursors for the synthesis of tens of thousands of secondary metabolites, which typically occur as by-products in low concentrations [39]. Secondary metabolites are relevant to the interactions between plants and their environment. They can act as UV filters or provide color to flowers, which attract pollinators. They are responsible for the medicinal properties of plants, but their distribution is

very limited compared to that of primary metabolites [40, 41]. The level of secondary metabolism is affected by a variety of factors including the age of the plant, the season, microbial attack, grazing, radiation, competition, nutritional condition, and various environmental stresses [32, 42, 43].

Biosynthesis of SMs begins with fundamental metabolic pathways, such as glycolysis, the Krebs cycle, or the shikimate pathway, and then broadly diversifies according to cell type, developmental stage, and environmental influences. In addition, plant growth and development are often triggered or inhibited by different environmental conditions. Therefore, adaptations of plant morphology, anatomy, and physiological functions to changes in the biotic and abiotic domains can affect the accumulation of secondary metabolites. The SM pathway and its regulation are highly sensitive to environmental changes, as the expression of genes involved in the SM pathway can be altered by different stresses [44]. Most plausible hypotheses for SM biosynthesis have been published, as shown in Figure 1 and 2 [45].

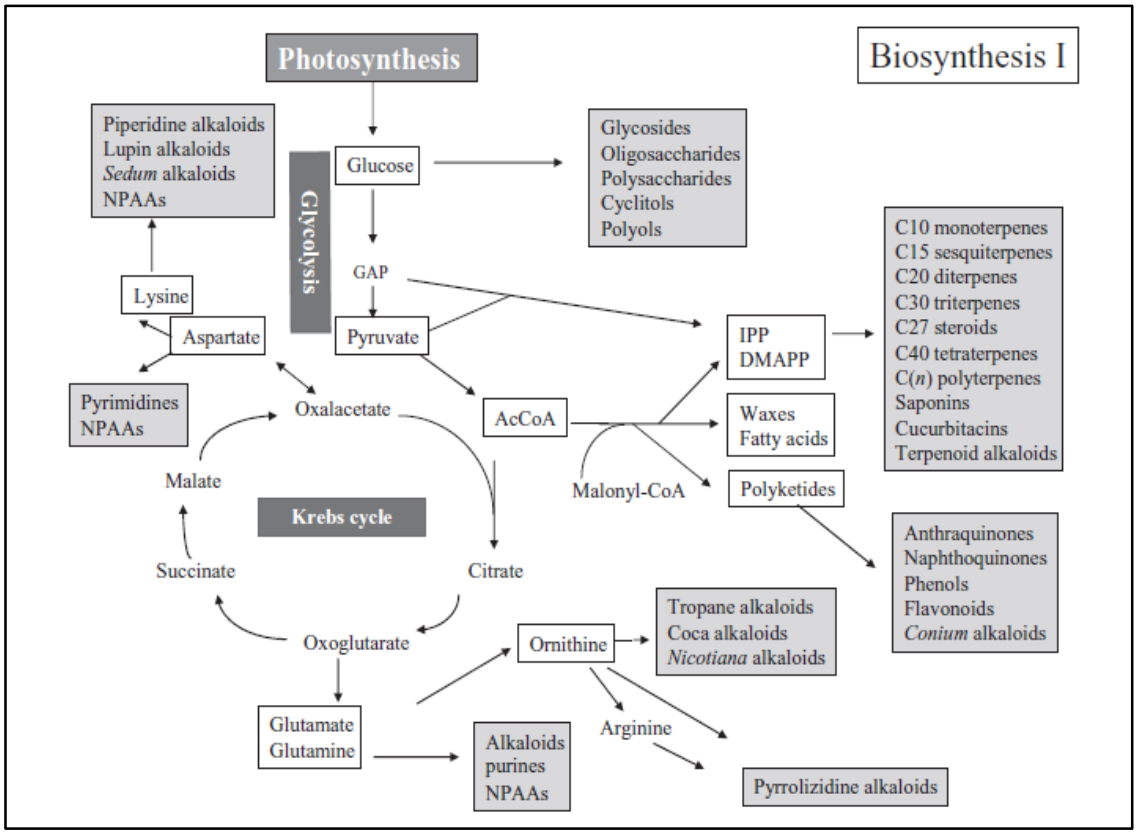

Figure 1: Main pathways leading to secondary metabolites

(IPP: isopentenyl diphosphate. DMAPP: dimethyl allyl diphosphate. GAP: glyceraldehyde-3-phosphate. NPAAs: non-protein amino acids. AcCoA: acetyl coenzyme)[45]

There are approximately 100,000 SMs in the plant kingdom, restricted to specific taxa. It is estimated that the isolated secondary metabolites are less than 10% of the total available in plants. Their classification can be based on chemical composition (with or without nitrogen), chemical structure (ring-containing, sugar containing), biosynthetic pathway (eg, tannin-producing phenylpropane), or their solubility. Phytochemicals are roughly classified into alkaloids, terpenes, saponins, and polyphenols according to their chemical structures, chemical properties and the presence of functional groups [50,51].

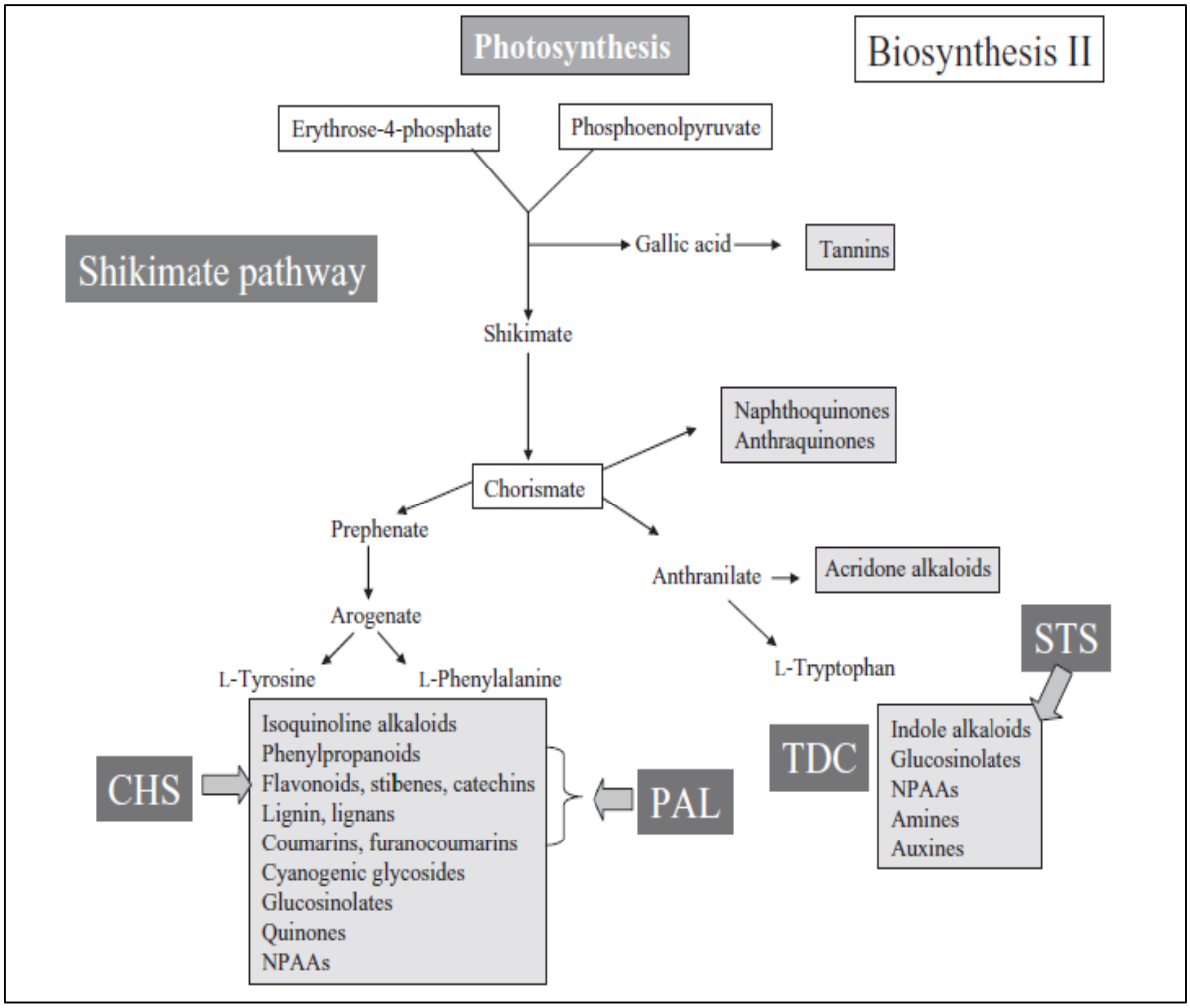

Figure 2: Several pathways of secondary metabolites derive from precursors in the shikimate pathway. *(*NPAAs: non-protein amino acids. PAL: phenylalanine ammonia lyase. TDC: tryptophan decarboxylase. STS: strictosidine synthase. CHS: chalcone synthase)[45].

I.1.3.1 Phenolic compounds:

Phenolic molecules are secondary metabolites, found ubiquitously in plants and in plant-based foods and beverages. Since phenolic compounds are frequently referred to as polyphenols, this term should only be used to refer to molecules with at least two phenolic rings. More than 8,000 different phenolic structures, ranging from simple compounds like phenolic acids to highly polymerized substances like tannins, are currently known. They are widely distributed throughout the plant kingdom and are the most prevalent secondary metabolites of plants. They are present in all plant organs and are thus an integral part of human nutrition. Phenolics are common constituents of plant foods (such as fruits, vegetables, grains, olives, beans, chocolate, etc.) and beverages and are responsible in part for the overall organoleptic characteristics of plant foods [46]. These compounds are often involved in the attraction of pollinators, the performance of structural functions, the defense against UV radiation and the protection of plants against microbial invasions and herbivores [47]. From a biogenetic perspective, phenolics are secondary metabolites derived from the pentose phosphate, shikimate, and phenylpropanoid pathways in plants [48]. Most phenolic compounds in plants are synthesized via the phenylpropanoid pathway[49].

Structurally, phenolic compounds contain aromatic rings bearing one or more hydroxyl substituents and range from simple phenolic molecules to highly polymeric compounds. The majority of naturally occurring phenolic compounds can be found as conjugates of monosaccharides and polysaccharides attached to one or more phenolic groups, but also as functional derivatives such as esters and methyl esters [48]. Although such structural diversity is the reason for the wide range of phenolic compounds found in nature. They can basically be categorized into several classes as shown in Figure 3 and Table 1.

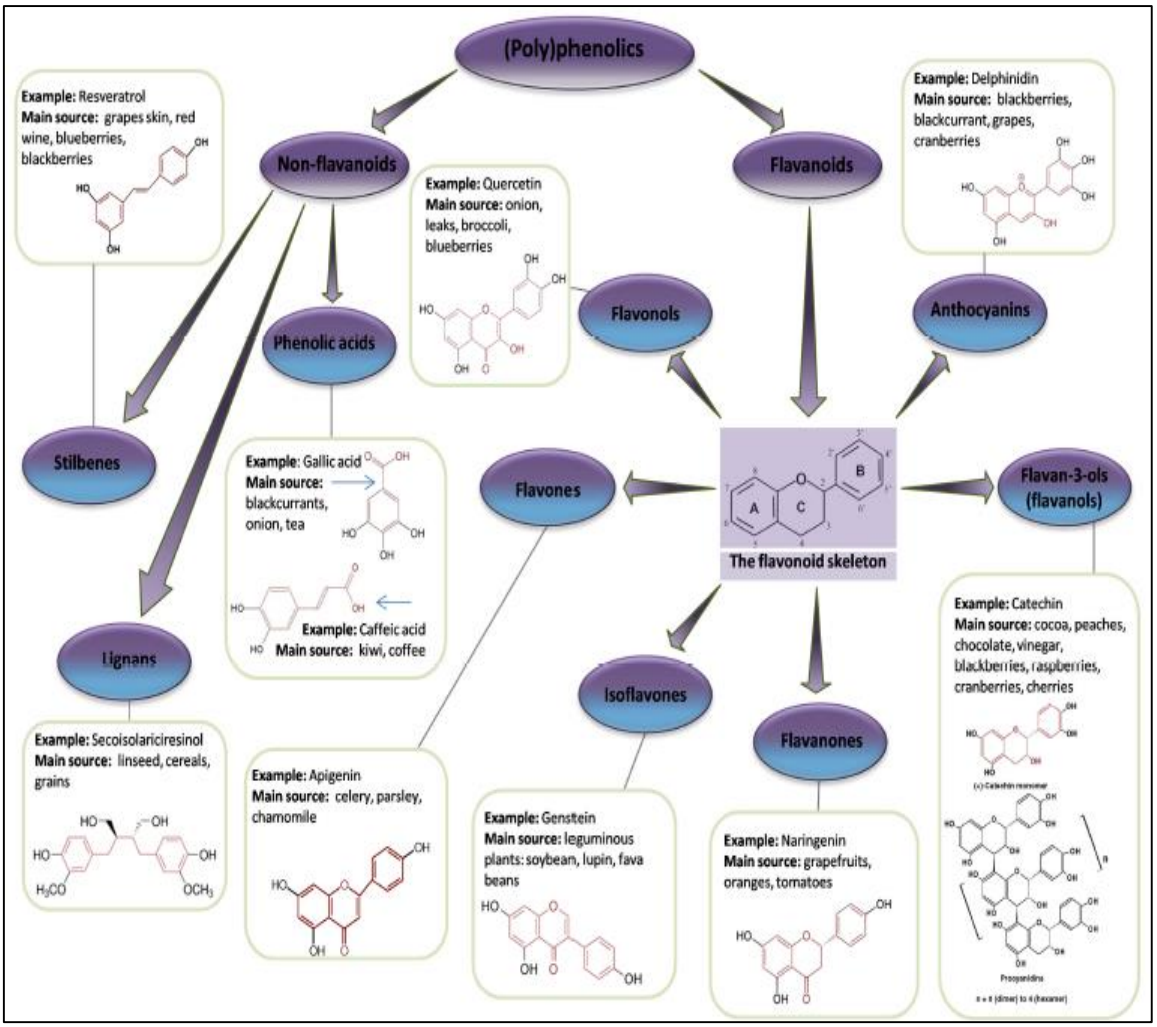

Figure 3: Phenolic compounds [50]

Table 1: Classes of phenolic compounds in plants [51]

Class	Structure
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	$C_6 - C_1$
Acethophenones, phenylacetic acids	$C_6 - C_2$
Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	$C_6 - C_3$
Napthoquinones	$C_6 - C_4$
Xanthones	$C_6 - C_1 - C_6$
Stkibenes, anthraquinones	$C_6 - C_2 - C_6$
Flavonoids, isoflavonoids	$C_6 - C_3 - C_6$
Lignans, neolignans	$(C_6 - C_3)_2$
Biflavonoids	$(C_6 - C_3 - C_6)_2$
Lignins	$(C_6 - C_3)_n$
Condensed tannins (proanthocyanidins)	$(C_6 - C_3 - C_6)_n$

I.1.3.2 Terpenes and terpenoids:

Terpenes are a diverse and large group of hydrocarbons that are produced by a variety of plants and some animals. These compounds are naturally occurring substances found in abundance in flowers, fruits and vegetables [52]. Terpenes are the largest class of secondary metabolites and are primarily composed of five-carbon isoprene units (many isoprene units) linked together in thousands of ways. Terpenoids are a class of modified terpenes with various functional groups and oxidized methyl groups moved or removed at different positions whereas terpenes are simple hydrocarbons. Terpenoids are classified based on the number of isoprene units they comprise; isoprene itself is synthesized and released by plants and consists of 1 unit and is classified as hemiterpenes; monoterpenes contain 2 isoprene units, sesquiterpenes contain 3 units, diterpenes contain 4 units, triterpenes contain 5 units, triterpenes contain 6 units, and tetraterpenes contain 8 units [53].

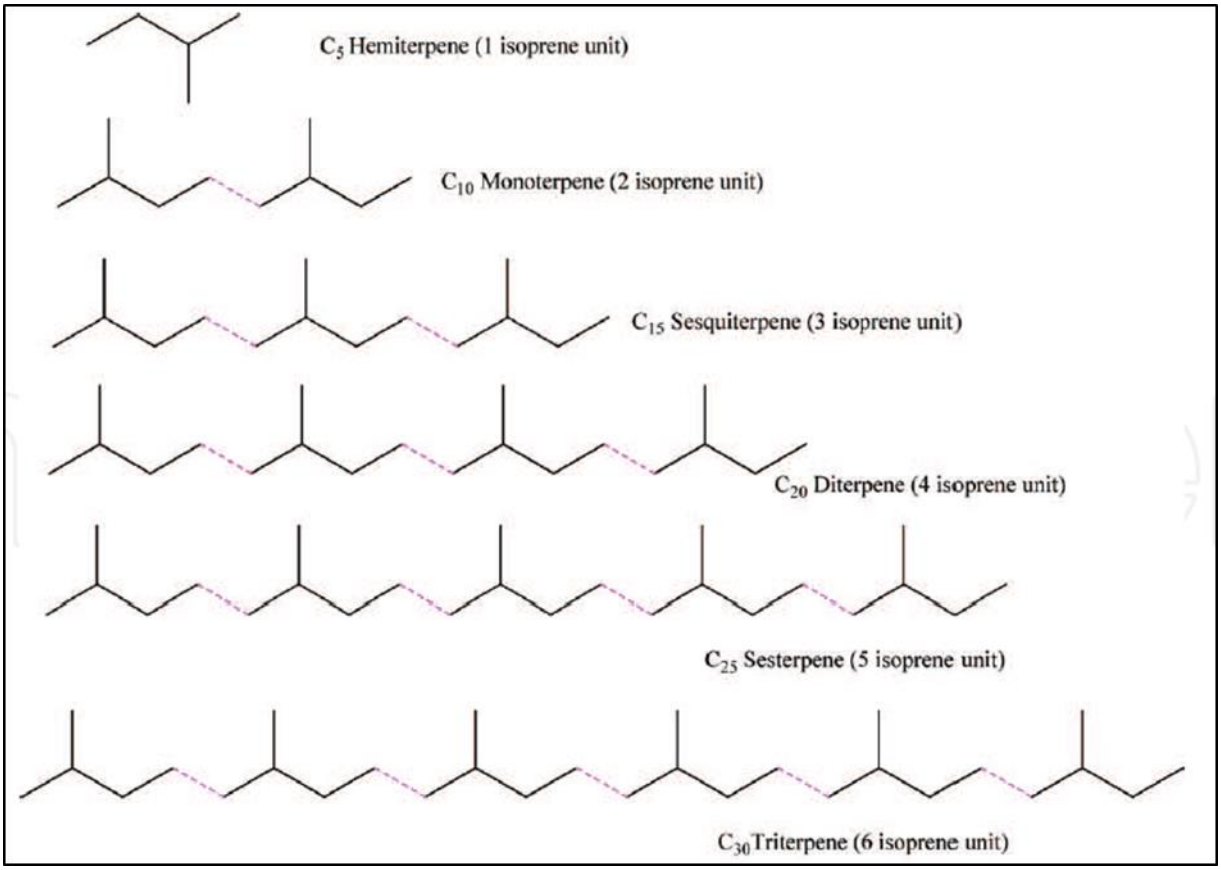

Figure 4: Classification of terpenes

As the biggest class of natural products, terpenoids are composed of approximately 25,000 different chemical structures and have potential practical applications in the flavors and condiments industry, especially in the pharmaceutical and chemical industries [54]. The diversity of structures and functions of terpenoids has caused an increased interest in their commercial application. Terpenoids have been discovered to have antibacterial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, antihyperglycemic, anti-inflammatory, and immunomodulatory characteristics, making them effective in the prevention and treatment of a number of illnesses, including cancer. They can also be used as natural pesticides and storage protection for agricultural products [55].

I.1.3.3 Essential oils and hydrosol extracts:

The volatile liquids produced by the secondary metabolism of aromatic plants are identified as essential oils. Since Antiquity, essential oils are known for their medicinal properties, and are very valuable and potent natural plant products. They are still of outstanding importance today. For thousands of years, essential oils have been used to flavor spices, food and beverages, or to heal the mind and body [56, 57]. Out of the 3000 essential oils (EOs) that

are now recognized, 300 are thought to be commercially significant and employed by the flavor and fragrance industries [58].

The word "essential oil" was first used in the sixteenth century and is derived from the medicine Quinta essentia, which was given that name by the Swiss physician Paracelsus von Hohenheim. Many authors have tried to define essential oils. The French Standardization Agency: Agence Française de Normalization (AFNOR) gives the following definition (NF T 75-006): "Essential oils are products obtained from plant raw materials by steam distillation or by mechanical processes from the epicarp of citrus or "dry" distillation. The essential oils are then physically separated from the aqueous phase [59].

Essential oils (Eos), also known as ether oils or volatile oils are only found in 10% of the plant kingdom. They are stored in plants in specialized brittle secretory structures such glands, secretory cavities, secretory hairs, secretory ducts or resin ducts. The overall essential oil content of plants is typically low and seldom exceeds 1%. However, in exceptional situations, such as clove (*Syzygium aromaticum*) and nutmeg (*Myristica fragrans*), it can reach more than 10% [60, 61]. Essential oils are non-polar or weakly polar hydrophobic aromatic oils that are soluble in alcohol and non-polar or polar solvents, waxes and oils, but only slightly soluble in water, with relative density less than 1, with a few exceptions (sassafras, cinnamon and vetiver). They can be liquid at room temperature, although some of them are solid or resinous and display different colors from pale yellow to emerald green and blue to dark brown-red [62, 63].

Different methods are used to extract essential oils: Expression, extraction with solvents, aqueous infusion, cold or hot pressing, effleurage, supercritical fluid extraction, steam-distillation, hydro-distillation, hydro-cum-steam distillation, and hydro-diffusion processes. The most commonly used method is the steam-distillation [64].

Essential oils are extremely complex mixtures of volatile components, and many of them contain around 20 to 60 different compounds, although some may contain more than 100 individual components. Gas chromatography and mass spectrometry of EOs or their headspace are used to analyze the chemical composition in detail. The main volatile components are hydrocarbons (such as limonene, pinene and bisabolene), alcohols (such as linalool, santoprolol), aldehydes (such as citral), cyclic aldehydes (such as cumin aldehyde), acids (such as benzoic acid, geranic acid), phenols (eugenol), phenolic ethers (anethole), ketones (such as camphor).), lactones (bergamot), oxides (1, 8-cineole), and esters (geranyl acetate). All these

compounds can be divided into two main groups: terpenoids and phenylpropanoids, or hydrocarbons and oxygenated compounds [65-67].

The chemical composition of an essential oil can be influenced by many factors. Numerous studies have shown that the chemical composition of oils, both quantitative and qualitative, varies depending on the technique of extraction. For instance, hydrodistillation and steam distillation processes produce oils rich in terpene hydrocarbons. In contrast, , a greater proportion of oxygenated molecules were present in the essential oils extracted by supercritical technique [68, 69].

The chemical composition of essential oils can vary depending on geographic location and growing conditions (soil type, climate, altitude, and water availability), seasonal changes, plant organs and plant maturity (e.g., before or after flowering), and the time of day when harvesting is accomplished.[70-72]. In addition, another important factor can severely affect the chemical composition of essential oils, which is the genetic composition of the plant. Therefore, the same plant species can produce a similar essential oil, but with a different chemical composition, resulting in different therapeutic activities. Such variations in chemical composition led to the notion of chemotypes [72].

Essential oils have several biological activities. In phytotherapy, they are used for their antibacterial, antiviral, antifungal, insecticidal, antimycotic, antioxidant, anticancer, anti-inflammatory, anti-staphylococcal, and antidiabetic effects. The mechanism of action of essential oils depends on the part of the plant and the different active substances they contain, which have different modes of action and therefore varying biological effects [73-76].

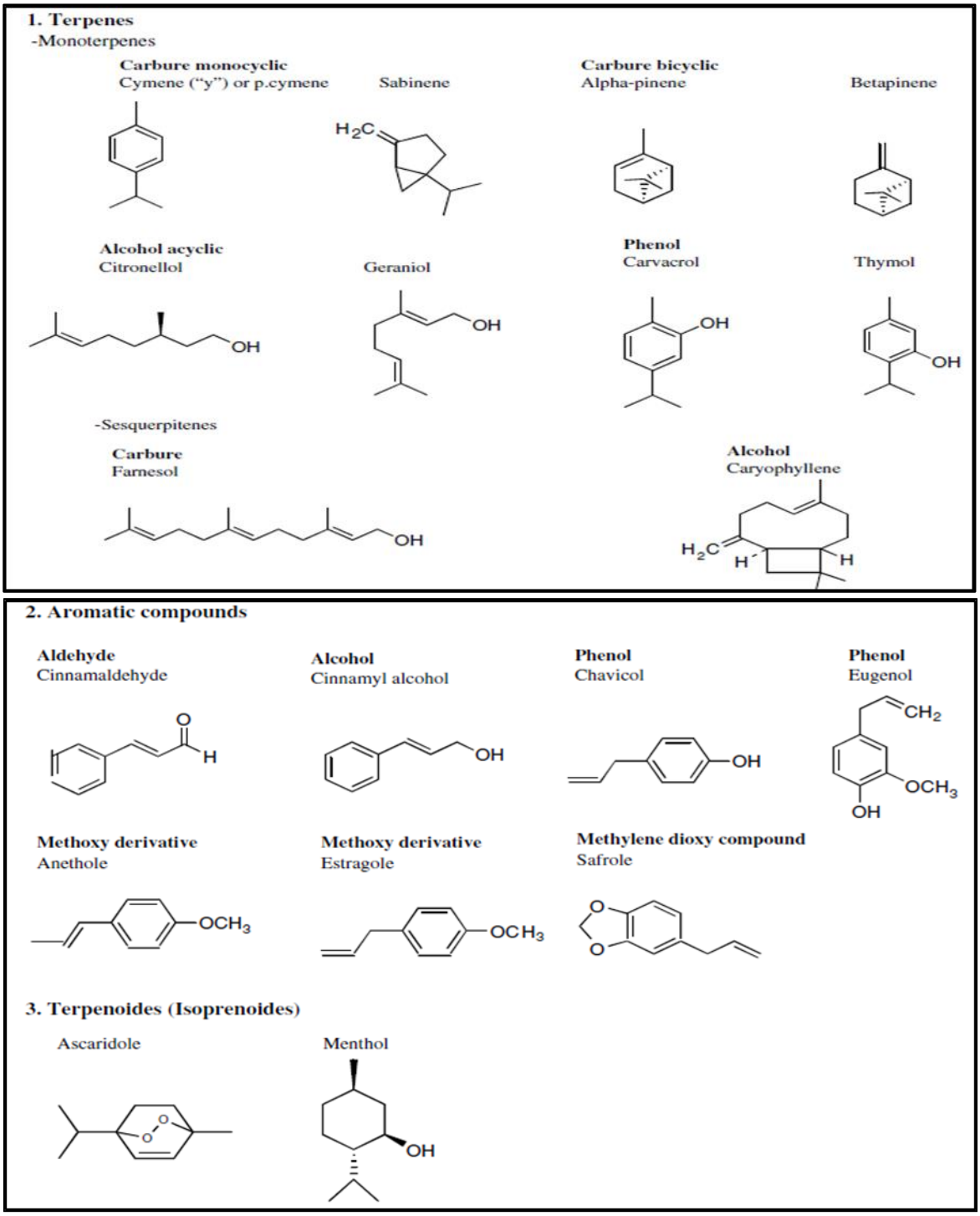

Figure 5: Chemical structures of selected components of essential oils

In general, the distillation of any aromatic plant yields two products: the essential oil (primary oil), which is the main one, and the hydrosol (water of distillation), which is the byproduct of the distillation process. In the field of aromatherapy, hydrosols are also referred to as floral waters, plant waters and hydrolates. During the process of distillation, the slightly

hydrophilic portion of the Eos remains dissolved in the aqueous phase and imparts hydrosols their fragrance. Generally, the essential oil fraction contained in the hydrosol is very aromatic and its composition is usually different from that of the primary essential oil. However, the main components are usually the same as those present in the oxygenated portion of the essential oil. As a result, hydrosols are very fragrant, highly flavored with pH values of 4.5 to 5.5. Although the main constituents of the hydrosols are the same as the constituents in lower concentrations in the oxygenated fractions of the corresponding Eos, they can be used as antioxidant, antibacterial, and antifungal agents in the cosmetic and food industries. Hydrolats can also be used in organic farming to combat phytopathogenic pathogens, molds and insects and for soil fertilization. Some researchers attribute the biological properties of hydrosols to the main compound and its functional groups [77-80].

I.2 Methods Used for Bioactive Compound Extraction:

The field of extraction of bioactive compounds (BACs) and their identification and production represents a huge wealth of opportunities for the food and pharmaceutical industries. It is important to choose the best extraction technique before isolating, purifying and characterizing bioactive compounds. In this sense, the extraction procedure is the most crucial step in the analysis of biologically active compounds from natural matrices. The process of extracting BACs from plant material may be challenging task due to the wide range of physiochemical properties of the BACs and the complexity of plant matrices (for example, many BACs are thermally unstable). Therefore, in order to produce high-quality extracts, it is it is technically imperative to identify and optimize the extraction parameters [81].

The term "extraction," as it is used in the pharmaceutical industry, refers to the typical processes used to separate medicinally active chemicals from plants using selected solvents. The objective of standard extraction procedures for the crude drugs (medicinal plant parts) is to obtain therapeutically desired compounds and to remove unwanted substances by treatment with selective solvents. During the extraction process, solvents attract soluble molecules, leaving behind insoluble materials [82]. The efficacy and efficiency of the chosen extraction methods have a significant impact on the extraction of bioactive chemicals from natural sources. All techniques used have common objectives: to isolate targeted bioactive compounds from complex plant samples; to improve the selectivity and sensitivity of the bioassay method by increasing the concentration of targeted compounds, to convert these compounds into a simpler form allowing quantitative and qualitative analysis, and to improve the reproducibility of the

studies regardless of sample composition variables. [83, 84]. The matrix characteristics of the plant materials, the solvent, the temperature, the pressure, and the time are the most frequent variables that affect the extraction process. Undoubtedly, by using the appropriate extraction techniques, the plant material (such as type of plant material, origin, degree of processing, moisture content and particle size), production equipment, extraction method (such as type of extraction, extraction time, flow rate, and temperature and pressure) and solvent (such as nature of solvent, concentration and polarity) as well as good manufacturing practices will result in an ideal extraction quality [83, 85].

Conventional extraction techniques are classical extraction process based on the solvation power of different solvents in use and the application of heat and/or mixing. the existing classical techniques are: Soxhlet extraction, Solvent extraction and hydrodistillation [86]. Soxhlet extraction is widely used to extract valuable biologically active compounds from various natural sources. It is used as a model for comparing new extraction alternatives. The principle of Soxhlet extraction (SOE) means frequent processing of fresh solvent with the sample after each solvent reflux cycle, followed by evaporation and solvent condensation at high temperature. Solvent extraction is a solid liquid extraction (SLE) or a liquid- liquid extraction (LLE) process. Solid liquid extraction relying on the migration of solid particles into the liquid by the principle of diffusion and mass transfer after the processing with the liquid solvent [87, 88].

A variety of solvents are commonly used to extract phytochemicals, and scientists often use dried plant powders to extract biologically active compounds while eliminating the interference of water. The chemical nature of the extraction solvent is of paramount importance in promoting the solubility of the compound, that is, the recovery and selectivity of the extraction [81]. Knowledge of such chemical properties forms the basis for the interactions between solvents and compounds, guiding the selection of appropriate solvents to maximize yield or purity of target molecules. In general, solvents used to extract biomolecules from plants are selected according to the polarity of the target solute. In this regard, a solvent of polarity similar to that of the solute will successfully dissolve the solute [89].

Various solvents can be used in sequence to limit the amount of similar compounds in desired yields. The polarity of some common solvents, from less polar to more polar, is as below: hexane < chloroform < ethyl acetate < acetone < methanol < ethanol < water [90]. It is reported that the most commonly used solvents are acidified methanol or ethanol. However,

because of its toxicity, methanol is not used in the food industry, and therefore ethanol is preferred for extraction [81].

Table 2: Solvents used for bioactive compounds extraction[91].

Methanol	Ethanol	Water	Chloroform	Ether	Dichloro-methanol	Acetone
anthocyanins Terpenoids Saponins Tannins Flavones Polyphenols	Tannins Polyphenols Flavonol Terpenoids Alkaloids	Anthocyanins Tannins Saponins Terpenoids	Terpenoids Flavonoids	alkaloids Terpenoids	terpenoids	Flavonoids

Liquid-Liquid Extraction (LLE) is a process that involves treating a liquid capable of dissolving one or more specific compounds with a nearly immiscible solvent. Therefore, the selectivity of liquid solvents due to density differences is crucial. At the end of the extraction process, two distinct phases are obtained. The first phase known as the extract, contains the desired compound, while the second phase known as the raffinate consists of a residue with a lower concentration of the compound of interest [92].

Hydrodistillation is a conventional method of extracting biologically active compounds and essential oils from plants. It does not involve organic solvents and can be carried out before dehydration of plant materials. Three types of hydrodistillation exist: water distillation, water and steam distillation, and direct steam distillation. Hydrodistillation includes three major physic-chemical process; hydrodiffusion, hydrolysis and thermal decomposition [83].

The main challenges and disadvantages of a classical or a conventional extraction technique are the long extraction times, the need for expensive and high-purity solvents, the evaporation of large quantities of solvents, the low extraction selectivity, and the thermal decomposition of thermolabile components. New and promising extraction approaches are introduced to address these limitations of traditional extraction methods. These methods are known as non-conventional or non-traditional extraction methods. Some of the most promising techniques include ultrasonic-assisted extraction, enzyme-assisted extraction, microwave-assisted extraction, pulsed electric-field-assisted extraction, pressurized liquid extraction and supercritical fluid extraction [93]. These innovative technologies offer great potential to significantly decrease or completely eliminate the usage of dangerous chemical solvents,

increase the effectiveness of the extraction process, and improve the yields and quality of the extract. These techniques are also referred to as "cold extraction techniques." because the temperature during extraction is relatively low and will not affect the stability of the extracted compound. The new technology can also be used as pretreatment or combined with environmentally friendly and safe organic solvents to improve extraction efficiency by enhancing cell membrane permeability, which is the parameter that determines extraction efficiency [94].

I.3 Isolation and Purification of Bioactive Molecules from Plants:

Since plant extracts often arise as a combination of different types of bioactive compounds or phytochemicals with different physical, chemical and biological properties, their isolation remains a major challenge in their identification and characterization process. Obtaining pure biologically active compounds with only one isolation procedure is not easy; more than one protocol is often used. In isolating these biologically active compounds, it is common practice to use a number of different separation techniques to obtain pure compounds. Pure compounds are subsequently used to determine their structure and biological activity. Various chromatographic techniques can be employed to separate and purify biologically active compounds. Different types of spectroscopic techniques such as UV-visible, Infrared (IR), Nuclear Magnetic Resonance (NMR), and mass spectroscopy can be used to identify the purified compounds [90, 95].

I.3.1 Chromatographic techniques :

Various chromatographic techniques have been used and most liquid chromatographic separations are performed using liquid-solid isolation techniques involving migration of the mobile phase through a stationary phase by capillary forces, such as various forms of planar and column chromatography.

Adsorption column chromatography is widely employed for separating natural products, especially at the preliminary separation stage, because of its simplicity, high capacity and low cost of adsorbents like silica gel and macroporous resins. The separation is primarily based on the difference between the adsorption affinities of the natural products to the surface of the adsorbents. The choice of adsorbent (stationary phase) and mobile phase is critical to achieve successful separation of natural products, to maximize recovery of target compounds, and to prevent irreversible adsorption of target compounds on the adsorbent [96]. The most commonly

used adsorbent is silica gel, which is employed for separating lipophilic and hydrophilic mixtures of substances. It is estimated that almost 90% of phytochemical separations (preparative scale) are performed using silica gel which is a polar absorbent with silanol groups. Molecules are retained by the silica gel via both hydrogen bonding and dipole-dipole interactions. Thus, the use of several mobile phases of different polarity is useful for high value separations [97].

I.3.2 High-performance liquid chromatography (HPLC):

High performance liquid chromatography is a chromatographic technique that separates mixtures of compounds. It is used in biochemistry and analytical chemistry to identify, quantify, and purify individual constituents of a mixture. The type of HPLC usually depends on the phase system being used in the process. These types include: normal phase high performance liquid chromatography (NP-HPLC), which employs a polar stationary phase and a non-polar mobile phase; Reversed phase HPLC (RP-HPLC) with an aqueous, moderately polar mobile phase and a non-polar stationary phase; size exclusion chromatography (SEC), also known as gel permeation chromatography, separates particles primarily based on size. This technique is widely used for molecular weight determination of polysaccharides. Ion exchange chromatography is another type of HPLC where the attraction between solute ions and charged sites bound to the stationary phase is the basis for retention. All ions of the same charges are excluded [98, 99].

I.3.3 Gas Chromatography (GC):

The GC is a chromatographic method that uses a carrier gas to transport the sample in the vapor state through a narrow column consisting of fused silica tubes (0.1-0.3 mm inner diameter) whose surface is bonded and cross-linked with a thin film of refined stationary phase films (0.1-5 µm) to improve thermal stability. The column is mounted in a temperature-controlled oven and can be slowly heated from ambient temperature to 350-450 °C for separation of a wide variety of compounds. The carrier gas is typically hydrogen or helium under pressure, and eluting compounds can be detected by a variety of means, including flame (flame ionization detectors), changes in carrier properties (thermal conductivity detectors), or mass spectrometry. The availability of "universal" detectors makes GC a suitable tool for the study of essential oils. On the other hand, GC is limited to molecules that exhibit sufficient stability and volatility to pass intact through the GC system at operating temperatures [100].

I.4 Structural Clarification of the Bioactive Molecules:

The final step in the process of natural product extraction is to unequivocally identify the specific molecule that is responsible for a given biological activity. The chemical composition can be determined using advanced spectroscopic techniques, including UV-visible, Infrared (IR), Nuclear Magnetic Resonance (NMR), and mass spectroscopy (MS), MS-MS, and LC-NMR-MS.

I.4.1 Nuclear Magnetic Resonance spectroscopy:

Chemists typically use nuclear magnetic resonance spectroscopy to study the chemical structures of simple molecules using simple one-dimensional (1D NMR) techniques. Two-dimensional techniques (2D NMR) are used for determining the structure of more complex molecules. Nuclei of many fundamental isotopes have characteristic spins. The isotopes commonly used in organic chemistry are ^{1}H and ^{13}C. NMR behaviors of ^{1}H and ^{13}C nuclei have been exploited by organic chemists as they provide valuable information that can be used to determine the structure of organic compound. One-dimensional proton spectroscopy (^{1}H NMR) is too complex or difficult to interpret in plant product analysis due to the strong overlap of most signals. By introducing an extra spectral dimension, these spectra are simplified and additional information is obtained. The invention of two-dimensional nuclear magnetic resonance was a major leap forward in nuclear magnetic resonance spectroscopy. The different types of 2D NMR experiments include the correlation spectroscopy (COSY), the total correlation spectroscopy (TOCSY), the nuclear enhancement spectroscopy (NOESY), the rotating frame nuclear enhancement spectroscopy (ROESY), the single quantum heteronuclear correlation spectroscopy (HSQC), and the multi-bond heteronuclear correlation spectroscopy (HMBC) [101, 102].

I.4.2 Mass spectroscopy:

The mass spectroscopy is an analytical technique in which charged particles (ions) are generated from the molecules of the analyte. The resulting ions are analyzed to obtain information about the compound's molecular weight and chemical structure. In mass spectrometry, organic molecules are bombarded with electrons or lasers, which convert them into highly energetic charged ions. A mass spectrum is a plot of the relative abundance of fragment ions versus the mass-to-charge ratio of those ions. Using mass spectrometry, molecular weights (molecular mass) can be determined with high precision, and precise

molecular formulas can be determined by knowing where a molecule has been fragmented [103]. Recently the combination of mass spectroscopy with other chromatographic techniques such as HPLC and GC facilitates rapid and accurate identification of chemical compounds in medicinal herbs.

I.5 Antioxidant and antimicrobial activity of phytomedicines:

I.5.1 Antioxidant activity:

The need to control oxygen levels has been recognized since the discovery of oxygen by Antoine Laurent Lavoisier in the early 18th century. Oxygen is an elusive molecule that plays contradictory roles, one essential to life and the other toxic. It is essential for the metabolism of organisms, the production of energy required for biological effects, and the purification of exogenous substances [104].

Free radicals are defined as molecules with unpaired electrons in outer orbitals. In general, they are unstable and highly reactive. They are produced in the human body as important mediators of essential processes mainly in neurotransmission and inflammatory responses, or as by-products with no role in the actual process. Examples of oxygen radicals are superoxide, hydroxyl, peroxyl radicals ($RO_2^{\bullet}$), alkoxyl radicals ($RO^{\bullet}$), and hydroperoxyl radicals ($HO_2\bullet$). Nitric oxide and nitrogen dioxide ($^{\bullet}NO_2$) are two nitrogen radicals. Oxygen and nitrogen radicals can be converted to other non-radical reactive species such as hydrogen peroxide, hypochlorous acid (HOCl), hypobromous acid (HOBr), and peroxynitrite (ONOO). ROS (reactive oxygen species), RNS(reactive nitrogen species), and reactive chlorine species are formed in animals and humans under physiological and pathological conditions [105, 106]. An overview of reactive oxygen and nitrogen species is given in Table 3 [104].

Table 3: Typical physiological reactive oxygen and nitrogen species

Radicals	Non- radicals
Reactive oxygen species (ROS):	
Superoxide ($O_2^{\bullet -}$) Hydroxyl ($HO^{\bullet}$) peroxyl ($RO_2\bullet$) alkoxyl ($RO\bullet$)	Hydrogen peroxide (H_2O_2) Hypochlorous acid (HOCl) Ozone (O_3) Singlet oxygen, ($^1O_2$)
Reactive nitrogen species (RNS):	
Nitric oxide ($NO^{\bullet}$) Nitrogen Dioxide ($NO_2^{\bullet}$)	Nitrous acid (HNO_2) Nitrosyl cation NO^+) Nitroxyl anion (NO^-) Peroxynitrite ($ONOO^-$) Alkyl peroxynitrites (ROONO)

The term "oxidative stress" is used to describe the condition of any disturbance in the equilibrium of reactive species (FRs, ROS, and RNS) and antioxidants in favor of the former as a result of a variety of factors, including aging, trauma, inflammation, pollution, poor diet, colds, infections, increase in oxidative metabolism, strenuous physical activity, radiation, toxicity, and drug actions [107]. The primary mechanism of action of free radicals is the perturbation of the balance of biological systems by damaging their major constituent molecules, leading to cell death. Free radicals cause their deleterious reactions by peroxidizing cell membrane lipids, leading to changes in membrane structural integrity and/or changes in the membrane microenvironment. They cause single-or double-strand breaks in nuclear and mitochondrial DNA and may also lead to DNA-protein cross-linking, leading to cellular damage and subsequent malignant transformation of cells [108, 109]. Therefore, damage caused by free radicals is often suspected contributes to the patho-physiology of a variety of diseases such as hypertension, asthma, atherosclerosis, , Alzheimer's disease, Parkinson's disease, liver disease, AIDS, blood diseases , rheumatoid arthritis, diabetes, Influenza, lung disease , myocardial infarction, radiation injury, autoimmune disease ,skin disease, , aging, cancer, kidney injury, heart failure, gastrointestinal disease, etc [108, 110, 111].

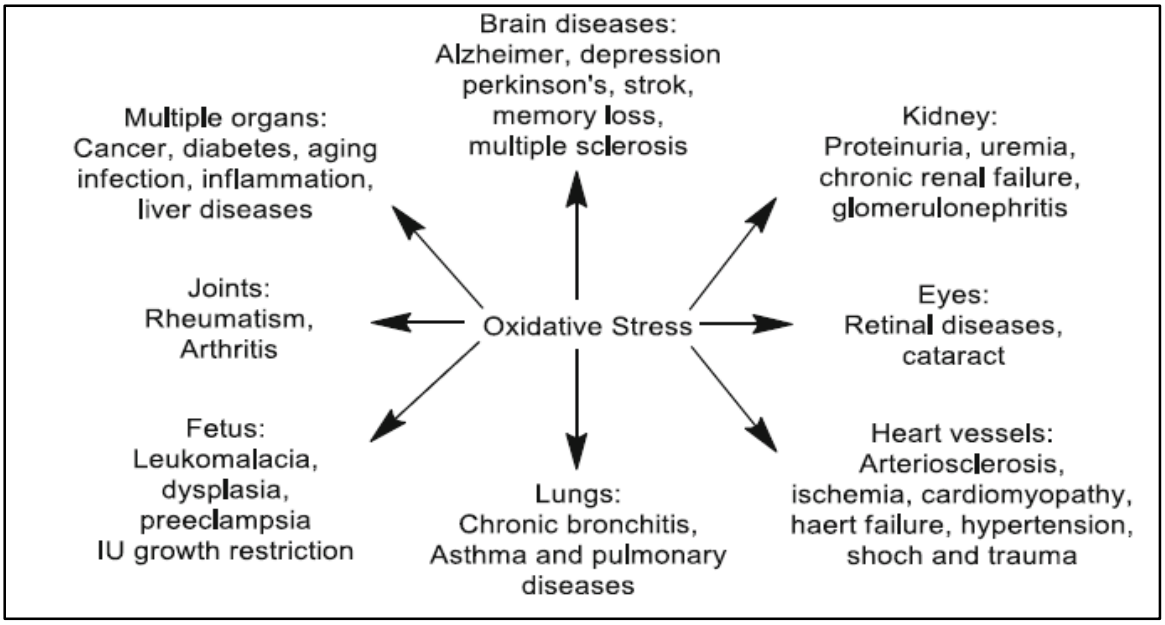

Figure 6:Various diseases associated with oxidative stress condition[112]

Fortunately, the human body has a complex antioxidant defense system that protects cellular components from damage caused by free radicals, reactive oxygen species, and other reactive species. Antioxidants have been defined as "any substance which, at a concentration lower than that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate". A more bio-relevant definition of antioxidants are synthetic or natural substances that resist oxidation or inhibit reactions mediated by oxygen or peroxides [113, 114]. Antioxidants that directly react with free radicals or some other reactive species to inhibit cellular components from being oxidized can be divided into enzymatic and non-enzymatic antioxidants, as shown in the table 4 [115]**.**

Consequently, the body contains an extensive network of antioxidants that can be chemically reduced by each other, thus reducing the reactivity of the formed antioxidant free radicals and restoring the reduced antioxidant for use in the body's defense against reactive species. In this manner, antioxidants act synergistically to destroy active substances [116-118]. Under normal conditions, the aforementioned endogenous antioxidant network provides sufficient protection against reactive species like ROS and RNS [119]. Nonetheless, a number of health claims have been made for using exogenous dietary antioxidants to reduce oxidative stress and related damage through supporting and strengthening the body's natural antioxidant defenses. Exogenous antioxidants mainly come from food and medicinal plants including fruits, grains, vegetables, flowers, mushrooms, beverages, spices and traditional medicines. These

natural plant-derived antioxidants are mainly phenolic compounds (such as phenolic acids, flavonoids, quinones, coumarins, lignans, stilbene, tannins), nitrogen compounds (alkaloids), vitamins (vitamins E and C) and terpenoids (including carotenoids) [120-122].

Table 4: Enzymatic and non- enzymatic antioxidants[115].

Enzymatic antioxidants	**Reaction**
Enzyme Superoxide dismutases (SOD) Catalases Glutathione peroxidases (GPx) **Non enzymatic antioxidants**	 $2\ O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$ $2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$ $2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$
Hydrophilic	Hydrophobic
Glutathione (GSH) E) Ascorbate (vitamin C) Uric acid	α-Tocopherol (vitamin E) Caretenoids Ubiquinol-10

Polyphenolic compounds are the most abundant antioxidants in the plants kingdom and have excellent ability to scavenge oxidative free radicals. Numerous earlier investigations established a link between antioxidant activity and total phenolic content in medicinal herbs, vegetables, and fruits, and they also found that phenolic compounds significantly influenced the antioxidant activity attained [123]. The chemical activity of polyphenols is related to their reducing properties as hydrogen or electron donors, suggesting their potential as free radical scavengers (antioxidants). Any antioxidant's activity is determined by: (a) its reactivity as a hydrogen or electron-donating agent, (b) The fate of the derived radical is controlled by its capacity to stabilize and delocalize the unpaired electron, (c) Its reactivity with other antioxidants, and (d) transition Metal-chelation potential. In vitro studies have demonstrated that polyphenols are more potent antioxidants than vitamins E and C because they have the appropriate structural chemistry for free radical-scavenging activity [124, 125]. Phenolic compounds are capable of neutralizing free radicals by donating electrons or hydrogen atoms to various reactive oxygen, nitrogen and chlorine species. As potent free radical scavengers, they can interrupt the propagating pathway of lipid autooxidative chain reactions, or act as metal chelators for converting hydroperoxides or metal prooxidants into stable compounds [126].

The antioxidant capacity of phenolic compounds has been related to their chemical structures. Some phenolic molecules, such as flavonoids, phenolic acids, and tannins, have been studied for the relationship between their chemical structure and their activity. It was found that generally the free radical scavenging and antioxidant activities of phenols mainly depend on the number and position of hydrogen-donor hydroxyl groups on the aromatic ring of phenolic molecules, and are also affected by other factors, such as aglycones glycosylation. , other donor groups (-NH, -SH). For example, flavonol aglycones with many hydroxyl groups, such quercetin, myricetin, and kaemperol, exhibited stronger antioxidant activity than their glycosides, like myricitrin, rutin, and astragalin [123, 127].

Essential oils are other plant components that are considered powerful natural antioxidants. They can act as natural antioxidants in various modes such as prevention of chain initiation, free radical scavengers, reducing agents, termination of peroxides, quenchers of singlet oxygen, catalysts for the synthesis and binding of transition metal ions. The antioxidant activity of essential oils is largely determined by their chemical composition. However, due to the wide variety of compounds, their antioxidant effects cannot be attributed to a single mechanism of action. However, in order to facilitate the study of the antioxidant effects of essential oils, some researchers have linked the antioxidant activity of the main constituents to the overall activity of the essential oils, such as eugenol in cloves, carvacrol in oregano, metathyme in thyme Citronellol or beta-citronellal in phenols and beta-citronella [128, 129].

Despite the enormous chemical diversity observed, the major constituents of common essential oils can be divided into two structural families in terms of the hydrocarbon backbone: terpenoids and phenylpropanes [130]. Numerous studies in this field have provided some conclusions on the hydrogen-donating or free-radical scavenging activities of monoterpenes and diterpenes and their effectiveness in inhibiting lipid peroxidation. Carvacrol, Thymol, Menthol, (γ)-Terpinene, Linalool, Terpinolene, 1,8-Cineole, Isopulegol, ρ-Cymene, Alpha-terpineol and terpinen-4-ol are various monoterpenes with antioxidant activity (figure7) [112, 130].

The difference in antioxidant activity of different essential oils is mainly due to the type and quantity of the major antioxidant components in the essential oil, the method of extraction used and the time of plant harvesting, that determine the concentration of the main components of the oil [156].

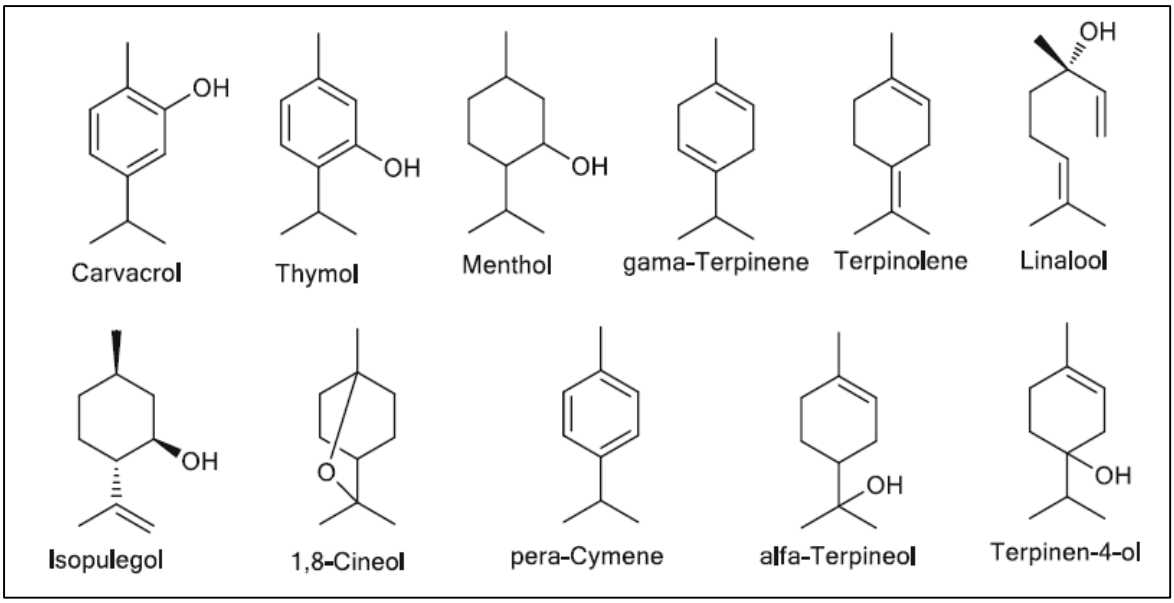

Figure 7: Different volatile monoterpenes that have antioxidant potential

Today, studies on antioxidants in plants and foods are one of the most important topics in the field of food and agriculture. The different antioxidants present in nature have different mechanisms of action. As a result, many tests for the study of antioxidant activity have been developed and implemented. These antioxidant tests can be divided into two main groups: *in-vitro* and *in-vivo* tests. *In-vitro* antioxidant methods are generally simple and relatively easy to perform compared to *in-vivo* methods. Such *in- vitro* methods can be categorized into different groups according to their mechanism of action, such as simple electron transfer (SET) based methods, hydrogen atom transfer (HAT) based methods, mixed approaches (with both HAT and SET mechanisms), chelation and lipid oxidation based methods, and various methods. Table 1 in annex 1 shows the classification of the different *in-vitro* antioxidant assays into different groups based on mechanism of action [112, 131, 132]. *In-vivo* methods are generally carried out by administering samples containing natural antioxidants to laboratory animals, like rats, mice and rabbits, at a defined dose as shown in the corresponding method. After a predetermined period, the animals are usually sacrificed and the blood, blood serum and tissue are used for the study. For this reason, *in-vivo* methods are more difficult to perform than *in-vitro* methods. Currently, about ten types of in vivo methods are used, namely plasma iron reducing power, glutathione peroxidase (GSHPx) estimation, reduced glutathione (GSH) estimation, glutathione reductase (GR) test, glutathione-S-transferase (GSt), lipid peroxidation test (LPO), superoxide dismutase test (SOD), low-density lipoprotein (LDL) test, catalase method (CAT), and gamma-glutamyl transpeptidase (GGT) activity test [112, 131].

I.5.2 Antimicrobial activity:

Worldwide, microbial infections cause millions of deaths each year. Antimicrobial resistance is now one of the greatest challenges in global public health. The emergence of multidrug-resistant isolates in many infections, commonly referred to as diseases of poverty, has caused devastation in developing countries [133, 134]. The most prevalent multidrug-resistant bacteria are methicillin-resistant Gram-positive bacteria, *Staphylococcus aureus*, enterococci, and Gram-negative bacteria: members of the Enterobacteriaceae and others such as *Pseudomonas aeruginosa*, *Mycobacterium tuberculosi*s. The most common multidrug-resistant fungal pathogens, belong to the genera *Candida, Aspergillus, Rhizopus, Penicillium, Fusarium, Cryptococcus* and *Mucor,* and are highly resistant to antifungal agents [73, 135].

Antibacterial resistance mechanisms mainly include activation of the efflux pump, destruction of antibacterial agents by destruction enzymes, modifying antibiotics with modifying enzymes and altering target structures in the bacterium that have a lower affinity for antibacterial recognition [133, 136]. Figure 8 summaries major resistance mechanisms.

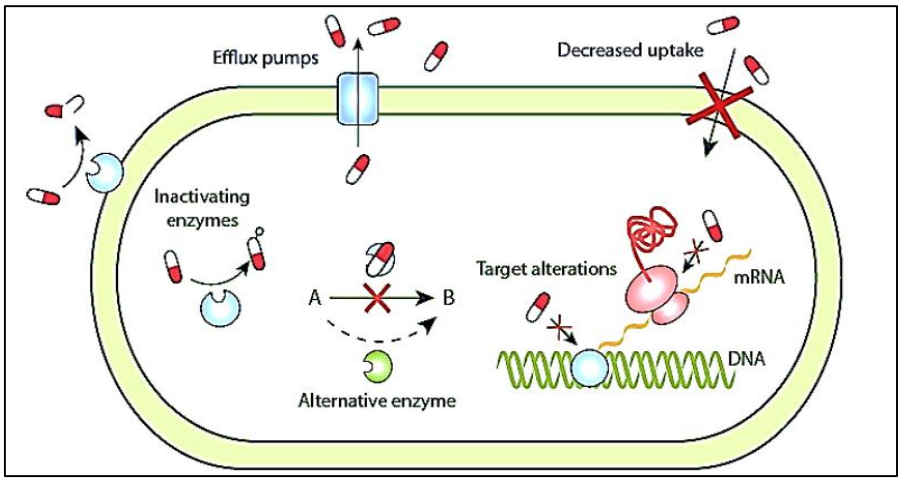

Figure 8: Schematic diagram of major antibiotic mechanisms of resistance[137].

Invasive fungal infections pose an ongoing and serious threat to human health and are associated with at least 1.5 million deaths worldwide annually [138]. The literature estimates a mortality of 30-40% for invasive candidiasis, 20-30% for disseminated cryptococcosis and a similar percentage for invasive aspergillosis. These infections are very common in immunocompromised patients following aggressive therapies (like cancer chemotherapy, long-term corticosteroid therapy and organ transplantation) and immunosuppressive infections such as HIV/AIDS. Approximately 90% of these deaths are attributed to species belonging to the

fungal genera *Candida, Aspergillus, Cryptococcus, Pneumocystis, Mucor* and *Rhizopus* [138, 139].

Although there are many antifungal agents in clinical use, none of them fulfills all the requirements to be a fully effective, nontoxic antifungal drug, and all have significant drawbacks. Fungal resistance leads to clinical failures of antifungal chemotherapy that render fungal infections extremely difficult to eradicate. As with antibacterial, resistance can be classified as microbiological and clinical and in turn, resistance can be either primary (intrinsic) or secondary (acquired). Strains of the genera *Candida, Cryptococcus* and *Aspergillus* are the most likely to develop resistance.[139, 140].

The scientific community and public health authorities are currently under pressure to provide effective plans and techniques to address the antimicrobial resistance dilemma, and it is now vital to enforce and put into action concrete measures to reduce this drug resistance problem. Controlling and rationalizing the use of antibiotics, identifying and understanding the genetic mechanisms underlying drug resistance, discovering new antimicrobial compounds from medicinal plants, and combining antibiotics with non-antibiotic medications and with natural naturally occurring antimicrobial compounds are a few of the potential strategies that have been suggested. It is hypothesized that certain plant extracts, with different target sites than conventional antibiotics, will have improved activity against microbial pathogens that are usually resistant to antibiotics without the same side effects [141]. Furthermore, according to previous published reports, potentiation of antimicrobial activity by a natural product can be attained by several mechanisms including: a multi-target effect, in which each compound targets a different site in the microbial cell; pharmacokinetic or physicochemical effects by improving the solubility and bioavailability of the antimicrobial drug and the targeting of a specific resistance mechanism of the microorganisms, which is the main challenge in combination therapy [142].

On a global scale, phytochemicals have been tested as possible sources of new antimicrobial compounds, food preservation agents, and alternatives for treating infectious disorders because of their antifungal, antibacterial, and antiviral properties. The main classes of secondary metabolites found in plants that have antimicrobial properties are phenols, phenolic acids, flavonoids, quinones, tannins, saponins, coumarins, terpenoids, and alkaloids. The structural and chemical variations of these compounds result in differences in their antimicrobial action. The impact of the antibacterial action that plant-derived chemicals

produce against microbes depends on the vast structural variety of these compounds. The impact of the antimicrobial action that plant-derived chemicals produce against microbes depends on their vast structural configuration [143, 144].

Essential oils have been reported to have substantial antibacterial, antifungal, antiseptic, antiviral, antiparasitic, and insecticidal properties. An important characteristic of essential oils and their constituents is their hydrophobicity, which allows them to partition with lipids found in bacteria and mitochondrial cell membranes making them more permeable by disturbing the cellular structures. This ultimately leads to the death of the bacterial cell due to the massive leakage of critical molecules and ions from the bacterial cell to a considerable extent. Other common impacts include cell content leakage, loss of motive proton force, coagulation of the cytoplasm, and damage to membrane proteins (such as enzymes). Some essential oil constituents are shown to modulate drug resistance through targeting the efflux mechanisms of several Gram-negative bacteria. Inhibition of enzyme production or activity may also be a target of Eos [145, 146].

EO components derived from terpenes and their oxidized derivatives have been extensively tested *in-vitro* for their antimicrobial potency. The results obtained showed an effective inhibition of bacterial growth with variable amplitudes. Gram-negative bacteria are generally less sensitive than Gram-positive bacteria. The outer membrane of Gram-negative bacteria contains hydrophilic lipopolysaccharide (LPS), which forms a barrier to macromolecules and hydrophobic compounds, making Gram-negative bacteria more resistant to hydrophobic antimicrobial compounds such as those found in Eos [147].

With regard to the antifungal activity of essential oils, it has been found that when essential oils and isolated components are tested for fungi-static activity, it appears that such activity is directly related to the chemical composition of essential oils. The mechanisms of antifungal activity of thymol and carvacrol has been little studied, but researches have shown interactions with the cellular envelope and intracellular targets. In *Candida* strains, they damage vesicles and cell membranes and impair ergosterol biosynthesis, thereby compromising membrane integrity, as ergosterol regulates membrane fluidity and asymmetry, similar to cholesterol in animal cells [147, 148].

Phenolic compounds demonstrated a variety of mechanisms of action when used against various strains of microorganisms, ranging from synergistic activity via inhibition of the efflux pump, interacting with the cell membrane, and inhibition of cell wall biosynthesis to inhibition of a few key enzymes like urease, sortase A, and dihydrofolate reductase [133]. The inhibitory action is thought to be caused by the hydroxyl (-OH) groups of phenolic compounds, as these groups may interact with the cellular membrane of bacteria to disrupt the membrane structures and cause the leakage of cell components. An active group like -OH promotes electron delocalization which in turn acts as proton exchangers and reduces the gradient through the bacterial cell cytoplasmic membrane. This causes the proton motor force to collapse and the ATP reserve to deplete, ultimately leading to cell death. In addition, it has been reported that these -OH groups may easily bind to the enzyme active site altering the cellular metabolism of microorganisms. This effect demonstrates the importance of -OH groups in antimicrobial activity. In addition to this, phenolic compounds act as antioxidants by the suppression of the production of reactive oxygen species and by the scavenging free radicals, thereby reducing the redox potential of the growth medium [143, 149].

To assess or screen the in vitro antimicrobial activity of an extract or a pure chemical, a range of laboratory techniques can be applied. The disk-diffusion and broth or agar dilution procedures are the most well-known and fundamental techniques. In many clinical microbiology laboratories, the Agar disk-diffusion and Agar well diffusion procedures are frequently employed for standard antibiotic susceptibility testing. The best procedures for determining the minimum inhibitory concentration values (MIC) are dilution methods because they provide the possibility of estimating the concentration of the antimicrobial agent tested in the agar (agar dilution) or broth (microdilution) medium. Other techniques such as flux cytofluorometry and bioluminescence are not frequently used as they require specific equipment and subsequent re-evaluation for reproducibility and standardization, although they can provide rapid results on the effects of the antimicrobial agent and a deeper comprehension of their impact on cell viability and damage to the microorganism tested [150, 151].

I.6 Mouthwash as oral care product:

The buccal cavity most commonly known as the mouth or the oral cavity is the first part of the digestive system. It is made up of several anatomical aspects that work effectively and efficiently together to perform a number of different functions [152]. Its main function is to serve as an entrance to the digestive tract and to start the process of digestion by salivating and propelling the food bolus into the pharynx. In addition to the vital role that the mouth plays in the digestion and absorption, it is also critical for normal breathing, taste, speech formation, exchange, and pleasure. The mouth also plays an essential role in facial appearance and expression [153].

A good oral health refers to a person's capacity to adapt to physiological changes during their lifetime and to maintain their teeth and mouth by taking care of themselves. Oral health, according to the World Health Organization (WHO), is the absence of chronic mouth and facial pain, oral and throat cancer, oral sores, birth defects like cleft lip and palate, tooth decay and tooth loss, periodontal (gum) disease, and all other diseases and disorders affecting the oral cavity [154, 155].

Oral diseases represent a prominent global public health concern, with high prevalence and significant detrimental effects on people's lives, communities, and society. According to the 2017 Global Disease Burden Study, 3.5 billion individuals worldwide, or approximately 50% of the population, had an oral disease in 2016 [156]. In general, oral diseases are characteristically chronic and progressive in their nature. They typically start in early infancy and progress through adolescence, maturity, and later life [157]. The most common oral diseases are periodontal diseases, gingivitis, dental caries, oral candidiasis, oral mucosal lesions, tooth loss and oro-dental trauma. Besides, bad breath, dry mouth and halitosis can also be regarded as buccal disorders.

Like other surfaces of the body, various microorganisms from birth colonize the human oral cavity. The microorganisms that live in the human oral cavity are known as the "oral microbiome," "oral microbiome," and more recently, "oral microbiome." This is a complex ecological environment with as many as 750 recognized microorganisms, of which bacteria are the most common group of microorganisms, archaea, protozoa, fungi and viruses. The oral microbiota maintains a symbiotic relationship with the host, growing on surfaces as a community of interacting species organized structurally and functionally, known as dental plaque[158, 159].

Although the study of plaques has been going on for over a hundred years, the consideration of plaques as biofilms and microbial ecosystems is nearly new. The formation of a biofilm is a step-by-step process that begins with the adhesion of planktonic microorganisms to a surface. During this stage, the surface of the teeth is covered with a conditioning film composed of proteins and glycoproteins that provides binding sites for adhesion of the first bacterial colonizers of the dental surface. The next steps are colonization and co-adhesion, growth and maturation. During the maturation step, a matrix is formed consisting of bacterial polymers that are excreted into the external environment. It keeps the biofilm together, helps retain it on the surface and provides increased resistance to antimicrobial agents. It has been found that when microbes were organized into biofilms, they were less susceptible to antimicrobial agents and more resistant to immune defense mechanisms. The concentration of an antimicrobial agent that is effective against planktonic microorganisms must be increased by a factor of 10 to 1,000 to have the same effectiveness on microorganisms in a biofilm [158, 160, 161]. Therefore, effective biofilm control is essential to maintain oral health.

Healthy oral tissues require the maintenance of good oral hygiene, which is achieved through regular home oral hygiene routines and using various oral care products. Oral hygiene products include a wide range of formulations and devices primarily produced to improve oral health. They can be categorized as follows: toothbrushes (manual, electric, or wooden sticks), interdental cleaning aids (dental floss, interdental brushes, single-tuft brushes, and wooden sticks), toothpaste (dentifrice), mouthwashes, sprays, and gels [162]. Recommendations from dental practitioners on oral hygiene practices have focused largely on mechanical methods of daily oral care, such as brushing and interdental cleaning as the standard to achieve and maintain a healthy oral cavity. However, several studies have reported that mouthwashes may provide greater benefit than just mechanical oral hygiene in the prevention of different oral disorders [163].

Particularly, mouthwashes are frequently used in conjunction with traditional teeth brushing in order to try to decrease oral plaque accumulation. In recent decades, the use of mouthwashes has become usual, especially after the mechanical control of plaque biofilm. Given that they are simple to use, reduce plaque biofilm, and freshen breath, mouthwashes are appreciated by the public and they provide an ideal medium for incorporating chemicals [164].

Mouthwashes are defined as non-sterile aqueous solutions primarily used for deodorant, refreshing or antiseptic properties. Mouth rinses are usually classified as cosmetic or therapeutic

or as a combination of these two. Cosmetic mouthwash is a commercial product that removes oral debris before and after brushing, suppress bad breath temporarily, reduces oral bacteria, and leave the mouth fresh with a pleasant taste. Therapeutic mouthwashes often have the same benefits as their cosmetic counterparts, but also contain an additional active ingredient (such as fluoride or chlorhexidine) that helps prevent certain oral diseases. The amount of each ingredient in mouthwash varies from one product to another. Some have almost the same composition as toothpaste, even though they do not include abrasives. Unlike toothpastes, most mouth rinses contain alcohol, as a preservative and as a semi-active ingredient [165]. In general, mouthwashes are prescribed for two different purposes, one is to maintain oral health in patients with good hygiene and no acute or chronic alteration of the local and organismal immune defenses, and the second is to cure various diseases in many clinical situations at different therapeutic and prophylactic purposes [166].

Mouthwashes contain active and inactive ingredients. The active ingredients are the ingredient that provide therapeutic effect, such as antiseptics and disinfectants, whereas the inactive ingredients are non-therapeutic components that provide physicochemical properties, such as pH, feel, taste, sweetness and appearance. Typically, the formulation of a mouthwash is a combination of several chemically active agents, and the final product should have the following properties: effective against pathogenic microorganisms, can disrupt the effect of dental plaque without affecting the normal, healthy oral flora, safe for humans and the environment, having minimal and reversible side effects and pleasant taste [167, 168].

The most commonly used pharmacological antimicrobial agents in mouthwashes formulation are chlorhexidine, hexetidine, fluoride compounds (FC), delmopinol, cetyl pyridinium chloride, triclosan, octeneidine, polyvinylpyrrolidone (PVP), Oxygenating agents, hyaluronic acid (HA) essential oils and natural compounds.

Chlorhexidine-based formulations are considered the "gold standard" antiplaque mouthrinses because of their long-term, broad-spectrum antimicrobial activity and the potential for plaque inhibition. CHX is a broad-spectrum antiseptic agent effective against yeast, viruses gram-positive and gram-negative bacteria. Because CHX mouthwash's activity is pH dependent, it need to be used for a minimum of 30 minutes after any other dental care products. CHX used for a long period is associated to local side effects, including transitory taste changes (dysgeusia) and discoloration of the teeth, tongue, and restorative materials, which adversely impacts patients' compliance [167, 169].

Today, oral microorganisms have become resistant to synthetic chemicals and antibiotics. As a result, many antibiotics and synthetic oral care products have reduced clinical efficacy, particularly in people with HIV/AIDS. Additionally, synthetic drugs can cause nausea, diarrhea, and tooth discoloration, among other unpleasant side effects. Furthermore, the daily use of mouthwashes containing chemical agents negatively affect the oral mucosa, damage the cell membrane and break the double helix, resulting in DNA damage. Due of these drawbacks, plants and other naturally occurring antibacterial chemicals are already gaining popularity as beneficial antimicrobial alternatives that can be added to toothpastes and mouthwashes [170, 171].

In field of dentistry, phytomedicines are used as anti-inflammatory agents, analgesics, antibiotics, sedatives, and as an endodontic irrigant. There is a long history of using natural compounds from plants, animals, microorganisms and marine organisms to treat oral diseases and many natural product mouthwashes (NCCMs) have been shown to be effective in treating periodontal disease. Natural compound-containing mouthwashes (NCCMs) are divided into three categories: (1) Product containing components from a single natural product, (2) product containing compounds from multiple natural products, and (3) product containing both natural compounds and additional active chemical agents. While the chemicals ingredients control plaque and gingivitis through antimicrobial mechanisms, certain natural compounds possess additional anti-inflammatory and antioxidant effect that are beneficial to gingival health [172, 173].

Currently, mouthwashes containing essential oils (EO) are widely used as NCCMs by both orthodontic patients and the public. For many years, essential oil (EO) mouthwashes have been used as a supplement to brushing to maintain oral hygiene. In the literature, their effectiveness in reducing plaque and gingivitis is well established. They not only reduce plaque gingivitis, and bad breath but they can also reach areas that are hard to access or highly prone to plaque formation [174, 175]. The most commonly used essential oils are thyme oil, peppermint oil and eucalyptus oil. Therefore, many essential oil-based mouthwashes solutions are available. The most well-known of these is Listerine, which contains eucalyptol (0.092%), thymol (0.064%) menthol (0.042%) and methyl salicylate (0.060%) as active constituents. eucalyptol has antibacterial and antifungal effects, thymol acts as antiseptic agent and menthol is recognized for its local anesthetic and anti-irritant properties [176, 177].

In addition to essential oils, medicinal plants are rich in a variety of secondary metabolites that have been found to have antiplaque and anti-gingivitis properties. Phytoconstituents such as, terpenes, flavonoids, tannins and phenolic acids play a significant role in the biological activity of plants and may have positive effects on dental health. Their efficacy against oral diseases is mainly due to their anti-inflammatory, antimicrobial and antioxidant properties [172].

It is important to ensure that the formulated liquid mouthwash has a pleasant mouth feel during use and must have a pleasant taste to maintain customer acceptance. Based on the requirements, there are some basic properties for formulating mouthwashes, provided by various excipients such as surfactants humectants, solubilizers, flavoring agents, preservatives, solvents, colorants and antifoaming agents, as required [178].

The purpose of using humectants in the formulation of mouthwash to prevent water separation and evaporation. The most common humectants used in mouthrinses formulations are polyalcohols like glycerol, glycerin, , sorbitol, polyethylene glycol, propylene glycol and xylitol [165].

The surfactant excipients act as a solubilizer, wetting, emulsifier, dispersant agent and viscosity control agents. The most commonly used is an anionic surfactant which is the Sodium lauryl sulfate (SLS). In general, nonionic surfactants such as tween and poloxamer 407 are preferred in mouthwash formulations because of their low foaming capacity [165, 179].

The most common solvent used in mouthwash is water. It dissolves all other excipients and enables them to be thoroughly mixed. Preferably, the water used in mouthwashes is deionized, distilled, free from organic impurities and bacteria and essentially free of metal ions. Water makes up between 50% and 90%, preferably between 70% and 85% of the mouthwash compositions [180].

Hydroalcoholic mixtures are commonly used as carriers or solvents in mouthrinse formulations. Pharmaceutical grade denatured alcohol or ethanol is the most widely used. Isopropanol may also be used. Most mouthwashes are classified as alcohol-containing mouthwashes if they contain 6% to 26.9% alcohol. Typically, alcohol is used in mouthwashes to boost flavor effect, solubilize flavor and some active chemicals, act as a preservative, and enhance the delivery of the active ingredients into the plaque biofilm. The benefits of ethanol include its preservative and antiseptic properties, low cost and its ease of production. However, alcohol mouthwashes are counter-indicated for use by children, patients with mucositis, those

with dry mouth, immunocompromised patients, alcohol sensitive patients, and patients with composite restorations. There is concern that the alcohol in the mouthwash could be converted to acetaldehyde in the buccal cavity, which could damage DNA and lead to mutations [162, 164, 181]. The researchers also showed that ethanol, which is used as a solvent in most alcohol-based mouthwashes, caused surface softening and increased wear of dental resins and composites. People who use mouthwash with more than 25% alcohol on a daily basis are reported to have a higher risk of oral cancer. Recently, the demand for alcohol-free mouthwash has increased due to various reasons [182, 183].

Mouthwashes containing active ingredients that have antifungal activity are frequently used as daily oral care products to avoid fungal disorders of the buccal cavity or in conjunction with systemic antifungal medications. The most common human fungal infection is oral candidiasis. The word "candidiasis" is used to describe diseases caused by specific *Candida* species. *Candida* species are harmless commensals of the human body and are a typical component of the normal flora of the mouth, pharynx, intestine, vagina and perianal skin folds. The back of the tongue is the main oral reservoir for these microorganisms, although they can also live on mucosal surfaces and in dental plaque [184, 185].

Oral candidiasis (OC), commonly known as " thrush," includes infections of the tongue with other oral mucosa. It is characterized by fungal proliferation and superficial tissue invasion. The poplar term "thrush" is referred to the similarity between the white flecks found in some forms of candidiasis and the breast of the bird with the same name [186]. In healthy individuals, Candida is part of the normal oral flora. It is thought that between 45% and 65% of healthy newborns and between 30% and 55% of healthy adults have candida in their normal oral flora. *Candida albicans* is the most prevalent Candida species in humans, found in healthy oral mucosa and OC because of its adhesion abilities and higher level of pathogenicity [187, 188]. According to studies, the prevalence of *Candida albicans* isolated from the oral cavity ranges from 30% to 50% in the general healthy population, from 50% to 65% in those who wear dentures, from 65-88% in long-term care residents, and from 90% to 95% in individuals infected with the human immunodeficiency virus (HIV), as well as in patients receiving corticosteroids, chemotherapy, immunosuppressive drugs, or radiation for head and neck cancers [189].

The transition of *Candida albicans* from a commensal harmless state to the pathogenic state is highly dependent on numerous predisposing factors. Systemic factors involve conditions of immunosuppression, such as HIV (human immunodeficiency virus), leukemia, age-related decreased immunity malnutrition, endocrinal dysfunction such as diabetes, radiation therapy, systemic chemotherapy, the use of systemic corticosteroids, immunomodulatory drugs and broad-spectrum antibiotics. While local factors comprise: altered salivary gland function, smoking ,poor oral hygiene, prolonged wearing of dentures with inadequate denture hygiene, high dietary intake of sugar and carbohydrates and alcohol consumption [189, 190].

The management of oral candidiasis requires the elimination and treatment of any underlying causes or recognizable risk factors, accurate diagnosis through medical and dental records, the maintenance of good oral hygiene, and the adoption of antifungal treatment according to the severity of the infection and the sensitivity of the fungal species. It has been shown that maintaining good oral hygiene, combined with antifungal treatment, is of paramount importance to effectively prevent and treat OC. Oral hygiene includes proper cleaning of the teeth, dentures oral cavity and the tongue. Also, the use of anti-candida mouthwash. Antimicrobial mouthwashes have positive clinical outcomes when used in conjunction with other therapies to treat oral candidiasis. There are many medical treatment options, and the practitioner can choose the appropriate treatment option on the basis of the extent and the severity of the infection and taking into account possible adverse drug reactions and drug interactions. Topical antifungal agents include gentian violet, nystatin, amphotericin B, and imidazoles such as ketoconazole, clotrimazole, and topical miconazole. Systemic antifungal agents include oral fluconazole, itraconazole, posaconazole, and ketoconazole; however, they carry a risk of hepatotoxicity in addition to drug interactions and adrenal insufficiency [187, 191, 192].

Part II

EXPERIMENTAL PART

II. EXPERIMENTAL PART

II.1 Plant Material:

The plant *Plumbago europaea* belongs to family Plumbaginaceae of genus *Plumbago* that consists of 10–20 flowering plants species, native to warm temperate and tropical regions of the world. *Plumbago europaea* is a perennial plant native to the Central Asia and Mediterranean regions. It has a dark green color, very branchy, with slender, angular-striated branches. Its stem is angular and 50-150 cm long; it has got petiolate and amplexicaul leaves, with lanceolate or spathe shape, and dentate edge. The flowers are small, blue-purple colored, and are arranged in small terminal spikes.

Table 5: Scientific classification of *Plumbago europaea*.

Scientific classification	
Kingdom	Plantae
Division	Magnoliophyta
Class	Angiosperms
Order	Caryophyllales
Family	Plumbaginaceae
Genus	*Plumbago*
Species	*P. europaea*

The plant *P. europaea* is commonly known as la dentelaire or la dentelaire d'europe while vernacular names are: Tifezouj, Tife elgouze, Souak elraeyane. In traditional folk medicine, this plant is widely used to treat respiratory disorders, hepatitis, edema, leprosy, inflammations, scabies, toothache, warts, blisters, injury, calluses, and skin hardness. The roots of *P. europaea* have been used in local traditional medicine to treat dermatitis, herpes and toothache.

Naphthoquinones, phenolic acids and flavonoids are the groups of natural compounds isolated in some species of *Plumbago* genus and the main compound is plumbagin. Plumbagin was isolated for the first time in pure form by Dulong D'Astafort in *P. europaea*, and successively in *P. zeylanica, P. rosea* and *P. scadens.* In *P. europaea*, terpenoids have also been isolated in essential oil from the roots [193, 194].

II.2 Collection and Identification of Plant Material:

The roots of *P. europaea* were harvested from Ain Kebira, Tlemcen (Algeria) during the period extending from September to October 2017. The plant material was identified by Professor BENABADJI N. member of the laboratory of Ecology and Management of Natural Ecosystems, University Abou Bekr Belkaïd, Tlemcen (Algeria). The harvesting location, geographic parameters of the harvesting area of the plant are presented in the table 6 and figure 9.

The plant material is usually not used immediately after harvesting, it is necessary to know the best methods to preserve its active principles and therefore its therapeutic properties. The conservation of medicinal plants requires three steps: drying, packaging and storage. After each harvest, the plant material is cleaned (cleared of debris) and then spread out on cardboard laid on the ground and left to dry in the shade, away from dust and in well-ventilated places, away from humidity and at room temperature. The plant material was laid out in thin layers and stirred from time to time.

The drying time was from one week to ten days. The plant material used in the solvent extractions was ground and the powders obtained were sieved and stored in a well-sealed glass bottle in a cool, dry place for later use.

Table 6: **Geographic parameters of the harvesting location**

Plant material	Studied part	Longitude	Latitude	Geographic coordinates
Plumbago europaea	Roots	-1.67576	35.0304	35° 1′ 49″ North, 1° 40′ 33″ West

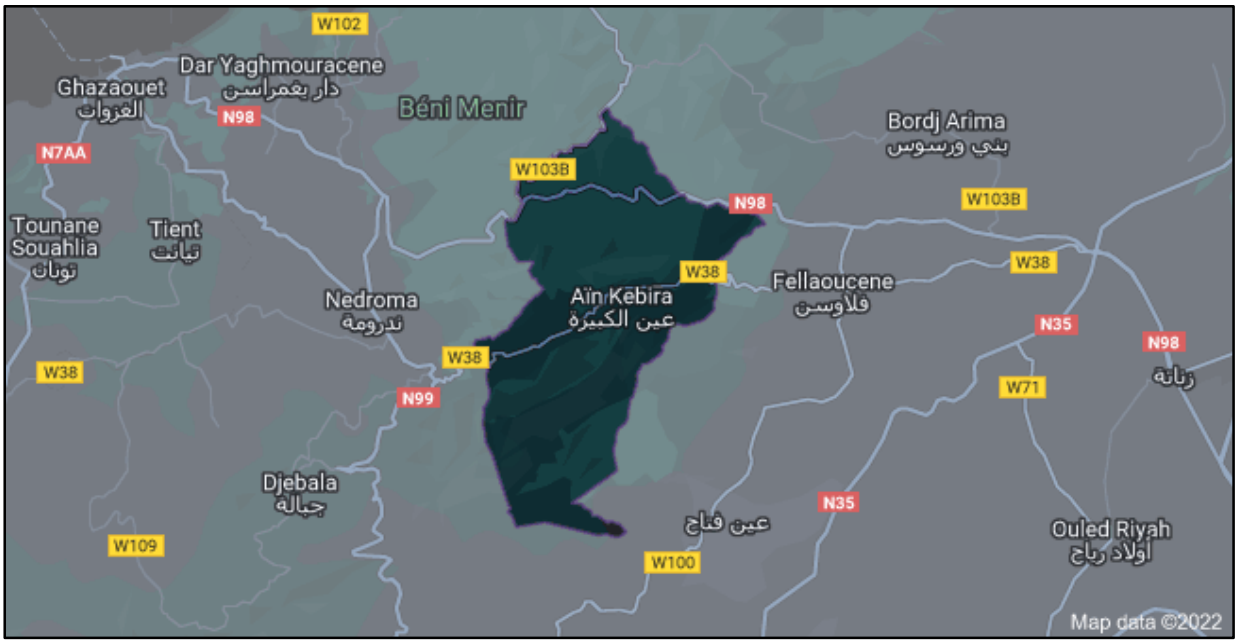

Figure 9: Geographic location of collection stations.

(a)

(b)

Figure 10: *Plumbago europaea*: (a) aerial part and (b) roots

II.3 Chemical study of essential oil and hydrosol extract:

II.3.1 Essential oil extraction:

There are various methods for extracting essential oils, the one most used in our laboratory being hydrodistillation. It is a widely used technique for the extraction of essential oils. The advantage of this technique resides in the reduction of the distillation temperature. The volatile compounds are thus entrained at temperatures much lower than their boiling temperature, which avoids their decomposition.

In this study, the hydrodistillations were carried out using a Clevenger type apparatus. 500 g of dried roots of *Plumbago europaea* were introduced into a 6L flask containing 4L of water and then the whole is heated for 5h. This flask is connected to a refrigerator which is used to condense the water vapor containing the extracted essential oil. The extraction starts when the first drops fall into the collector and continues for 4 hours. The resulting essential oil was dried with anhydrous magnesium sulphate and afterwards stored in a dark glass test tube at 4 °C until testing and analysis was carried out. The yield of essential oil was determined using the following equation:

$$\text{Extraction yield (\%)} = \frac{\text{the weight of the extracted essential oil}}{\text{the weight of dried roots}} \times 100$$

II.3.2 Hydrosol extract isolation:

Hydrosols or Hydrolats are by-products of steam distillation or the hydrodistillation of aromatic plants used to produce essential oils (Eo). During distillation, the water vapor passes through the plant material and causes the release of volatile molecules (Eo) which evaporate and then condense through a cooler. The essential oils are then separated from the water due to the difference in density which forms two phases, the upper phase is the essential oil and the lower phase is the floral waters, characterized by a specific smell characteristic of the plant material used.

The hydrosol extract was obtained using a liquid-liquid extraction method (LLE). Liquid-liquid extraction or solvent extraction is a physicochemical technique of separation and concentration of chemical compounds. It is based on the distribution of a solute between two

immiscible liquid phases, one of which is aqueous and the other organic. Subsequently, the 500 first milliliters of distillate water were collected and extracted three times with 200 milliliters of diethyl ether at room temperature to obtain the corresponding hydrosol extract. The organic layer was evaporated and dried with MgSO4 and stored in a dark glass test tube at 4 °C until testing and analysis was carried out. The yield was then calculated.

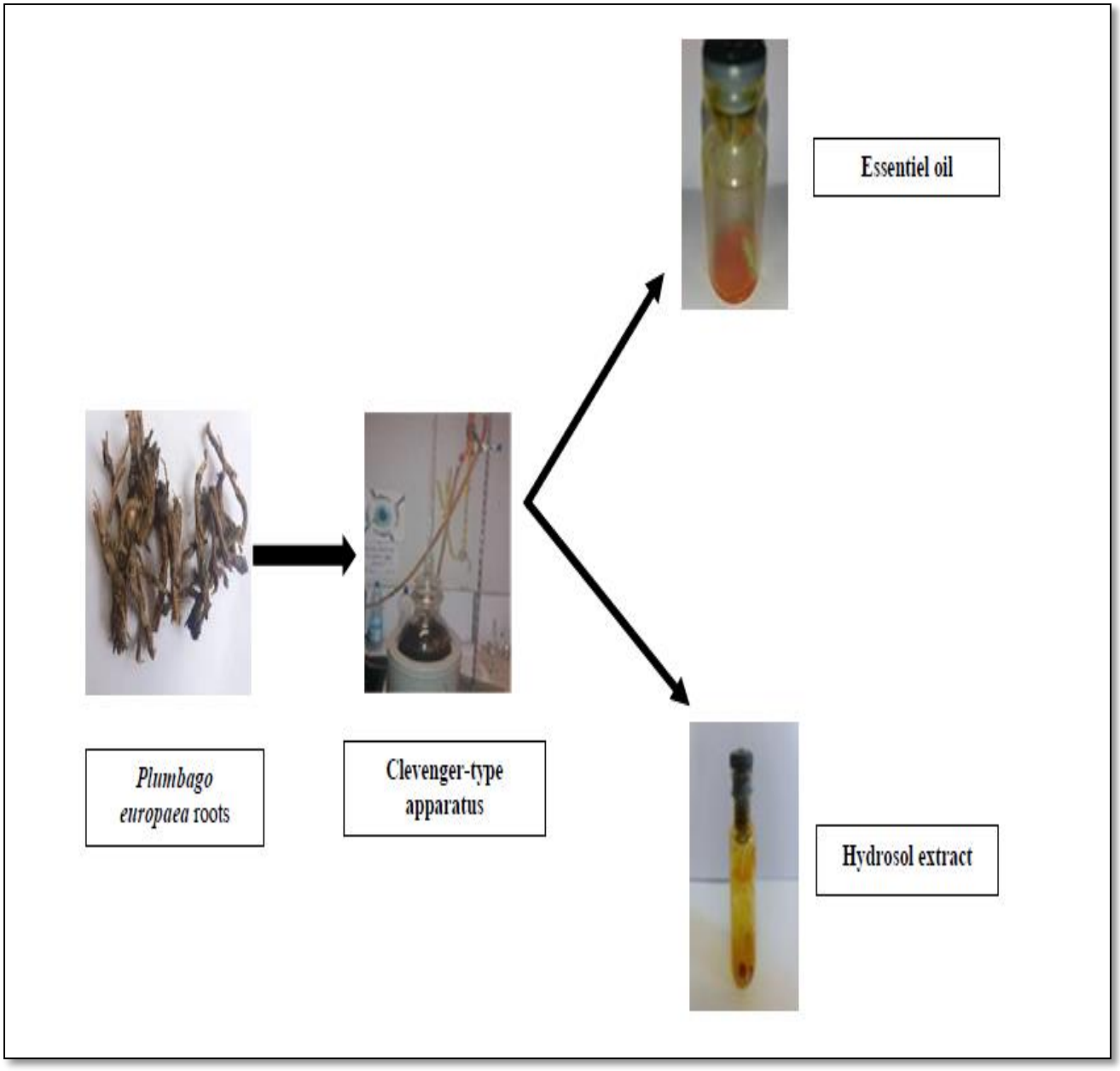

Figure 11: Essential oil and hydrosol extract isolation.

II.3.3 Chromatographic analysis of essential oil and hydrosol extract:

Essential oils have become a raw material for various sectors of activity such as perfumery, cosmetology, pharmaceutical and food industries. Whatever the sector concerned, the analysis and optimal knowledge of the chemical composition of essential oils remains an important step. Despite the considerable progress achieved in recent years in the field of analytical chemistry the characterization of essential oils remains a challenge that is then despite the constant developments of methods of separation and identification, remains a delicate operation requiring the implementation of various techniques.

The study of the chemical composition of an Eo is generally carried out by gas chromatography (GC) coupled with a spectral identification technique, generally mass spectrometry (MS). They make it possible to combine the separative capacity of chromatographic methods with the characterization power of spectroscopic and spectrometric methods. Actually, this technique is the reference method in the analysis of essential oils; it allows the analysis complex mixtures with varied nature and volatility [195, 196].

Once the essential oil and hydrosol extract are obtained, the analysis allows the identification and quantification of the chemical composition. In the current work, the analysis of the volatile part is carried out by two chromatographic techniques (CPG & CPG/SM).

II.3.3.1 Gas chromatography:

Analyses were carried out using a Perkin Elmer Clarus 600 GC apparatus equipped with a dual flame ionization detection system and two fused-silica capillary columns (60 m x 0.22 mm I.D., film thickness 0.25 μm), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethyleneglycol). The oven temperature was programmed from 60 °C to 230 °C 2 °C/min and then held isothermally at 230 °C for 35 min. The injector and detector temperatures were maintained at 280 °C. The essential oil and hydrosol extract were injected in the split mode (1/50), using hydrogen as the carrier gas (0.7 mL min-1); the injection volume was 0.2 μL. The retention indices (RI) of the compounds were determined from Perkin-Elmer software.

II.3.3.2 Gas chromatography-mass spectrometry (GC-MS):

Essential oil and hydrosol extract were analyzed with a Perkin–Elmer TurboMass quadrupole analyzer, coupled to a Perkin-Elmer Autosystem XL, equipped with 2 fused-silica capillary columns and operated with the same GC conditions described above, except for a split

of 1/80.In addition, the electron Impact (EI) mass spectra were acquired under the following conditions: Ion source temperature of 150 °C, energy ionization of 70 eV, and mass range 35-350 Da (scan time: 1 s).

II.3.3.3 Component identification and quantification:

Identification of individual components was accomplished by comparing their GC retention indices (RIs) on nonpolar and polar columns, determined relative to the retention time of a series of n-alkanes with linear interpolation, with those of authentic compounds or literature data [197, 198] and through computer matching with commercial mass spectral libraries [199, 200] and also by comparing the spectra obtained with those of the in-house laboratory library. The quantification of Eos and hydrosol extract components was performed using peak normalization (%) abundances calculated by integrating FID response factors relative to tridecane (0.7 g/100 g), used as an internal standard.

II.4 Chemical study of ethanolic extract:

Most scientists are interested in discovering new natural drugs with significant medicinal values, without side effects, to treat diseases. The crude extract of the plant contains various chemical compounds, which are responsible for the biological activities. The bioactivities of crude extracts obtained from plants depend entirely on the bioactive constituents [201]. The medicinal plant studied in this research project have been used in local traditional medicine to treat inflammation, dermatitis, herpes and toothache. The preparation of an ethanolic extract from roots of plumbago europaea and the determination of its chemical composition in order to to explain the relationship between chemical composition and biological activity are among the objectives of this study.

II.4.1 Preparation of ethanolic extract from root material:

The collected roots were washed and then dried in the shade at room temperature until all the plant parts became well dried. After drying, the plant materials were ground well into a fine powder using a mechanical blender and transferred into airtight containers with proper labeling for future use.

Before choosing maceration as solvent extraction in this research and for the optimization of extraction technique, we have performed a rapid screening of the antimicrobial activity of

extracts prepared by maceration and by soxhlet extraction. The extract prepared using maceration technique was more active against tested microorganisms.

About 10 g of the grounded roots of *P. europaea* were soaked in 100ml of ethanol and placed in a mechanical shaker (figure 12) with temperature control (37°C) at constant stirring rate at 100 RPM for 72h. After that, the extract was filtered using Whatman filter paper No. 1 and concentrated under vacuum on a rotary evaporator at 40 °C. The crude extracts were weighted in order to calculate the yield and then stored at 4 °C in the refrigerator for further use.

Figure 12: Mechanical shaker

II.4.2 Column chromatographic fractionation of ethanolic extract:

The ethanolic extract (EE) was subjected to Silica gel column chromatography for purification the active phytocompounds. A vertical glass column (40 mm width 60 mm length) made of borosilicate material was used. Hexane was poured into the column up to 3/4th level by closing the stopcock. 80 g of silica gel (60–120 mesh size) was used as the packing material. Silica slurry was prepared with hexane and was poured from the top of the column approximately 2/3rd of the column with simultaneous draining of the solvent to aid proper packing of the column. 4g of EE was mixed with minimum quantity of hexane and was poured down from the top of the column along the sides and was rinsed down with the solvent and added to the top of the extract to 1 cm height. Solvent level 6 cm from above the extract was

maintained to prevent drying of the column. Gradient elution method was followed to separate fractions from EE by using solvents from low polarity to high polarity. Two solvent system were used for the elution: hexane to ethyl acetate and in varying ratios and dichloromethane-methanol (50/50) and methanol (100%). The flow rate was adjusted to 5 ml/min and 15 ml solvent was collected for each fraction.

Figure 13: Column chromatographic fractionation of ethanolic extract.

II.4.3 Thin Layer Chromatography (TLC) of fractions:

The fractions were collected separately and subjected to TLC as described in literature [202, 203] with some modifications. The fractions were spotted on 1.5 cm of the bottom of the TLC plates and placed on the chromatography jar containing mixture of hexane-ethyl acetate (95: 5). The plates were lefts until the solvent a crossed to the top, then, removed, allowed to dry, and visualized under UV (254 nm) to determine R_f values. Fractions with the same R_f values were pooled and dried using rotary evaporator at 45°C.

Three main fractions named F1, F2 and F3 were obtained; the dry weight of the fractions was measured. The fractions F1 and F2 were further analyzed by GC-Ms.

II.4.4 Gas chromatography mass spectrometry (GC-MS):

The analyses of fractions F1 and F2 by gas chromatography were carried out in the center of Scientific and Technical Research in Physical - Chemical Analyses: Bousmail using a

chromatograph Hewlett Packard Agilent 6890 plus, equipped with Mass Spectrometer: Hewlett Packard Agilent 5973, and a capillary column Type: HP-5MS, Dimensions (30 m×0,25 mm×0,25 μm) with a stationary phase: 5% Phenyl 95% dimethylpolysiloxane. The following conditions were applied:

- Oven temperature: 60°C for 8min, 2°C/min to 250°C., isothermal for 10 min.
- Analysis time: 113 min
- The samples were injected in split mode (1:50) using helium as carrier gas purity: N6.0; GV flow rate: 0.5 ml/min

The molecules are bombarded in an ionization source of 230 °C by an electron beam of 70 eV, the detection is done by a quadrupole analyzer made of an assembly of four parallel electrodes of cylindrical section.

- The injected volume of the samples is 0.2 μL
- Analysis mode: Scan TIC (from 30 to 550)
- Solvent delay: 3.5min
- Interface temperature: 280 °c
- Type of ionization: Electronic impact

Figure 14: GC/MS Chromatograph.

II.5 Biological study of essential oil, hydrolsol extract, ethanolic extract and purified fractions:

II.5.1 Antioxidant activity:

Today, there is a growing interest in the biology of free radicals. This is due to their role in acute phenomena such as trauma and ischemia, as well as their involvement in many chronic conditions associated with aging, such as cancers, inflammatory and cardiovascular diseases, and degeneration of the immune system. Although synthetic antioxidants are effective and their approved doses are considerably limited to avoid any toxicity problems, there has been a fascination and growth in natural products and the development of plant extracts for antioxidant use since the 1980s, which will have biological properties that help reduce the risk of certain diseases [204].

Given the complexity of oxidation processes, there is no single method that can reflect the antioxidant profile of a sample. It is therefore necessary to combine the results obtained with different and complementary tests. This is why we chose to use three chemical tests: the 2,2-Diphenyl-1-Picryl-Hydrazyl (DPPH-) radical scavenging, the ferric ion reducing power (FRAP) and the β-carotene bleaching assay to evaluate the antioxidant activity of essential oil, hydrolsol extract, ethanolic extract and purified fractions F1, F2, F3.

II.5.1.1 DPPH free-radical scavenging assay:

The free-radical scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to assess the antioxidant activity of essential oils and hydrosol extract of the plant under study, as described in the literature [205]. This radical was one of the first radicals used to study the structure/antioxidant activity relationship of phenolic compounds.

The DPPH free-radical scavenging assay is a spectrophotometric test, in which the stable radical DPPH is utilized as a reagent. This reagent is a free radical which is recognized for its remarkable stability due to the delocalization of the radical in aromatic ring. It has an intense deep purple color. During this assay, an antioxidant species (or reducing agents) act as donor of a hydrogen atom or an electron to the free radical which will be neutralized and converted to its reduced form (DPPH or DPPH-H) by accepting either the hydrogen atom or

the electron at the end of the reaction and its initial color fades to a pale yellow (figure15) [206].

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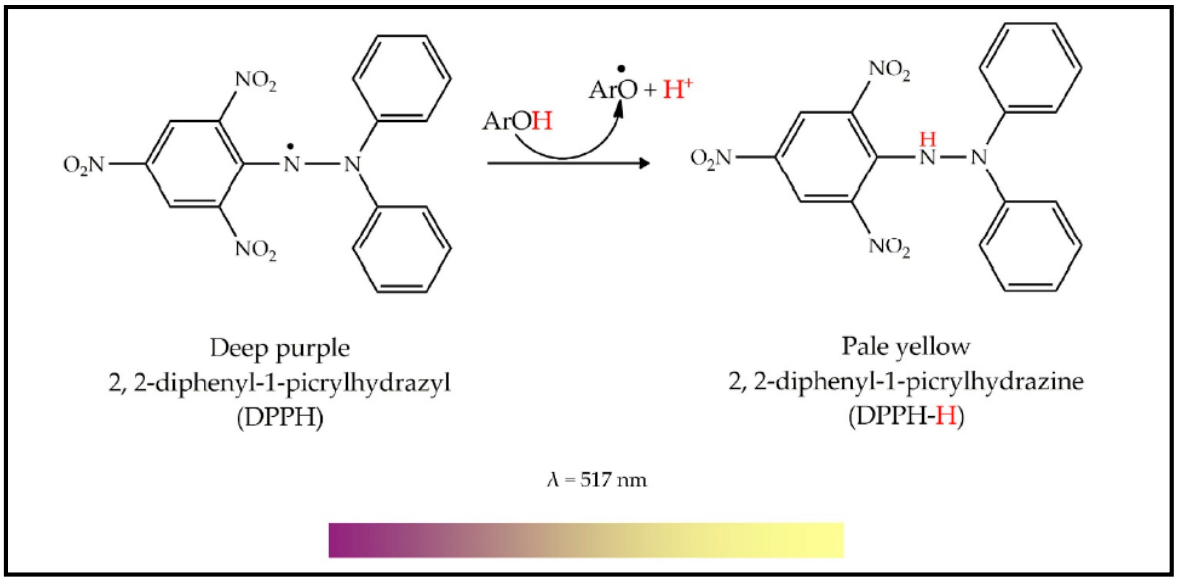


Figure 15: 2, 2-diphenyl-1-picrylhydrazyl (DPPH) reaction mechanism

In this method, one milliliter of different concentrations of the tested essential oil and hydrosol extract prepared in ethanol was added to 1 mL of DPPH ethanolic solution at a concentration of 0.2 mmol/L (prepared by dissolving 7.886 mg of DPPH in 100 ml of ethanol). Regarding ethanolic extract and fractions F1, F2 and F3, one milliliter of different concentrations prepared in methanol was added to 1 mL of a 0.2 mmol/L DPPH methanolic solution.

The obtained mixture was vigorously agitated and left standing for 30 min at room temperature. The absorbance was then measured at 517 nm for the resulting solution [205, 207]. Ascorbic acid was used as standard and DPPH mixture without any sample served as blank. The following equation was used to calculate the inhibition of the free radical DPPH in percent (I %).

$$I\% = (A\ blank - A\ sample / A\ blank) \times 100\%$$

Where A blank is the absorbance of the control reaction (without extracts), and A sample was the absorbance in the presence of extracts. IC_{50} values were determined from the graphs of scavenging activity against the concentration of the extracts. These values are provided in mg/ml and are defined as inhibitory concentration of the extract required to decrease the initial concentration of the DPPH radical by 50%. Triplicate measurements were performed.

II.5.1.2 Ferric-reducing antioxidant power assay (FRAP):

As the antioxidant activity of a substances is often in correlation with their reducing capacity, the ferric-reducing power (FRAP) assay provides a reliable method to assess the antioxidant activity of plant metabolites. It is used to evaluate the ability of a substance to reduce $Fe^{+3}$to Fe^{+2}, which is measured by the formation of a colored complex with potassium ferricyanide that can be read spectrophotometrically at 700 nm (figure 16). It is a rapid, simple, direct and reproducible technique based on the presence of a reductant in the extracts that causes the reduction of an oxidant (Fe^{3+}) by giving up an electron to form (Fe^{2+}) and can be evaluated by monitoring the increase in the density of the blue color in the reaction medium at 700 nm [208].

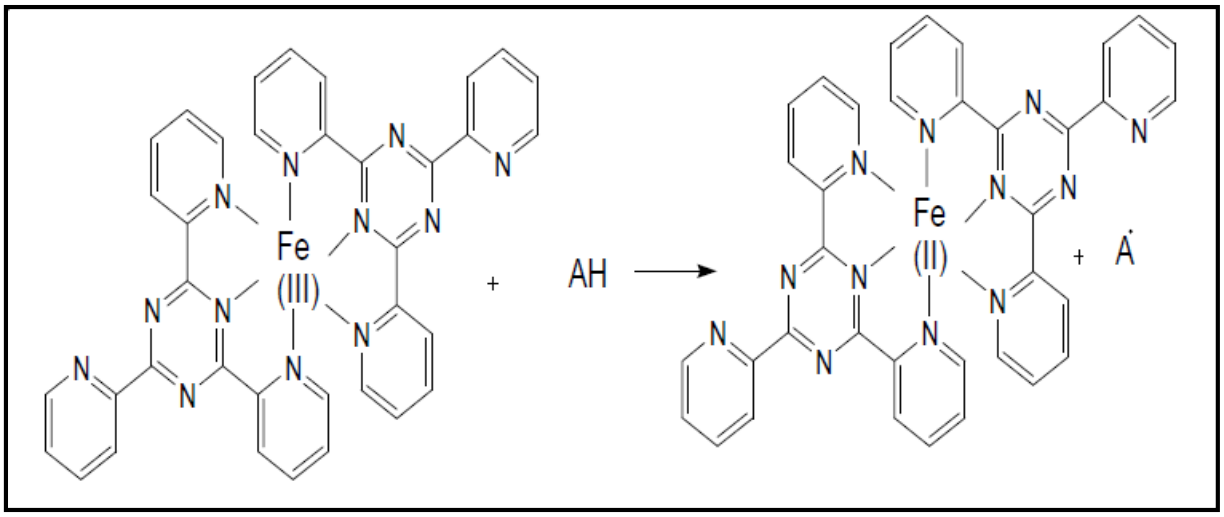


Figure 16: FRAP assay

In this study the FRAP assay was carried out to evaluate the total antioxidant capacity of tested samples as described by Oyaizu (1986). [209, 210]. Different concentrations of essential oil and hydrosol extracts, ethalonic extract (mg/mL) in ethanol and different concentrations of crude extract and fractions F1, F2, and F3 in methanol were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50°C for 20 minutes. After that, trichloroacetic acid (2.5 mL, 10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%).

Absorbance was read at 700 nm against a blank (ethanol for the essential oil and hydrosol extract and methanol for the ethanolic extract and purified fractions). Analyses were achieved in triplicates. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the standard compound.

II.5.1.3 *β*-carotene bleaching assay:

The *β*-carotene bleaching test assesses the ability of antioxidants to inhibit lipid peroxidation in both the initiation phase and in the propagation phase. The technique consists in measuring at 470 nm the decolorization of *β*-carotene oxidized by peroxide radicals regenerated by the degradation of linoleic acid. The oxidation of linoleic acid generates peroxide radicals following the abstraction of hydrogen atoms from the methylene groups of linoleic acid. These free radicals will then oxidize the highly unsaturated β-carotene, which loses its double bonds thus causing the disappearance of its red color, which is followed spectrophotometrically. However, the presence of an antioxidant could neutralize the free radicals derived from linoleic acid and thus prevent the oxidation and bleaching of β-carotene[211, 212].

The antioxidant activity of the tested essential oils and hydrosol extract was assessed using a *β*-carotene-linoleate model system following the method described in literature with some modifications [213, 214] . 2 mg of *β*-carotene was solubilized in 10 mL of chloroform. 1 mL of this solution was taken in a beaker containing 20 µL of linoleic acid and 200 mg of Tween 40. After evaporation of the chloroform, 50 mL of oxygenated water were added to the flask under vigorous shaking. Aliquots (5 mL) of this emulsion were transferred into a series of tubes containing 500 µL of the solution of the essential oils, hydrosol extract or the synthetic antioxidant (BHT) at different concentrations. The tubes are then placed in a water bath at 50°C for 120 min. The absorbance of each extract was measured at 470 nm at t = 120 min. Antioxidant activities (Inhibition %) of all tested samples were calculated using the following equation:

$$\textbf{Inhibition (\%)} = = [(A_{S\,(120)} - A_{B\,(120)}) / (A_{B\,(0)} - A_{B\,(120)}] \times 100$$

Where:

$A_{S\,(120)}$: Absorbance of sample at 120min.

$A_{B\,(120)}$: Absorbance of the blank at 120 min.

AB $_{(0)}$: Absorbance of the blank at 0 min.

This activity is also expressed in IC_{50} as for the DPPH test.

II.5.2 II.6.2. Antimicrobial activity:

II.5.2.1 Microbial strains:

The microorganisms used in this study are belonging to the "American Typed Culture Collections" (ATCC). They were brought from the stock culture of the Laboratory of Food, Biomedical and Environmental Microbiology (LAMAABE) University of Tlemcen, Algeria. These microorganisms comprised nine (9) bacteria: *Escherichia coli* (ATCC 25912), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 15313), *Micrococcus luteus* (ATCC 9341), *Bacillus cereus* (ATCC 10876), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Klebsiella pneumoniae* (ATCC 700603), *Enterococcus faecalis* ATCC 49452 and three yeast *Candida albicans* (ATCC 26790), *Candida albicans* (ATCC 10 231), *Candida albic*ans (IP444).

The antimicrobial activity was determined using the disc diffusion and the micro-well dilution methods. Concentrations of these microorganisms were prepared to contain approximately 1 to 2×10^8 CFU/ml of bacteria and 1×10^6 to 5×10^6 CFU/ml of yeast).

In this study the antibacterial and antifungal activity are determined for both essential oil and hydrosol extract while for ethanolic extract and purified fractions only the antifungal activity has been evaluated.

II.5.2.2 Disc diffusion assay:

Disc diffusion method was carried out according to the recommendations of the Clinical and Laboratory Standards Institute CLSI [215]. Mueller-Hinton agar was used for bacteria and Sabouraud was supplemented with glucose (2%) for yeast, the medium of choice was inoculated on the surface with the appropriate microorganisms using a cotton swab. The inoculums were then allowed to dry. Then sterile filter paper discs (6 mm diameter) soaked with the tested sample (essential oil, hydrosol extracts (15µl/disc) were placed on the plates.

Gentamicin (10µg/disc) and amphotericin B (0.2 mg/disc) were used as positive controls for antibacterial and antifungal tests, respectively. Plates were incubated at 37°C for bacteria

and 28°C for yeast for 24 h. Each test was performed in triplicate. Afterwards, the zones of inhibition were measured.

II.5.2.3 Micro-well dilution assay:

Minimum inhibitory concentrations (MICs) of the plant extracts studied were performed using the method of microplate (96 wells) (40). The test microorganisms mentioned above were inoculated onto Mueller Hinton broth for bacteria and Sabouraud broth supplemented with glucose (2%) for yeasts. After 24 h, 100 µl of each inoculum (5×10^5 CFU/ml for bacteria and 1 to 5×10^4 CFU/ml for yeast strains respectively) were prepared. Then, 100 µl of each sterile broth were placed in each line of the microplate. Afterwards, 100 µl of the extract were introduced into the first well.

After having thoroughly mixed the contents, 100 µl were removed from the first well and placed in the second well so on, until reaching the 10^{th} well; the remaining100 µl were then eliminated. As a result, a ½ dilution was obtained. In the end, 100 µl of each inoculum were added. The last two wells represent negative controls; the 11^{th} well contains the medium and inoculum, and the 12^{th} one contains only the medium. The microplates were sealed and incubated at 37 °C for bacteria and at 28°C for yeasts, during 20 h. Each assay was carried out in triplicate incubation. The MIC was defined as the lowest concentration of plant extract that inhibits the visible growth.

II.5.2.4 Determination of the synergistic activity:

In order to evaluate the synergistic effect, a combination of hydrosol extract and two antimicrobial drugs (Gentamicin and amphotericin B) and a combination of ethanolic extract with amphotericin B were studied using the checkerboard method as described by White et al [216]. In this technique, appropriate plant extract concentrations were mixed with each antimicrobial agent dilution to produce a series of combinations. The dilutions of antimicrobial drugs and plant extract antimicrobial drugs were prepared by the same method, and subsequently the MIC values were evaluated. For both the studied plant extract and the tested antimicrobial drugs, the concentrations prepared ranged from 1/32 to 4 times the MIC.

To assess the effect of the studied combinations, the fractional inhibitory concentrations (FICs) and the fractional inhibitory concentration indices (FICIs) were then calculated for both

plant extract (PE) and antimicrobial drugs (AD) in every combination, using the two following formulas:

$$\text{FIC of PE} = \frac{MIC\ PE\ combination}{MIC\ HE\ alone}$$

$$\text{FIC of AD} = \frac{MIC\ AD\ combination}{MIC\ AD\ alone}$$

The MIC values for the PE-AD combinations were defined as the lowest concentrations at which no visible growth of microbial strains could be detected as compared to their growth in the control well.

FIC index = FIC of PE + FIC of AD

Synergy was defined as a FIC index value ≤ 0.5. Indifference was defined as a FIC index value> 0.5 but ≤ 4. Also, antagonism was defined as a FIC index value > 4.

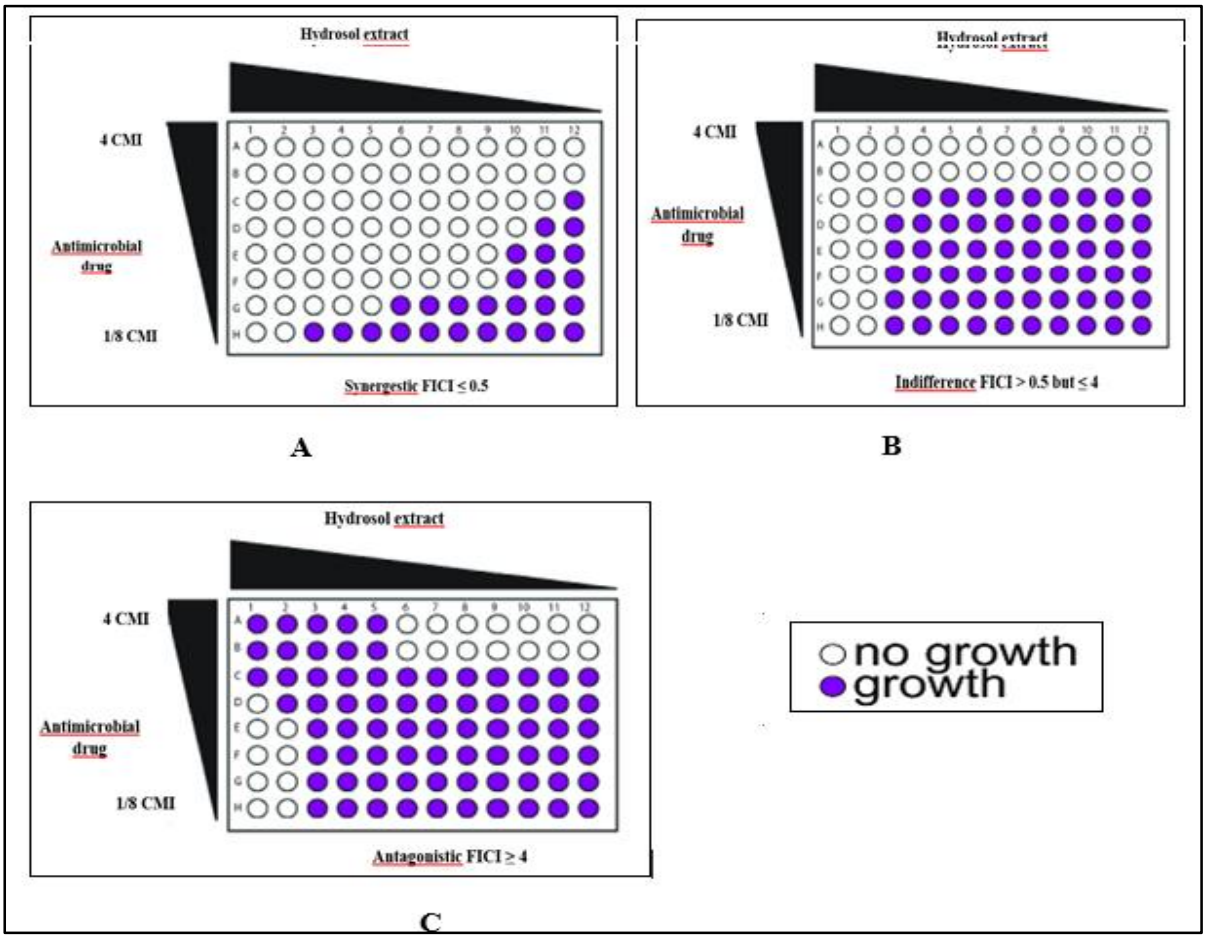


Figure 17: Graphical results of Checkerboard Assay. Illustrations of synergistic, indifference, and antagonistic interactions are depicted in A, B, and C, respectively.

II.5.3 Hemolytic activity:

In-vitro hemolytic activity of ethanolic extract was performed according to Guo-Xiang et Zai-Qun [217] for 5 concentrations (4, 2, 1, 0.5 and 0.25 mg/ml) on suspension of human erythrocyte in PBS (phosphate buffered saline) at pH = 7.4 ± 0.2 [sodium chloride (137 mM), potassium chloride (2.7 mM), sodium hydrogen phosphate (8 mM), potassium dihydrogen phosphate (2 mM)].

The human blood collected in heparin tubes from a healthy donor and centrifuged at 2500 rpm for 10 minutes to prepare the erythrocyte suspension. After removal of the plasma, the pellet was washed twice with PBS and then re-suspended and diluted to 20 times of the original volume with PBS. This was referred to as the stock erythrocyte suspension. In hemolysis tubes, 20 µl of each extract at different concentrations are added to 1980 µl of the erythrocyte suspension prepared. These tubes are then incubated at 37 °C for 60 minutes. After incubation, samples of 250 µl are taken for each tube to be taken up in 750 µl of PBS. These are gently mixed and put in an ice bath to stop the reaction, then centrifuged at 2500 rpm for 10 minutes. The absorbances are read at 548 nm using a spectrophotometer, against a blank containing PBS. Under the same conditions and the same experimental procedures, we have prepared a total hemolysis tube which contains 100 µl of the erythrocyte suspension and 1900 µl of distilled water and a negative control tube composed of 250 µl of erythrocyte suspension and 750 µl of PBS buffer solution. The hemolysis rate of the different extracts is calculated as a percentage relative to the total hemolysis, after 60 minutes of incubation, according to the following formula:

$$\textbf{Hemolysis rate (\%)} = \frac{A\,(extract) - A(\,negatif\ control)}{A\,(positif\ control)} \times 100$$

II.5.4 In-vitro anti-inflammatory activity:

The *in-vitro* anti-inflammatory activity of ethanolic extract was evaluated by using inhibition of albumin denaturation technique, by the standard protocols as reported in literature [218-220]. To study anti-inflammatory activity, the reaction mixture (5 mL) comprised of 0.2 mL of egg albumin, 2.8 mL of phosphate buffer solution (pH= 6.4) and 2 mL of ethanolic extract

Solution at different concentrations (1 mg/ml, 0.5 mg/ml, 0.25 mg./ml, 0.125 mg/ml) . A double volume of distilled water was used as a control. The mixtures were incubated at 37±2°C for 15 min and then heated to 70°C for 5 min in water bath. After cooling, absorbances were measured at 660 nm by using a UV spectrophotometer. All the readings were observed in triplicate. Diclofenac sodium was used as reference drug and treated similarly for determination of absorbance. The % inhibition of protein denaturation is calculated, using the formula:

$$\% \text{ Denaturation inhibition} = (1 - A_s / A_c) \times 100$$

As: Absorbance of sample

Ac: Absorbance of negative control

II.6 Preparation of a free alcohol mouthwash:

II.6.1 Chemical reagents:

- **Active ingredient:** Ethanolic extract (EE) was used as active ingredient.
- **Excipients [221]:**

    1. Glycerin
    2. Menthol
    3. Saccharin
    4. Sodium Benzoate

- **Solvent:** purified water used as vehicle.

II.6.2 Formulation:

Six different formulations were prepared. Formulations: F1-F5 were developed with different EE concentration (5MIC, 10MIC, 15MIC, 20MIC, 30MIC), where MIC was the minimum inhibitory concentration of EE against *Candida albicans.* The sixth Formulation F6 was prepared as negative control without the active ingredient. 100 ml of each formulation was prepared by solubilizing weighted amount of EE in purified water under mixing until the solution became clear. After that, Glycerin was added drop by drop with maintaining mixing for at least 5 min.The remaining ingredients were then added to the prepared solution. The final volume was adjusted to 100 mL by purified water.

For better homogenization, samples were sonicated for 10 minutes using a bath sonicator. The obtained solutions were transferred into airtight containers with proper labeling and the macroscopic aspect of the prepared mouthwash is noted and monitored over time.

Tableau 7: Composition of mouthwash formulations

Ingredient	Function	F1	F2	F3	F4	F5	F6
Ethanolic extract	Active ingredient	5MIC	10MIC	15MIC	20MIC	30MIC	-
Glycerin	Humectant	10%	10%	10%	10%	10%	10%
Menthol	flavoring	0.001%	0.001%	0.001%	0.001%	0.001%	0.001%
Saccharin	sweetener	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%
Sodium Benzoate	Preservative	0.05%	0.05%	0.05%	0.05%	0.05%	0.05%
Purified Water	Vehicle	100	100	100	100	100	100

Color and Odor: Physical parameters like odor and color were tested by visual examination.
pH: pH was measured of each prepared mouthwash solution and of the pH meter was calibrated using standard.

II.6.3 Antifungal activity of the prepared mouthwash:

Antifungal activity of the prepared mouthwash was evaluated against three yeast of *Candida albicans* using disc diffusion technique as described previously. Sterile filter paper disks (6 mm diameter) soaked with the tested mouthwash were placed on the Sabouraud supplemented with glucose (2%) plates. The plates were incubated at 28°C during 24 h. Each assay was carried out in triplicates. Then, the inhibition zones were measured. The evaluation of the antifungal activity of the most active prepared mouthwash in comparison with Listerine® and Xethol® two commercialized mouthwashes based on essential oil was carried.

II.6.4 Stability study:

Different mouthwash formulations were subjected to stability test. Stability test aims to ensure that the mouthwash formulations are usable and can maintain the same characteristics in the long term. The formulation and preparation of any pharmaceutical product is incomplete without proper stability studies of the prepared product. This is done in order to determine the physical and chemical stability of the prepared product and thus determine the safety of the product. A general method for predicting the stability of any product is accelerated stability

studies, where the product is subjected to elevated temperatures as per the ICH guidelines [222]. A short term accelerated stability study was carried out for the period of 3 months for the prepared formulation. The samples were stored at under the following conditions of temperature in the refrigerator at 3-5 °C, at room temperature at around 25 °C and in the oven at round 40°C. Finally, the samples kept under accelerated study were withdrawn on monthly intervals and were analyzed in term of their antifungal activity, physical stability and microbiological stability. The visual appearance, physical separation, homogeneity, and pH of the tested mouthwashes were all recorded as part of the physical stability test. The purpose behind the evaluation of the microbiological stability of the stored mouthwashes was the verification of the presence or absence of growth of micro- organisms in the tested formulations. The presence and the absence of growth was analyzed by counting colony-forming units [223, 224]. The antifungal activity and the microbiological stability are determined at the end of the third month only for the formulation F5 which was the most active against the three tested strain of *Candida albicans.*

II.6.5 Determination of the Oral cavity residence factor:

The Oral Cavity Residence Factor (OCRF) is defined as the summed hedonic response (competing pleasure and pain perception) expressed as:

$$OCRF = \frac{Voluntary\ retention\ time}{10}\ (sec) \times\ urge\ to\ rinse$$

The experiment consists first of all in choosing at least ten volunteers (preferably 5 women and 5 Man) who do not present any irritation of the oral mucosa, any canker sores, any periodontal pockets, any oral cavity cuts, bruises or other abnormalities. 10 ml of each tested mouthwash was diluted in a glass water and each subject is asked to introduce 15 ml of the test mouthrinse into their oral cavity and flush the liquid vigorously throughout the mouth with a "swishing", rinsing motion for as long as the test material is comfortable to their mouth, teeth, gums and tongue. Each subject is instructed to expectorate. The voluntary retention time is measured by a stop watch and starts when the test material is introduced into the mouth and concludes upon expectoration.

Immediately upon expectorating, each subject is asked to determine their urge to rinse with water on a scale of 1 to 10 where 1 equal "urgently, as soon as possible" and 10 is "no urge

to rinse". The average or mean OCRF for the test subjects is determined for each tested mouthwashes (prepared mouthwash, listerine®, Xethol®) per the equation set out above.

II.6.6 Choice of Mouthwash color:

Product design is seen as an opportunity to gain a differentiating advantage in the market. The appearance of a product affects consumer product choice in a number of ways. Many companies successfully use product design as a competitive tool [225].

The visual aspects of a product may influence a consumer's purchasing decision because they provide an attractive way of conveying information about the product's features at the point of purchase. These visual elements influence product selection (or selection within a product category), and color is often an important component. In fact, color represents one of the most crucial elements in visual merchandising and can affect consumer behavior and purchasing decisions [226].

In the case of mouthwashes, color is an important parameter thereby by improving the appearance of the product it increases the acceptability of the consumer. It also plays a role in the course of the formulation by allowing control of the homogeneity of the preparation although it does not affect the mouthwash efficiency. For this reason, two mouthwash solutions were prepared with and without dye. The dye-free mouthwash had a yellow color due to the ethanolic extract of *Plumbago europaea*, the second mouthwash solution was prepared as described above by adding 0.002% of colorant.

Part III

Results and discussions

III. Results and discussions

III.1 Objectives

Infectious diseases and microbial pathologies are responsible for millions of deaths each year. They continue to be the leading cause of death in low-income countries and the third leading cause of death worldwide. Invasive fungal infections pose a serious and ongoing threat to human health and are associated with at least 1.5 million deaths worldwide each year. Unfortunately, the treatment of these infections has been hampered by the emergence of antimicrobial resistance, which is considered one of the most serious threats to the global health system in this century. In this context, many researchers are interested in medicinal plants as an alternative and important source of natural compounds to supplement the body with exogenous bioactive molecules with therapeutic effect.

Traditional medicinal practices using medicinal plants continue to play a significant role in the world's primary health-care delivery system. They are frequently employed as a normative basis for sustaining good health. The plant kingdom is a presumed inexhaustible source of a huge variety of potential drugs. Botanic products are extremely important to humanity because of their phyto-compounds, which are active principles with therapeutic potential. Phytocompounds have been identified as a promising research focus in the search for new pharmaceutical compounds because they have a wide range of chemical structures and biological properties such as hypoglycemic, anti-diabetic, antioxidant, anti-microbial, anti-inflammatory, and anti-carcinogenic properties. The extraction and chemical analysis are the most important steps in exploring and valuating these natural phyto-compounds.

Algeria is the largest country bordering the Mediterranean. It is recognized by its varietal diversity in medicinal and aromatic plants, as well as their various popular uses in all the country's soils. In a country like Algeria, where the flora is abundant, the development of the medicinal plants sector has become critical. Several scientific research laboratories have been created in order to develop this sector for use in various fields, including the manufacture of pharmaceutical, agricultural and cosmetic products.

Our work is designed and implemented as part of an effort to valuate plant resources from Tlemcen region through scientific research in order to determine the active molecules carrying

the therapeutic effect of the selected medicinal plant and to prepare an antifungal mouthwash contain natural plant extract as active ingredient.

In order to isolate new substances from plants and thus find new ways of application in both the pharmaceutical and cosmetic fields, and in order to make the isolation strategy as efficient as possible, the plants to be studied must be carefully selected. The selected plant in our research was *Plumbago europaea*. The choice of plants studied is simply based on an ancestral tradition of use.

Our research study was divided in three parts. The first part is devoted to the chemical study of essential oil, hydrosol extract and ethanolic extract from roots of *Plumbago europaea*. The second part is intended to the biological study of the prepared extracts (antioxidant, anti-fungal, hemolytic and anti-inflammatory activities). Finally, the third part is devoted to the formulation of a free alcohol mouthwash containing ethanolic extract as active ingredient and the *in- vitro* evaluation of its efficacy against three yeast of *Candida albicans*.

III.2 Chemical study of essential oil and hydrosol extract:

III.2.1 Essential oil and hydrosol extract isolation:

In the present work, the root part of *Plumbago europaea* was hydrodistilled for 5h using a clevenger type apparatus to isolate the essential oil. A liquid liquid extraction with diethyl ether at room temperature was used to obtain the corresponding hydrosol extract. The yields and organoleptic characteristics of these two extracts are mentioned in the table 8.

Table 8: Yield and organoleptic characteristics of Essential oil and Hydrosol extract.

Characteristic	Essential oil	Hydrosol extract
Aspect	Viscous	Needle-like crystals
Color	Yellow	Yellow to orange
Smell	Weak smell	A particular strong smell
Yield	0.016%	0.024%.

The criteria for appreciating and evaluating an essential oil are its organoleptic properties such as taste, color, and smell. These properties give only very limited information about the essence. In the present work, the essential oil was obtained with a yield of 0.016% and a viscous aspect, a yellow color and a weak smell. The essential oil extracted from the root part of the same plant in Iran was yellow in color with a yield of 0.2% [194]. Previous research has shown that environmental conditions in different geographical regions such as temperature, precipitation, soil composition and altitude can significantly influence the yield of essential oil. The yield of essential oil can also be strongly affected by the genotype, the harvesting period, the place, the duration and the temperature of drying [227, 228].

It also reported that when plants grow in favorable conditions, they do not need to develop mechanisms to adapt to the environment, which favors the production of primary metabolites. On the other hand, a stressful environment can stimulate the regulation of the production of secondary metabolites including essential oils in plants [229].

In the current study and for the first time, the hydrosol extract was isolated from the roots of *Plumbago europaea* with a yield of 0.024%. It has a Needle-like crystals aspect with yellow to orange color and a particular strong smell.

III.2.2 Chemical composition :

The essential oil and hydrosol extract produced by hydrodistillation and liquid-liquid extraction techniques, respectively from dried roots of *P. europeae*, were analyzed using gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) techniques. The chemical compositions of both essential oil and hydrosol extract are shown in table 9.

Nine components representing 92.4% were determined in the essential oil (Table 9). Essential oils are complex mixtures made up of several compounds and among these many compounds, one usually dominates; it is called the main or the major compound. In general, an essential oil is a mixture of hydrocarbons and oxygenated compounds derived from these

hydrocarbons. In the current research, phenolic compounds constitute the main class of components accounting for 68.9% followed by non terpenic compounds (16.2%), monoterpene hydrocarbons (4.5%) and oxygenated monoterpenes (2.8%). The phenolic compound that has been identified was the plumbagin with a percentage of 68.6%. Limonene (3.5%), α-pinene (0.5%) and myrcene (0.5%) represent the monoterpene hydrocarbons class. Linalool (2.8%) was the only oxygenated monoterpenes class components found in the essential oil of *P. europeae*.

To the best of our knowledge the chemical profile of the essential oil from roots of *Plumbago europaea* has only been described in one research which investigated the chemical composition of the essential oil from *P. europaea* roots collected in Iran and disclosed the presence of 15 components[194]. The main components have been found be Plumbagin (69.1%), 1-octen-3-yl acetate (9%), limonene (5.7%), nonanal (2.5%) and *β*-bisabolene (2.1%) (34). Regarding the results of our study, the same major constituent (plumbagin) was found with practically the same percentage. However, there are some differences in the other components, which can be attributed to the geographical region, species and age of the plant. This should certainly result in a different chemical composition of the essential oils. Therefore, it can be stated that the same species/variety can give essential oils with different compositions [230, 231]. According to natural products research institutes, the chemical composition of essential oils is very fluctuating. Numerous research works indicate the impact of macroscopic and microscopic effects on the chemical composition of an essential oil. Indeed, its chemical composition can vary considerably depending on several parameters. It depends on many natural factors (genetics, location, maturity, soil, climate, etc.) and / or technical factors (the nature of storage, the extraction procedure) [232].

Table 9: Chemical composition of essential oil and hydrosol extract of *P. europea*

No [a]	Compounds	*Lit*/RI [b]	RIa[c]	RI_p[d]	*Essential oil*	*Hydrosol extract*	Identification [e]
1	α-Pinene	931	931	1022	0.5	-	RI, MS
2	Myrcene	980	979	1159	0.5	-	RI, MS
3	p-Cymene	1011	1013	1270	0.1	-	RI, MS
4	Limonene	1020	1023	1196	3.5	-	RI, MS
5	Linalol	1081	1094	1544	2.8	-	RI, MS
6	Nonanal	1083	1090	1394	16.2	tr	RI, MS
7	Thymol	1266	1278	2189	0.2	4.8	RI, MS
8	Carvacrol	1281	1278	2289	Tr	12.4	RI, MS
9	Plumbagin	1580	1600	2822	68.6	80,2	RI, MS, ref
% Identification					**92.4**	**97.4**	
% Oxygenated monoterpenes					2.8	-	
% Monoterpene hydrocarbons					4.5	-	
% Phenolic compounds					68.9	97.4	
% Non terpenic compounds					16.2	-	

[a]:Order of elution is given on apolar column (Rtx-1), [b]: Retention indices of literature on the apolar column (IRIa) reported from könig et al 2001, [c]: Retention indices on the apolar column Rtx-1 column RIa, [d]: Retention indices on the polar Rtx-wax column (RIp)

In general, hydrosol extract are rather complex mixtures containing traces of essential oils and several water-soluble components. It contains less than 1 g/L of dispersed essential oils, which give them organoleptic properties. In particular, they are composed of water condensed during distillation polar, oxygenated, aromatic oily components, hydrophilic, volatile components that form hydrogen bonds with water [233]. According to the reports of many researchers, the chemical composition of hydrolats and essential oils of plants always seems to be different, not only quantitatively but often qualitatively as well [233, 234].

In the current study, the hydrosol extract was produced using the liquid-liquid extraction method and GC-RI and GC-MS techniques were used to determine its chemical composition. As shown in (Table 9) four components were identified in the hydrosol extract of *P. europeae*, representing 97.4 % of the total extract composition. Hydrolate extract was solely represented by phenolic compounds with the percentage of 97.4%. The main components were plumbagin with a percentage of 80.2% followed by carvacrol (12.4%) and thymol (4.8%). A broad search in the literature review revealed that there was no research published on the chemical composition and biological activities of hydrosol extract from the roots of *Plumbago europea*. Our work is the first to address the extraction, chemical composition, and biological activities of the hydrosol extract from the roots of this local medicinal plant.

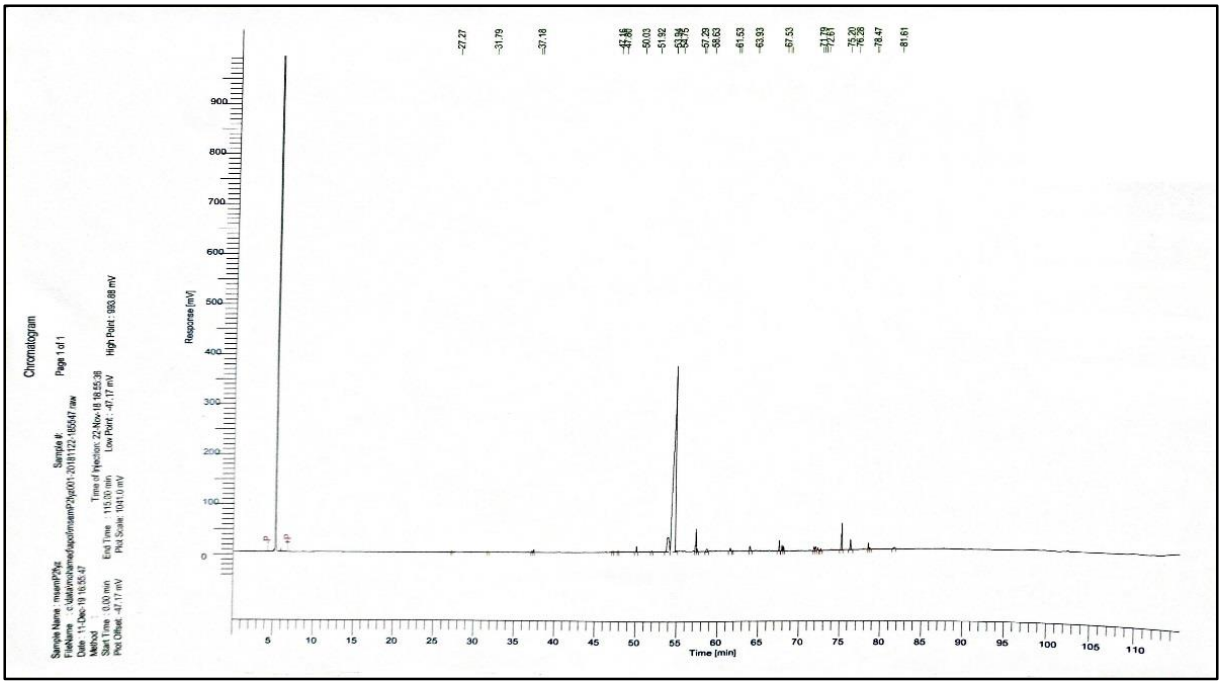


Figure 18: GC Chromatogram of Hydrosol extract.

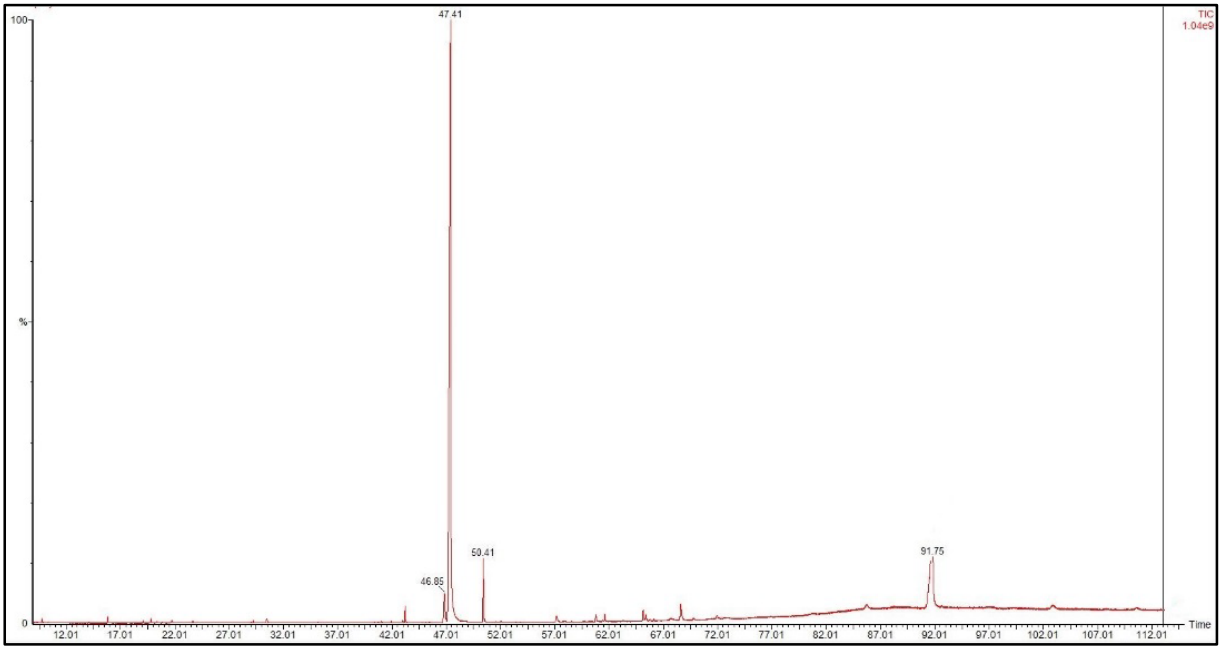


Figure 19: GC-MS Chromatogram of Hydrosol extract.

III.3 Chemical study of ethanolic extract:

III.3.1 Ethanolic extract preparation:

The selection of an adequate extraction method, followed by appropriate separation and identification processes, is essential for qualitative and quantitative analysis of bioactive compounds from plant materials. In the investigation of bioactive chemicals from natural matrices, the extraction method is the most important step. It has a substantial and critical impact on the ultimate result and outcome. It consists on the separation of medicinally active chemicals from plants using specific solvents and following standard procedures. The chemical properties of the extraction solvent are critical in favoring the compound solubility, the extraction selectivity. A variety of solvents have been used to extract phytochemicals. The polarity of the solute of interest is taken into account when choosing and selecting them [83].

Conventional extraction methods with organic solvents, as maceration or hot extraction in Soxhlet apparatus, are widely used to obtain plant extracts. However, prolonged heating time has been shown to promote plumbagin degradation [235].The amount of dried plant extract depends on the analytical technique that will be employed. Other parameters should be considered such as type of solvent, the organic solvent volume, the temperature and the duration of extractive process duration.

In this part of our work, the extraction from the plant materials was performed by maceration at 37°C for 72h in ethanol. The volume of solvent must be sufficient to keep the plant material immersed during the entire extraction process. The optimum solid: liquid ratios most often found in the literature are generally between 1/10 and 1/50 [236]. The ratio of 1:10 was chosen for the current study. The ethanolic extract obtained was brown in color with a yield of 10%.

III.3.2 Fractionation and isolation of compounds from the ethanolic extract:

The crude extract obtained by different extraction techniques are very complex and contain a large variety of different natural compounds that require further purification and separation to obtain the pure active fraction of the natural products. The separation process depends on the physical and chemical properties of each natural product. Different chromatographic techniques

such as thin layer chromatography (TLC), column chromatography (CC) and preparative TLC were commonly used for obtaining pure natural products from a complex mixture. In this separation technique, silica gel particle is used as the stationary phase and solvent with different polarity are used as mobile phase. The polarity of the mobile phase plays an important role in the isolation of pure chemical components from crude extracts using various chromatographic systems.

In the present work, fractions of 15 ml were collected separately and subjected to TLC to detect the presence of phytocompounds. Similar fractions (with the same Rf value) were pooled and dried using rotary evaporator at 45°C.This fractionation resulted in three main fractions named F1, F2 and F3 (as shown in table10). The dry weight of the fractions was measured., the F2 fraction was the main fraction with a weight of 1.78g. It is a dark brown solid. Fraction F3 was a light brown solid with a mass of 0.827g. The fraction F1 was yellow viscous fraction with a mass of 0.741 g.

Tableau 10: Results of separation by column chromatography of the ethanolic extract.

Fraction	Weight (g)	Color and consistence
F1	0.741	Yellow, viscous
F2	1.78	Dark brown, solid
F3	0.87	Light brown, solid

III.3.3 GC-MS analysis of Fraction F1 and F2:

Various bioactive compounds in medicinal plants possess stimulating pharmacological effects, such as antibacterial, antifungal, anticancer, anti-inflammatory and antioxidant activities. Herbal medicines are usually made from raw plant extracts, which are complex mixtures of different phytochemicals. These phytochemicals have unique and complex structures and can be used to treat persistent and infectious diseases. A large number of biologically active secondary metabolites exist in various plant species, but only a small fraction of them have been studied and identified as significant sources of biologically active agents. It is important to develop suitable screening methods when searching for new compounds [237, 238].

In the present study, the GC-MS technique was adopted for the detection and identification of phytochemicals present in the ethanolic extract prepared from roots of *Plumbago europaea*. GC-MS analysis of fraction (F1) revealed the presence of thirty-eight compounds representing 100% of the total fraction (Table 11, Fig 24).

Table 11: Chemical composition of the fraction F1 of the ethanolic extract isolated from the roots of *Plumbago europaea*

P.N°	Compound	Retention time (RT)	% Content
1	Dodecane	11.775	0.20
2	Butylated Hydroxytoluene	12.147	0.51
3	Phenol, 2,4-bis(1,1-dimethylethyl)	12.256	0.81
4	Hexadecane	13.193	0.21
5	**Plumbagin**	13.862	**11.32**
6	4-Methyl Benzamide, N-(3,4-methylenedioxy benzyl idenamino)	14.022	0.18
7	2,6,11-Trimethyl dodecane	14.273	0.28
8	Heptane, 2,2,4,6,6-pentamethyl-	14.342	0.36
9	4'-Butoxyacetophenone	14.805	1.49
10	3,5-Di-tert-butyl-4-hydroxybenzaldehyde	15.233	0.39
11	Octadecane	15.433	0.49
12	(2-Hydroxy-3-oxo-1,4,6-cycloheptatrienyl)-p-benzoquinone	15.719	0.23
13	6-Methylthieno[2,3-b]pyridine	16.256	0.52
14	1-Iodoundecane	16.508	0.57
15	pentadecanoic acid 13-methyl- methyl ester	16.776	0.43
16	Octadecane, 5,14-dibutyl	16.936	0.25
17	**n-Hexadecanoic acid**	17.331	**1.38**
18	Undecanoic acid, 2,4,6-trimethyl- methyl ester	17.451	0.60
19	**6,11-eicosadienoic acid methyl ester**	19.034	**3.40**
20	Octadecanoic acid	19.183	0.48
21	Docosane	19.308	0.34
22	Decane, 3,8-dimethyl-	20.177	0.15
23	methoxyacetic acid 2-pentadecyl ester	20.360	0.16
24	Triacontane	21.012	0.40
25	**Eicosane**	21.817	**1.01**
26	Tetradecane, 2-methyl-	22.040	0.65
27	**1,2-benzenedicarboxylic acid mono(2-ethylhexyl) ester**	22.269	**40.32**
28	1-Heptadecanamine	22.395	0.53
29	**Phosphine imide, P,P,P-triphenyl-**	22.572	**17.50**
30	Heptacosane	23.332	0.59
31	Hexadecane, 2-methyl-	24.047	0.44
32	Nonacosane	24.744	0.59
33	Triacontane	25.430	0.25
34	Obscurinervan-21-one	26.264	0.53
35	Stigmastan-3,5-diene	26.441	0.51
36	gamma.-Sitosterol	28.888	0.83
37	**7-Benzyl-8-(methylthio)theophyllin**	32.391	**9.38**
38	**benzenepropanoic acid 3 5-bis(1 1-dimethylethyl)-4-hydroxy-octadecyl ester**	32.928	**1.30**

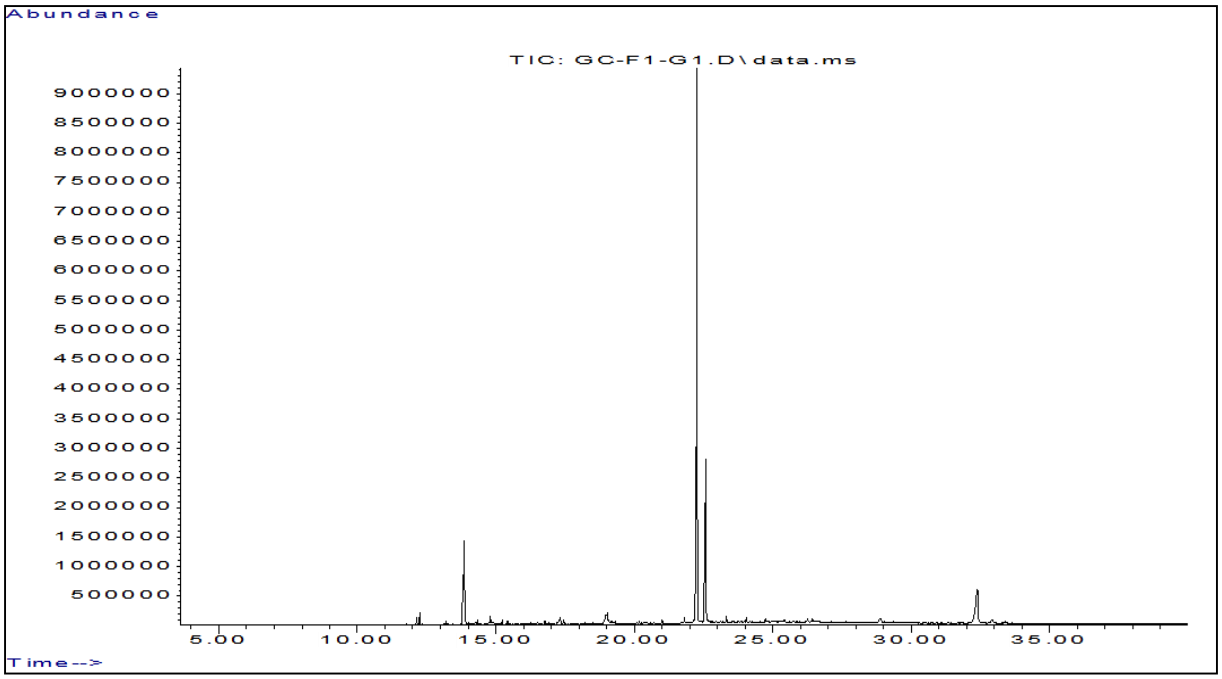


Figure 20: GC-MS Chromatogram of F1

The components with their retention time and percentage are presented in table 11. GC-MS analysis of F1 showed that the main components of this fraction were the mono(2-ethylhexyl) ester of 1,2-benzenedicarboxylic acid (40.32%), the imide of phosphine, P,P,P-triphenyl- (17.50%), plumbagin (11.32%), 7-benzyl-8-(methylthio) theophylline (9.38%), acid methyl ester 6,11-eicosadienoic acid (3.40%), n-hexadecanoic acid (1.38%), benzenepropanoic acid 3 5-bis(1 1-dimethylethyl)-4-hydroxy-octadecyl ester (1.30%) and eicosane (1.01%).

The GC-MS chromatogram of the compounds detected in fraction F2 was shown in Figure 21. The identified compounds are Stigmastan-3,5-diene as the major compound with a percentage of 43.03% followed by the (1,5) Naphthaleno (2,6) pyridinophane,17-(dimethylamino)-1,12-bis(methylthio)-, (Z) (32.14%). Ergosta-7,22-dien-6-one, 3-hydroxy, O-methyloxime, (3.beta.,5.alpha.,22E)- (14.08%) and N'-[(2-Hydroxy-1-naphthyl)methylene]-2-(4-octyloxyphenoxy)acethydrazide (10.75%) (Table 12).

Table 12: Chemical composition of the fraction F2 of the ethanolic extract isolated from the roots of *Plumbago europaea*

P.N°	Compound	Retention time (RT)	% content
1	Ergosta-7,22-dien-6-one, 3-hydroxy, O-methyloxime, (3.beta.,5.alpha .,22E)-	25.341	14.08
2	(1,5)Naphthaleno (2,6) pyridinophane,17-(dimethylamino)-1,12-bis(methylthio)-, (Z)-	26.249	32.14
3	**Stigmastan-3,5-diene**	26.427	**43.03**
4	N'-[(2-Hydroxy-1-naphthyl)methylene]-2-(4-octyloxyphenoxy)acethydrazide	28.838	10.75

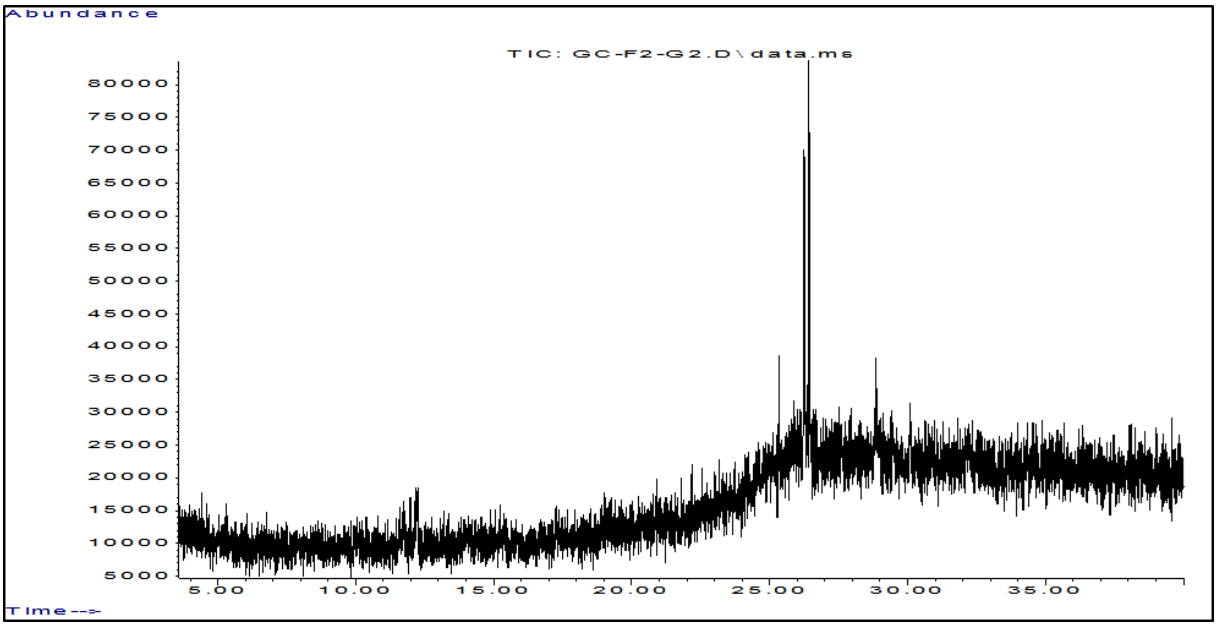


Figure 21: GC-MS Chromatogram of F2

To the best of our knowledge, our research is the first to address the fractionation and purification of the ethanolic extract prepared from roots *of Plumbago europaea.* The results obtained show that the studied roots extract contain compounds belonging to different chemical classes such as: naphthoquinones, fatty acids and esters, carboxylic acids, steroids, alkanes, alkaloids and other compounds. GC-MS analysis of whole plant extract collected from the Nablus region in Palestine declared the presence of 59 compounds of which Plumbagin, β-bisabolene, nonanal, limonene, and 1-octen-3-yl acetate were found to be the major components [239].

The plant *Plumbago europaea* belongs to family Plumbaginaceae of genus *Plumbago* that is characterized by the presence of Naphthoquinones. This class of phytochemicals and their derivatives containing hydroxyl group have been found in plants, such as *Juglandaceae, Plumbaginaceae, Ebenceae, and Lythraceae* etc. They exhibit wide range of pharmacological activities, such as antioxidant, antibacterial, antiviral, anticancer, antimalarial, and antifungal activities [240, 241]. Among them plumbagin was recognized as chemotaxonomic marker of the Plumbaginae tribe [193]. In the present study, the plumbagin was identified in fraction F1 with a percentage of 11,32%. It is reported that the roots and leaves of *Plumbago zeylanica*, which is a plant of the same family as the one studied in the present work, contain plumbagin, as the major component. It constitutes about 0.03% of dry weight of the roots, which has been identified as significant bioactive component related to several pharmacological activities[242]. The mono (2-ethylhexyl) ester of 1,2-benzenedicarboxylic acid was identified as the main compound of fraction F1 with a percentage of (40.32 %). This phthalate compounds was identified by GC-MS in the methanolic extract prepared from roots of *Plumbago zeylanica* collected in Nigeria with a percentage of 7.926% [243]. It has also been identified and isolated from different extracts of plants that belong to different botanical families [244-247]. It was detected as the main constituents of essential oil of *Moringa oleifera* [248], *Senna podocarpa* [249] and *Hertia cheirifolia* [245] where it was shown to exhibit good antimicrobial activities.

The phthalates are ubiquitous substances that have been used in polymers as plasticizers for many decades. They have been successfully isolated from many plants, algae, bacteria and fungi. In the isolation of natural products, phthalates are often considered as secondary metabolites of plants. As is often the case, after the isolation of the products from the plant, the bioactivity of these compounds was studied [250, 251]. At the same time, several studies in this field have shown that some phthalate compounds are very mobile in the environment, and have become invasive pollutants of our biosphere, penetrating water and soil [252]. Consequently, the presence of phthalates in plants may be related to environmental pollution. The production of phthalates in plants is still questionable, and the case for discussion among scientists and the detailed, clear and plausible biological genetic pathways if phthalates are natural products have not yet been submitted. It could be shown with a fungal enzyme that dibutyl phthalate could be produced from glucose, indicating that a natural shikimic acid pathway to phthalates may exist [250]. Further studies on the presence of phthalates in plants will be very interesting and will help to understand whether these compounds are environmental pollutants or can be considered plant metabolites.

According to the GC-MS results, the F2 fraction is characterized by the presence of only steroids with a percentage of 57.11% and naphthalene derivatives with a percentage 42.89%. Different compounds belonging to these two classes of phytochemicals have been identified previously in different parts of *Plumbago zeylanica* [242, 253].Several reports in the literature have shown that these two classes of phytochemicals displayed important biological activities. Plant steroids play important physiological functions within plants and exhibit a variety of pharmacological activities: antimicrobial, antifungal, anti-tumor, anti-inflammatory, hepatoprotective, hypocholesterolemic, immunosuppressive and neuroprotective activity [254-256]. Like steroid naphthalene derivatives also displayed important biological activities, such as wound healing, coronary vasodilating, HIV inhibitory, prothrombin decreasing, antimicrobial, antifertility, topoisomerase II inhibitory, antifungal, and anticancer activities [257-259].

The comparison of the chemical composition of plant extracts prepared from the same plant material growing in different regions or from different plants belonging the same botanical family revealed significant qualitative and quantitative differences between them. These variations can be attributed to genetic variability and/or different geographical/environmental conditions, different procedures used for extraction and different analytical procedures. In many investigations scientists confirm that the concentration and composition of secondary metabolites depend on the plant species and on external factors, such as climatic conditions, water and temperature stress, or infections [260]. It is revealed that the hard climatic conditions of the areas where the plants grow (high temperature, high sun exposure, drought and salinity) generally stimulate the biosynthesis of secondary metabolites [261]. For all these reasons, diversity of active compounds present in plants determine the chemical and biological activity of the plant extracts.

III.4 Biological study of essential oil, hydrolsol extract ethanolic extract and purified fractions:

III.4.1 Antioxidant activity:

Natural antioxidants can be found in the wood, bark, stems, pods, leaves, fruit, roots, flowers, pollen, and seeds of all higher plants. Typically, these are phenolic or polyphenolic chemicals. These plants have a wide range of antioxidant activity, from very low to very high.

Natural antioxidants have the ability to act as reducing agents, free radical scavengers, complexes of pro-oxidant metals, and quenchers of singlet oxygen production [262]. Natural antioxidants have been shown to be beneficial in a variety of complications such as cancer, burn, diabetes, hyper-lipidemia and amnesia [263, 264].

In this research, three most widely used assays, namely 2,2-diphenyl-l-1- picrylhydrazil (DPPH), ferric reducing antioxidant power (FRAP) methods and β-carotene bleaching assay were applied to evaluate the antioxidant potentials of the essential oil and hydrosol extracted from the roots of *P.europeae*. In the other hand, DPPH and FRAP assays were used to evaluate the antioxidant potentials of ethanolic extract and purified fractions F1, F2 and F3.

III.4.1.1 DPPH free-radical scavenging capacity, ferric reducing power and β-carotene bleaching capacity of essential oil and hydrosol extract:

The hydrogen atoms or electron-donating ability of the tested essential oils and hydrosol extract was determined from the bleaching of purple-colored ethanol solution of DPPH. The obtained results are shown in Table 13. The activity of the essential oil and hydrosol extract is proportional to the concentrations and the lower IC_{50} value reflects better protective action. The essential oil and the hydrosol extract were able to reduce the stable, purple-colored radical DPPH into yellow-colored DPPH-H, reaching 50% of reduction with an IC_{50} of 2.72 and 2.98 mg /ml respectively. The free radical scavenging capacity of both essential oil and hydrosol extract was lower than the free radical scavenging capacity of Ascorbic acid used as positive control or standard antioxidant (IC_{50}= 63µg/ml).

Table 13: 2,2-diphenyl-1-picrylhydrazil (DPPH) radical-scavenging activities (%) of essential oil (EO) and hydrosol extract (HY) from *P. europaea* roots

Extracts (mg/ml)	DPPH radical scavenging activity (%)	IC_{50}(mg/ml)*
Essential oil		
4.5	**76.44**	
2.25	**46 .57**	**2.72**
1.125	**23.55**	
0.5626	**17.33**	
0.28125	**7.46**	
Hydrosol extract		
6	**90.49**	
3	**57.8**	**2.98**
1.5	**30.94**	
0.75	**17.42**	
0.375	**4.39**	
Ascorbic acid		
0.0125	**86.92**	
0.00625	**57.80**	**0.0063**
0.003125	**30.94**	
0.001563	**17.42**	
0.0007815	**7.39**	

* the inhibitory concentration of the extract required to decrease the initial concentration of the DPPH radical by 50%.

In the present study the reducing power of both essential oil and hydrosol extract of *Plumbago europae*a was positively correlated to their concentrations as shown in figure 22 and absorbance was found to increase with increasing concentration. In comparison with ascorbic acid as positive control, both essential oil and hydrosol extract showed lower reducing power. The antioxidant activity could be attributed to different mechanisms, such as prevention of chain initiation, decomposition of peroxides, and prevention of continued hydrogen abstraction, free radical-scavenging, reducing capacity, and binding of transition metal ion catalysts [265]. In the present study both essential oil and hydrosol extract isolated from roots of *Plumbago europaea s*howed good to moderate free radical-scavenging and reducing capacity. In FRAP assay, they showed the presence of a reductive effects, which increased with an increase in concentration. The essential oil was slightly more active than the hydrosol extract as shown in figure 22. However, ascorbic acid used as positive control was more potent on reducing power compared with the tested extracts.

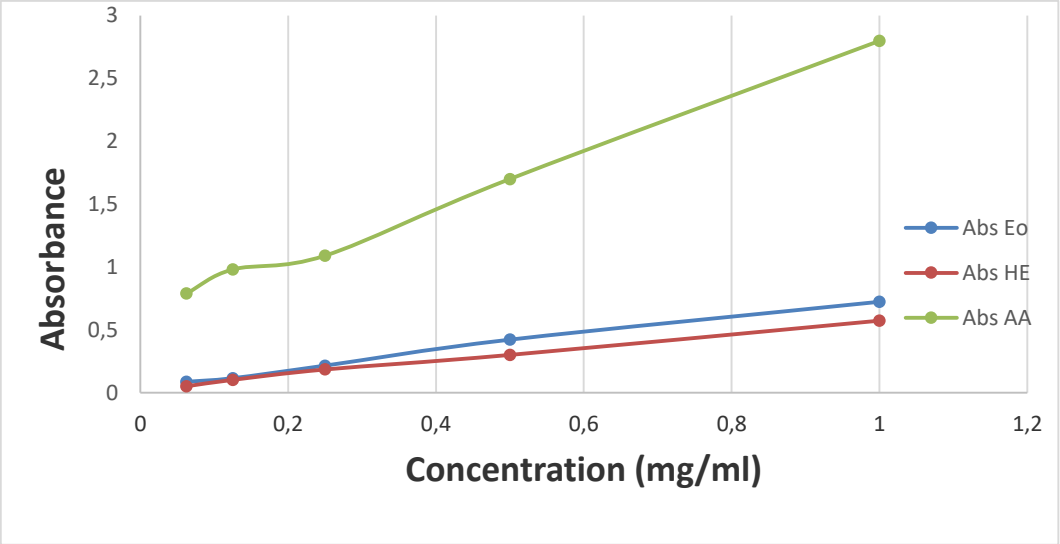


Figure 22: **Reducing power activities of Essential oil (Eo) and Hydrosol extract (HE) and ascorbic acid (AA)**

The antioxidant activity of essential oil, hydrosol extract and BHT as the antioxidant of reference was estimated also using the β-carotene bleaching assay by measuring the inhibition of the oxidative degradation of β-carotene by the oxidation products of linoleic acid.

The percentage of inhibition of essential oil, hydrosol extract and the synthetic antioxidant and the concentrations that inhibit 50% of the combined oxidation of linoleic acid and β-carotene (IC_{50}) are calculated graphically and are shown in table 14.

The results of table 14 reveal that the percentage of inhibition of the oxidation of β-carotene is proportional to the concentration. All the tested extracts (essential oil, hydrosol extract and BHT) inhibit β-carotene bleaching at different values through the scavenging of free radicals. The essential oil and BHT at different concentrations inhibit in a revealing way the free radicals with IC_{50} of 2.46 mg/ml and 0.084 mg/ml respectively, but the best antioxidant activity is attributed to the antioxidant of reference, an inhibition of 57.56% was recorded for a concentration of 0.1 mg/ml. The hydrosol extract was the less active in inhibiting the coupled oxidation of linoleic acid and β-carotene with an IC_{50} of 5.40 mg/ml.

As for the DPPH test. The calculated IC_{50} express the concentrations of extracts capable of reducing 50% of the peroxide radicals. The lower the IC_{50} value, the greater the antioxidant power.

Table 14: Antioxidant activity of essential oil, hydrosol extract and BHT expressed as IC_{50}

Extracts (mg/ml)	Inhibition (%)	IC_{50}(mg/ml)*
Essential oil		
4	**72.88**	**2.46**
2.5	**52.36**	
2	**41.24**	
1.5	**28.58**	
1	**12.39**	
Hydrosol extract		
6	**51.87**	**5.40**
4	**33.67**	
2.5	**20.88**	
2	**14.55**	
1	**9.2**	
BHT		
0.1	**57.56**	**0.084**
0.05	**31.56**	
0.025	**19.36**	
0.0125	**10.18**	

It is revealed that an extract that delays or inhibits β-carotene bleaching can be described as a free radical scavenger and a primary antioxidant. According to several authors, this test appears to be very useful as a mimetic model of lipid peroxidation in biological membranes. In addition to this, the decomposition of fatty acids is one of the main causes of food spoilage. The fight against the oxidation of lipids by the use of natural preservatives represents therefore a considerable challenge for the food industries. It is in this context that this test is commonly used to evaluate the antioxidant activity of natural products [211, 266].

Since this test is based on an emulsion system of lipids in water, Frankel and Meyer in 2000 proposed that apolar antioxidants exhibit greater antioxidant properties than polar antioxidants. In this system, apolar antioxidants are concentrated in the lipid-water interface, thus preventing the formation of lipid radicals and, consequently, the oxidation of β-carotene. While polar antioxidants remain diluted in the aqueous phase and are, therefore, less efficient [267]. This may explain the fact that the essential oil was more active than the hydrosol extract in this assay.

It is possible to explain the obtained antioxidant activity by the chemical properties of essential oil and hydrosol extract. Both of them contain plumbagin as the main component. Plumbagin or 5-hydroxy-2-methyl-1, 4-naphthoquinone is a plant-based secondary metabolite belonging to 1,4-naphthoquinone. It is the most important class in the quinone family. It is

mainly found in root, leaf and stem bark of some plant families including Plumbaginaceae, Ebenceae, Dioncophyllaceae, Ancestrocladaceae and Droseraceae. The chemical formula of plumbagin is $C_{11}H_8O_3$. It is a yellow crystal with a molecular weight of 188.18 g/mole. This natural component has a number of distinct biological activities including anticancer, anti-inflammatory, anti-oxidant, antifungal, neuroprotective, hypolipidemic, and antibacterial activities [268] **.** Antioxidant activity of plumbagin isolated from solvent extract of the aerial part and roots from *P. Europaea* growing in a Sardinian, Italy was investigated using DPPH technique and IC_{50} was found to be 4.5 mg/ml which is obviously superior to that obtained in our research with the tested essential oil and hydrosol extract from roots of *Plumbago europaea* collected in our region [193]**.** Otherwise, the essential oil and hydrosol extract were more effective in reducing the stable free radical DPPH than the isolated plumbagin in the study mentioned above, and this is may be due to the presence of plumbagin in combination with other compounds although they are present in small quantities they can act in synergy with the major component.

Previous research revealed that hydrocarbon and oxygenated monoterpenes exhibited remarkable in vitro antioxidant activity with a very promising IC_{50} values [269]. thymol and carvacrol were able to reduce the stable, purple-colored DPPH radical into yellow-colored DPPH-H, reaching 50 % of reduction with interesting IC_{50} value of 70.06 µg/mL and 79.75 µg/mL, respectively [270]. Also an additive antioxidant effect obtained when thymol and carvacrol were combined at low concentrations[271]. Finally, the moderate antioxidant activity of plumbagin can be explained by the fact that hydrogen atom of hydroxyl group is not available for interaction, as it is already involved in a strong intramolecular hydrogen bonding (between the hydrogen atom of –OH group and oxygen atom of the adjacent ketone group [240].

III.4.1.2 DPPH free-radical scavenging capacity, ferric reducing power of ethanolic extract, Fractions F1, F2 and F3:

The hydrogen atoms or electron-donating ability of the tested ethanolic extract and isolated fractions was determined from the bleaching of purple-colored methanol solution of DPPH. The obtained results are shown in Table 15.

Table 15: 2,2-diphenyl-1-picrylhydrazil (DPPH) radical-scavenging activities (%) of Ethanolic extract and purified fractions F1, F2 and F3.

Extracts (mg/ml)	DPPH radical scavenging activity (%)	IC_{50}(mg/ml)
Ethanolic extract		
0.020	**63.48**	
0.01	**40 .13**	**0.0146**
0.005	**22.08**	
0.0025	**16.55**	
0.00125	**11.97**	
Fraction (F1)		
3	**68,32**	
0,6	**30,44**	**1.96**
0,3	**21,62**	
0,15	**14,15**	
0,075	**9,81**	
Fraction (F2)		
0.016	**63.40**	
0.008	**4.58**	**0.0115**
0.004	**24.33**	
0.002	**16.80**	
0.001	**7.67**	
Fraction (F3)		
0.125	**77.14**	
0.0625	**47.33**	
0.0312	**33.40**	**0.0726**
0.0156	**18.14**	
0.0078	**8.60**	
Ascorbic acid		
0.0125	**82,42**	
0.00625	**51,42**	**0.0069**
0.003125	**28,35**	
0.001563	**15,14**	
0.0007815	**6,01**	

The activity of the EE and F1, F2 and F3 is proportional to the concentrations and the lower IC_{50} value reflects better protective action. The ethanolic extract was able to reduce the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) to the yellow-colored diphenyl picrylhydrazine with an IC_{50} of 0.0146 mg /ml. The pure fractions F1, F2 and F3were able to reduce the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) to the yellow-colored diphenyl picrylhydrazine with an IC_{50} of 1.96, 0.0115 and 0.0726 mg/ml respectively. The free radical scavenging capacity of both ethanolic extract and pure fractions was lower than the free radical scavenging capacity of Ascorbic acid (AA) used as positive control. The free radical

scavenging capacity of ethanolic extract was close to that of purified fraction F2 with interesting IC_{50} values of 0.0146 mg /ml and 0.0115 mg/ml respectively. Furthermore, fraction F2 was more effective in reducing the stable free radical DPPH in comparison with fraction F1 and F3 and F1 showed a moderate antioxidant activity with an IC_{50} significantly higher than that of ethanolic extract and F2 and F3. It is possible to explain the obtained antioxidant activity by the fact that the F2 fraction represents the majority part of the ethanolic extract (44%) in comparison with F1 (18.52%) and F3 (21.75%) also; these three fractions are combined in the crude extract and can act in synergy.

The antioxidant capacity of ethanolic extract and fractions F1, F2 and F3 was also evaluated using Ferric-reducing power (FRAP) assay. This approach evaluates the ability of a substance to reduce Fe^{+3} to Fe^{+2}, which is measured by the formation of a colored complex with potassium ferricyanide that can be read spectrophotometrically at 700 nm. The obtained results shown in figure 23 and revealed that the reducing power of both crude extract and purified fraction of *plumbago europaea* was positively correlated to their concentrations and absorbance was found to increase with increasing concentration. In comparison with ascorbic acid as positive control all tested samples showed lower reducing power with an interesting activity of crude extract and pure fraction F2.

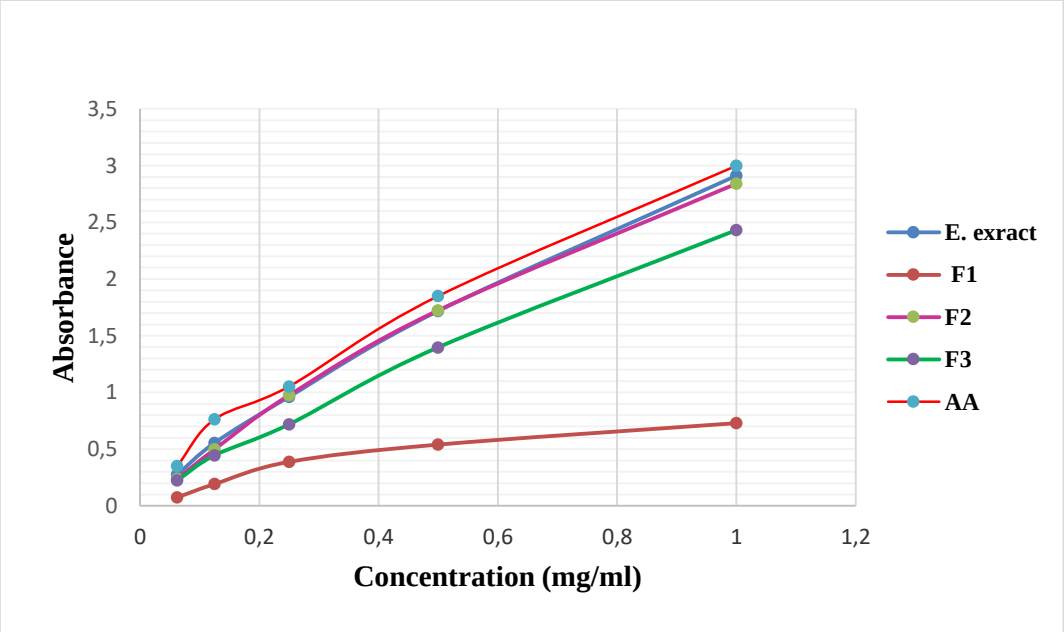


Figure 23: Reducing power activities of ethanolic extract and purified fractions F1, F2 and F3.

The moderate activity of fraction F1 was probably mainly due to its chemical composition. The main compound of this fraction was the mono (2-ethylhexyl) ester of 1,2-benzenedicarboxylic acid which was identified in different plant extracts such as *Polygonum chinense L, Desmodium gangeticum, Cadaba Trifoliata* and extracted from a marine derived *Streptomyces* sp and from an endophytic fungi and it has been reported to have an interesting antioxidant activity [247, 272-274]. This fraction also contains other compounds with different percentages and they can act in synergy with the major component such as the plumbagin that is known for its low antioxidant activity.

The interesting antioxidant activity of fraction F2 may be also attributed to it phytochemicals. This fraction is mainly composed of Stigmastan-3,5-diene which is a stigmasterol derivative. Stigmasterol is an unsaturated phytosterol that belong to the tetracyclic triterpene class. It represents one of the most commonly occurring plant sterols, found in a wide variety of natural sources. This plant sterol cannot be produced naturally by the human body thus, it can only be available through foods and diets such as vegetable oils, grains, seeds, and medicinal plants [275]. Numerous studies have been conducted that have demonstrated the antioxidant properties of herbal extracts containing this phytosterol. Stigmasterol has been found to attenuate excitotoxicity, mitochondrial dysfunction and DNA damage while decreasing ROS production. It increases the activities of antioxidant enzymes (catalase (CAT), superoxide dismutase (SOD)), and nitric oxide synthase enzymes (iNOS and nNOS), thereby providing neuroprotective and antitumor effects [276, 277].

III.4.2 Antimicrobial activity:

III.4.2.1 Antimicrobial activity of essential oil and hydrosol extract:

The antimicrobial activity of essential oil and hydrosol extract isolated from roots of *P. europaea* was determined against a large panel of microorganisms including 9 bacteria and 3 yeast. Their potencies were evaluated qualitatively by the presence or absence of the inhibition zones and quantitatively by the determination of their inhibition zone diameters and their minimum inhibitory concentrations values. The results obtained are presented in tables 16 and 17.

According to the obtained results, both essential oil and hydrosol extract possessed antibacterial activity of varying extent. The hydrosol extract was found to be more active than the essential oil against all tested bacteria. Moreover, disc diffusion data revealed that the essential oil showed the highest effectiveness against *M. luteus (ATCC 9341*) and *S. aureus (ATCC 25923)* with inhibition zones diameters of 15 and 23 mm respectively. However, no activity was exhibited against the three microorganisms *K. pneumoniae, P. aeruginosa and E. coli.* (Table16).

Table 16: Zones of inhibition of essential oil and hydrosol extract

	Microorganims	Zones of inhibition (mm)			
		EOs (0.5mg/disc)	**Hy (0.83mg/disc)**	**Gent (10 µg/disc)**	**Ampho B**
Gram-positive bacteria	*S. aureus*	15±1	40.33±0.577	33±0,577	-
	M. luteus	23±1	45±0.577	19±0.333	-
	L. monocytogenes	7±1	25±1	12±0,577	-
	E. faecalis	-	10.5±0,5	18,5±0,5	-
	B. cereus	10±0	31.66±0.577	22±0.70	-
Gram-negativebacteria	*E. coli*	0±0	11.66±0.577	23±0,5	-
	P. aeruginosa	6±1	10±1	25±0.0	-
	K. pneumoniae	0±0	11±1	15±0.70	-
	S. typhimurium	10±0.577	22.5±0.577	26.5±0.333	-
Yeast	*C. albicans ATCC 26790*	43±0.333	51±0.0	-	30±0.333
	C. albicans ATCC 10 231	39±0.666	51±0.333	-	32±0.333
	C. albicans IP444	0	15 ± 1	-	30±0.333

Furthermore, the hydrosol extract was found to inhibit the growth of bacterial strains, with inhibition zones diameters ranging from 10 to 45mm, depending on the susceptibility of the studied microorganism. In addition, Gram-positive strains were more susceptible to the hydrosol extract under testing than Gram-negative strains. *Micrococcus luteus ATCC 9341*, *Staphylococcus aureus ATCC 25923*, and *Bacillus cereus ATCC 10876* were the most sensitive species to the hydrosol extract, with inhibition zones of 45, 40, and 31 mm, respectively.

A potential activity against the three-tested yeast was observed. The highest activity of hydrosol extract was recorded against *Candida albicans ATCC 26790* and *Candida albicans ATCC 10 231* with inhibition zone diameter of 51mm. Even the essential oil inhibited the growth of these two yeasts with important inhibition zones diameters (43 and 39 mm respectively) but no activity against *C. albicans IP444* was recorded.

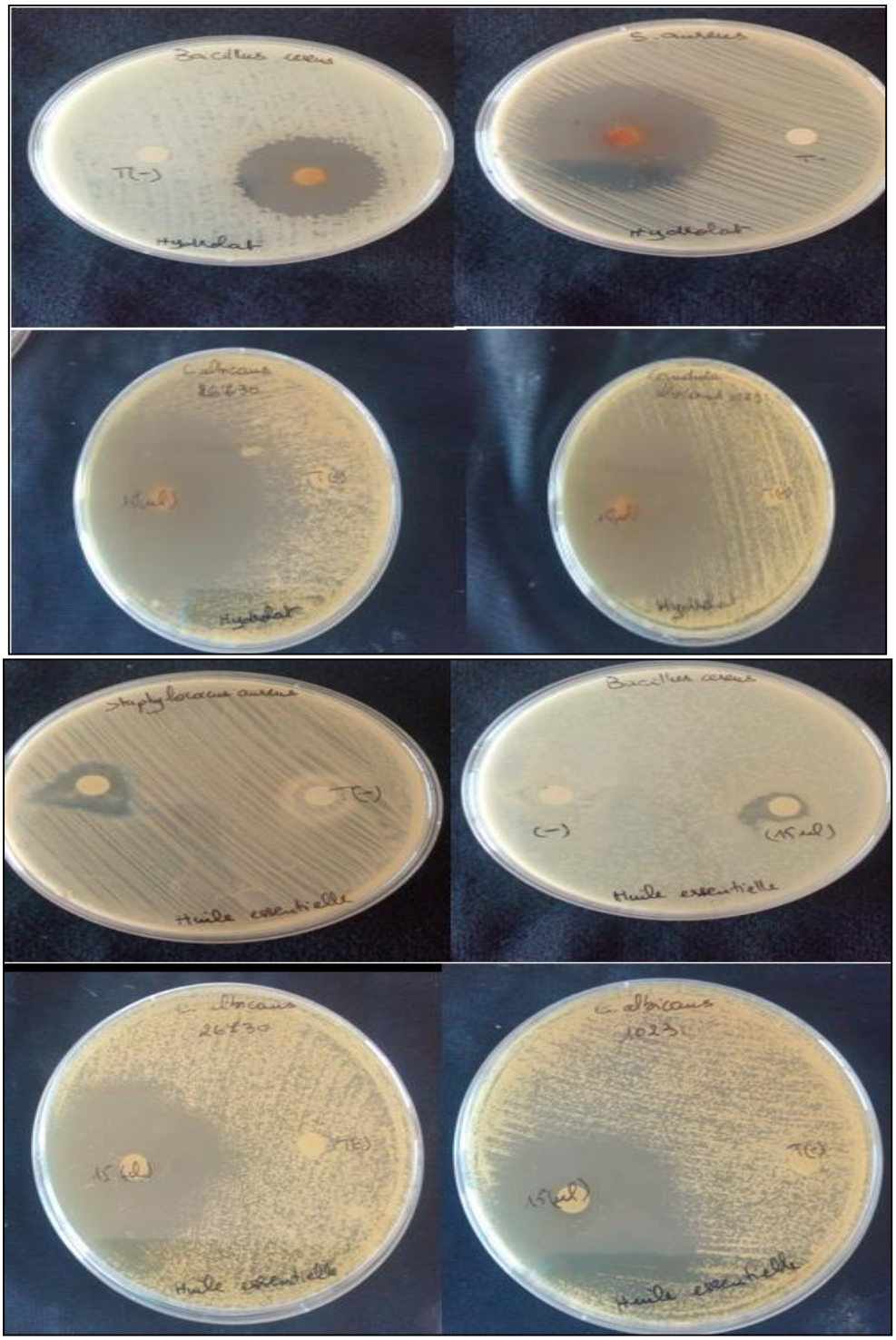


Figure 24: Inhibition zones diameters of essential oil and hydrosol extract

In this part of the present work, the minimum inhibitory concentrations (MICs) were determined using micro well dilution assay to provide more accurate data on the antimicrobial properties of the studied essential oil and hydrosol extract. When compared to Gentamicin and

Amphotericin B, the hydrosol extract of *P. europeae* had a notable inhibitory effect on the growth of the studied microorganisms.

The most pronounced inhibitory effect of hydrosol extract was obtained against *S. aureus, L. monocytogenes, M. luteus,B. cereus and C. albicans* with MIC values of 19 µg/ml. In contrast, the hydrosol extract showed the lowest antibacterial activity toward *S. typhimurium* (156 µg/ml). While, essential oil exhibited a moderate to weak activity against the all tested microorganisms with MICs exceeding 1000 µg/ml. Both essential oil and Hydrosol extract of *P. europea* roots showed the best antibacterial effect in the MIC assay on Gram-positive tested bacteria.

Tableau 17: Minimum inhibitory concentration of essential oil and hydrosol extract

	Microorganims	MIC (µg/mL)			
		Eos	**Hy**	**Gent**	**Ampho B**
Gram-positive bacteria	*S. aureus*	> 1000±0.000	19±0.000	0.5±0.000	-
	M. luteus	>2000 ±0.000	19±0.000	8±0.000	-
	L. monocytogenes	>4000±0.000	19±0.000	8±0.000	-
	E. faecalis	-	78±0.000	16±0.000	-
	B. cereus	>3120±0.000	19±0.000	0.5±0.000	-
Gram-negativebacteria	*E. coli*	-	78±0.000	0.5±0.000	-
	P. aeruginosa	-	78±0.000	0.5±0.000	-
	K. pneumoniae	-	78±0.000	8±0.000	-
	S. typhimurium	>4000±0.000	156±0.000	0.25±0.000	-
Yeast	*C. albicans ATCC 26790*	>4000 ±0.000	19±0.000	-	4±0.000
	C. albicans ATCC 10 231	>4000±0.000	19±0.000	-	8±0.000
	C. albicansIP444	-	19±0.000	-	8±0.000

The biological activity of plant extracts, including hydrosol extract, is well recognized and used since antiquity. It is attributed to the presence of constituents that characterize them by their functional groups. The capacity of hydrosols to control microbial growth differs depending on their chemical composition and the microbial target[233].

From the study and analysis of the above-mentioned results, as well as the observation of the chemical composition of both essential oil and the hydrosol extract (Table 9), it can be concluded that the effective antimicrobial activities of the hydrosol extract is most probably due to the presence of plumbagin as the major compound with a high concentration (80.2%). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a secondary metabolite founded in the families: Plumbagenaeace, Droseraceae and Ebenceae. Pharmacological screening of various

extracts from Plumbago species have revealed that this compound is a biologically active with a number of pharmacological properties [278-280].

The results of the current study provide evidence that the hydrosol extract can act as an antimicrobial towards *B. cereus, S. aureus, M. luteus* and *L. monocytogene* with MIC value of 19 µg/ml that is near to that obtained with the plumbagin isolated from *Aristea ecklonii* (16µg/ml) [281]. However, this minimum inhibitory concentration against *B. cereus, S. aureus, M. luteus* and *L. monocytogene* founded in the present work was *higher* than that obtained from the plumbagin isolated from other *Plumbago* species in previous research [282-284]. In the present study, it was found that the hydrosol extract contained the plumbagin in mixture with other phenolic compounds such as the carvacrol and the thymol, which may have an antagonistic effect on the antimicrobial activity of the plumbagin.

On the other side, the essential oil showed weak antibacterial and antifungal activity against all the tested microorganisms. The activity of an essential oil is directly related to its chemical composition. It is considered that essential oils rich in oxygenated compounds provide the best activities and conversely those rich in hydrocarbon constituents have a moderate activity. Numerous studies have demonstrated the correlation between antibacterial activity and the chemical profile of an essential oil. These works classify the activity exerted by the compounds of essential oils in the following order: phenols > alcohols > aldehydes > ketones > ethers > hydrocarbons [285, 286].

In the current study, the decrease in effectiveness of the essential oil extracted from roots of *P. europaea* may be explained by the fact that the major compound of the essential oil which is the plumbagin was found in mixture associated with non-active compounds such as nonanal. The mechanism of action of essential oils against microorganisms is complex and not fully understood. It is generally accepted that the antimicrobial activity of essential oils is related to their lipophilic or hydrophilic properties.

The mechanism of action of antimicrobial drugs also depends on the type of microorganism, mainly related to its cell wall structure and outer membrane arrangement. Gram-negative bacteria, such as *Pseudomonas aeruginosa*, exhibit intrinsic resistance to a variety of essential oils associated with the hydrophilic surface of their outer membrane, which is rich in lipopolysaccharide molecules. A permeability barrier is formed against toxic agents. Small hydrophilic molecules are not hindered from crossing the outer membrane due to the action of the abundant porin proteins. On the other hand, hydrophobic macromolecules, such as the constituents of essential oils, cannot penetrate the barrier [287, 288].

It has been shown that the efficacy of an antibacterial agent usually increases with its lipophilic properties due to its effect on cytomembranes. On the other hand, essential oils generally have low water solubility, which prevents them from attaining toxic levels in cell membranes, even though these oils have a good affinity for cell membranes [289].

Moreover, it is suggested that the limited water solubility of essential oils limits the diffusion of their components through the agar medium, which consequently diminish their antimicrobial activity [290]. Several different mechanisms of antimicrobial activity of plumbagin have been reported in the literature. According to Asche et al. plumbagin intercalates into the DNA, inducing single- or double-strand breaks[291]. Padhye et al. established the ability of plumbagin to chelate multiple trace metals, which may enhance its antimicrobial activities [280].

III.4.2.2 Antifungal activity of ethanolic extract and fraction F1, F2 and F3:

The antifungal activity of *P. europeae* ethanolic extract and isolated fractions F1, F2 and F3 were evaluated against 3 yeasts: *C. albicans (ATCC 26790), C. albicans (ATCC 10 231*) and *C. albicans (IP444).* Their potencies were qualitatively and quantitatively assessed by the presence or absence of the inhibition zones; moreover, their inhibition zone diameters and MIC values were determined. The results obtained are summarized in tables 18 and 19.

A very interesting activity against the three yeast *Candida albicans* was observed. The results obtained revealed that both ethanolic extract and purified fractions exhibited antifungal activity of varying magnitude. According to the inhibition zones diameter obtained, ethanolic extract showed highest antifungal activity against the tree tested yeast producing an inhibition zone diameter of 35.66 mm against *C. albicans (ATCC 26790),* 29.33 mm against *C. albicans (ATCC 10 231)* and 25.33±0,66 against *C. albicans (IP444*). With regard to the purified fraction, the fraction F1 turned out to be more active than the other tested. It showed highest growth inhibition against *C. albicans (ATCC 26790)* with inhibition zone diameter of 31 mm. The fraction F2 showed a similar antifungal activity as the crude extract. The fraction F3 seemed to be the least active with inhibition zone diameter of 19 mm against *C. albicans (ATCC 26790),* 12 mm against *C. albicans (ATCC 10 231)* and 15 MM against *C. albicans (IP444).*

Table 18: Zones of inhibition of ethanolic extract and fractions F1, F2 and F3

	Microorganims	Zones of inhibition (mm)				
		EE	**Fraction (F1)**	**Fraction (F2)**	**Fraction (F3)**	**Ampho B**
Yeast	*C. albicans ATCC 26790*	**35.66±0,3**	28±0.0	31±0.0	15±0.0	30±0.333
	C. albicans ATCC 10 231	**29.33±0,3**	23±0.333	25±0.0	12±0.0	32±0.333
	C. albicans IP444	**25.33±0,66**	20 ± 1	22±0.0	10±0.0	30±0.333

To have more precise data about the antifungal properties of the tested ethanolic extract and isolated fractions F1, F2 and F3, the minimum inhibitory concentrations (MIC) were determined and presented in table 19. The ethanolic extract of *P. europeae* exhibited remarkable inhibitory effect on the growth of tested yeasts compared to Amphotericin B. The most prominent inhibitory action of ethanolic extract was observed against *C. albicans IP444* with MIC values of 19 µg/ml followed by *C. albicans ATCC 10 231* and *C. albicans ATCC 26790* with MIC value of 39 µg/ml. Although the three purified fractions exhibited, a potential activity against the three-tested yeast with interesting MIC values (between 39 and 312 µg/ml) but it is still lower than that of the crude extract.

Table 19: Minimum inhibitory concentration of ethanolic extract and fractions F1, F2 and F3.

	Microorganims	Minimum inhibitory concentration (MIC) (µg/mL)				
		EE	**Fraction (F1)**	**Fraction (F2)**	**Fraction (F3)**	**Ampho B**
Yeast	*C. albicans ATCC 26790*	**19±0,00**	39±0.0	39±0.0	78±0.0	4±0.000
	C. albicans ATCC 10 231	**39±0,00**	78±0.333	48±0.0	96±0.0	8±0.000
	C. albicans IP444	**39±0,00**	78 ± 1	48±0.0	312±0.0	8±0.000

The present part of our study has been undertaken to determine the antifungal activity of ethanolic extract from roots of *P. europaea*. The significance of our study is particularly important keeping in view the growing resistance of the fungal species to commercially available drugs. Because of the emergence of many resistant strains against commonly used antimicrobial, the researchers are trying to evaluate some medicinal plants as the alternative of

antibiotics. In the current study, the potent antifungal activity obtained for the ethanolic extract and purified fractions against the three-tested yeast is mainly attributed to their effective phytochemicals and to the possibility of a synergy between the different compounds identified in the purified fractions.

III.4.2.3 Synergetic activity:

Combination drug therapy is a potential strategy for treating complicated disorders like cancer, inflammation, bacterial and fungal infections, and others. Combination therapy refers to the use of two or more components together to achieve a better therapeutic response against various clinical conditions, where one component may facilitate the pharmacological action of the other [292].

All infectious diseases are caused by bacteria, viruses, parasites and fungi, and are due to the complexity of the interaction that occur between the pathogen, the host and the environment. The development and discovery of antibiotics has made it possible to eradicate these infections that were ravaging humanity. However, their indiscriminate and inappropriate use has lead to the development of multi-resistant pathogens. One strategy used to overcome these resistance mechanisms is the use of drugs combination [293]. In a combination, the interaction between antimicrobials can result in three different outcomes i.e., synergistic, additive, or antagonistic. Synergy is obtained by combining two antimicrobial compounds producing antibacterial activity greater than the sum of the antibacterial activity of individual components. An additive effect is produced by combining antimicrobials producing an antimicrobial effect that is equal to the sum of the individual compounds. An antagonistic effect results in a decreased antimicrobial activity of two compounds in combination as compared with their individual antimicrobial activity [294].

In general, drug combinations have been demonstrated to be a critical feature of antimicrobial therapy, because they enhance the activity by acting synergistically or additively, reducing the required dose, reducing cost and adverse/toxic side effects and increasing range of activity [295]. Bertoni et al. (2006) showed that plants may contain antimicrobial components that can act synergistically with antibiotics, or other compounds that do not have inherent antibacterial activity but can sensitize pathogens to previously ineffective antibiotics. Combination therapy can be used to broaden the antimicrobial spectrum, preventing the

emergence of resistant strains, minimizing the toxicity, and achieving a synergistic antimicrobial activity [296].

Nowadays, studies on synergistic interactions between natural products and clinically relevant antimicrobial agents have been conducted and are showing increasing promise for the formulation of novel therapeutic strategies [297]. There are number of methods used to detect synergies. However, the checkerboard and time-kill curve methods are two of the most widely used techniques and the first one is a relatively easy to perform [219]. In the current research, the checkerboard method was used to evaluate the synergistic effect between the hydrosol extract and two antimicrobial drugs (Gentamicin and Amphotericin B) and between the ethanolic extract and Amphotericin B. In this technique, the assessment of the *in-vitro* synergistic effect between different antimicrobial drugs has been mainly based on evaluating and interpreting reported data from FIC index experiments. In many journals in the field of antimicrobials, synergy is defined as a FIC index value ≤ 0.5. In addition, indifference is defined as a FIC index value > 0.5 but ≤ 4. ($0.5 <$ FICI value ≤ 4), and antagonism is defined as a FIC index value > 4.

III.4.2.4 Evaluation of the synergistic effect of hydrosol extract with Gentamicin and amphotericin B:

The synergistic effect of hydrosol extract from roots of *P. europeae* with Gentamicin and Amphotericin B was studied and the obtained MICs and FIC index values are listed in tables 20 and 21. In the current study, an indifferent effect was produced between the hydrosol extract and Gentamicin against all tested microorganism, with a FIC index value between 1 and 1.5. An indifferent effect was also obtained between the hydrosol extract and Amphotericin B blend against *C. albicans* (ATCC 26790) and *C. albicans* (ATCC 10231), while a synergic effect was observed against *C. albicans* IP444 with FIC index of 0.375 (Table 20).

Although there was no synergistic effect, the association of hydrosol extract with gentamicin and amphotericin B improved their antimicrobial activity and growth inhibition of all tested bacteria and yeasts, and this was confirmed by the substantial decrease in MIC values of the antimicrobial agents. The MIC value of Gentamicin (0.5 µg / ml) against *S. aureus* (ATCC 25923), *B. cereus* (ATCC 10876) and *P. aeruginosa* (ATCC 27853) was lowered to 1/2 (combined MIC 0.25µg/mL), when used in combination with hydrosol extract of *P. europea* at MIC of 19, 9.5 and 39 µg/mL, respectively.

In the case of *E. coli* (ATCC 25912), *K. pneumoniae* (ATCC 700603) and *S. typhimurium* (ATCC 13311), this association was more effective as it lowered the MIC of Gentamicin to 1/8 of its initial value. Thus, the combined minimum inhibitory concentration of Gentamicin was reduced from 0.5 to 0.0625µg/mL for *E. coli*, from 8 to 1µg/mL for *K. pneumoniae* and *L. monocytogenes,* and from 0.25 to0.0312 µg/mL for *S. typhimurium*) (Table 20). Moreover, the MICs of amphotericin B against *C. albicans* (ATCC 26790) and *C. albicans* (ATCC 10 231), i.e. 4 and 8µg/mL respectively, were decreased to 1/2 and to 1/4 when associated with the hydrosol extract of *P. europea* at MIC values of 2.375 µg/mL and 9.5 µg/mL, respectively (Table 21).

Table 20: Hydrosol extract and Gentamicin - Fractional Inhibitory Concentration (FIC) and FIC Indices

	MICo	**MICc**	**FIC**	**FIC index**
Staphylococcus aureus (ATCC 25923)				
Hydrosol extract	19	19	1	1.5
Gentamicin	0.5	0.25	0.5	
*Micrococcus luteus (*ATCC 9341),				
Hydrosol extract	19	19	1	1.5
Gentamicin	8	4	0.5	
Listeria monocytogenes (ATCC 15313),				
Hydrosol extract	19	19	1	1.125
Gentamicin	8	1	0.125	
Enterococcus faecalis ATCC 49452				
Hydrosol extract	78	39	0.5	1
Gentamicin	16	8	0.5	
Bacillus cereus (ATCC 10876)				
Hydrosol extract	19	0.95	0.5	1
Gentamicin	0.5	0.25	0.5	
Escherichia coli (ATCC 25912),				
Hydrosol extract	78	78	1	1.125
Gentamicin	0.5	0.0625	0.125	
Pseudomonas aeruginosa (ATCC 27853)				
Hydrosol extract	78	39	0.5	1
Gentamicin	0.5	0.25	0.5	
Klebsiella pneumoniae (ATCC 700603)				
Hydrosol extract	78	78	1	1.125
Gentamicin	8	1	0.125	
*Salmonella typhimurium (*ATCC 13311)				
Hydrosol extract	1.56	1.56	1	1.125
Gentamicin	0.25	0.0312	0.125	
MICo= MIC of an individual sample, MICc,= MIC of an individual sample at the most effective combination; FIC= Fractional Inhibitory Concentration (see text); FICI = FIC of Hydrosol extract + FIC of Gentamicin.				

Table 21: Hydrosol extract and amphotericin B- Fractional Inhibitory Concentration (FIC) and FIC Indices

	MICo	MICc	FIC	FICI
Candida albicans (ATCC 26790)				
Hydrosol extract	19	2.375	0.125	0.635
Amphotericin B	4	2	0.5	
Candida albicans (ATCC 10 231)				
Hydrosol extract	19	9.5	0.5	0.75
Amphotericin B	8	2	0.25	
Candida albicans (IP444).				
Hydrosol extract	19	2.375	0.125	0.375
Amphotericin B	8	2	0.25	
MICo, =MIC of an individual sample, MICc,= MIC of an individual sample at the most effective combination; FIC= Fractional Inhibitory Concentration (see text); FICI = FIC of Hydrosol extract + FIC of Amphotericin B				

The combinations of antimicrobial compounds are crucial as they can reduce or prevent the emergence of resistant strains, diminish dose-related toxicity, and achieve a broad-spectrum antimicrobial activity [298]. Many studies on the interaction between plant extracts and antibiotics have shown a synergistic interaction. Some of the generally recognized mechanisms of antimicrobial interaction producing synergy include the sequential inhibition of common biochemical pathways, the inhibition of protective enzymes, the combination of membrane active agents, and the use of membranotropic agents to improve the diffusion of other antimicrobials [299, 300].

Gentamicin is an aminoglycoside antimicrobial agent commonly used to prevent and treat multiples infections caused by Gram-negative bacteria. This antibiotic drug, however, has a number of secondary effects that restrict its use. Nephrotoxicity, psychiatric disorders (depression, confusion, anorexia, visual hallucinations and disorientation) and toxicity to the sensory cells of the ear are the most important side effects to be mentioned [301].

In the present work, an interesting growth inhibitory effect of gentamicin was obtained when it was associated with the hydrosol extract against various strains of gram-negative bacteria tested (*Escherichia coli* (ATCC 25912), *Klebsiella pneumoniae* (ATCC 700603), *Salmonella typhimurium* (ATCC 13311)). This is confirmed by the substantial lowering of MIC values. These associations can decrease the minimum effective dose of Gentamicin and minimize its adverse effects.

Various hypotheses can be considered to elucidate the mechanisms leading to this interaction. In terms of chemical composition, plumbagin was found to be the main component of the hydrosol extract, accounting for 80.2%, which led us to believe that the high content of

this component favors and enhances the mechanism of action of Gentamicin by disrupting the protein synthesis by binding the 30S subunit of bacteria ribosome.

The polyene antibiotic Amphotericin B has been broadly used in the treatment of fungal infections since the 1950s. It has the ability to bind to the membrane of fungal cells and cause alterations in permeability. Its most frequent adverse effects are nausea, loss of appetite, gastric pain, weight loss, headache, fever and chills, muscle and joint pain, hepato- and nephrotoxicity [302, 303].

With the increasing, number of immunocompromised and immunosuppressed patients in recent years, the incidence of fungal infections has increased. [304]. *C. albicans* is among the most important opportunistic fungal pathogens typically found in the oral cavity, gastrointestinal tract, and genital region, as is the commensal flora found in more than half of the healthy population. When it has the opportunity to be pathogenic, *C. albicans* can cause more than 50% of human candidiasis and can lead to serious infections. [305, 306]. This research showed that the combination of amphotericin B with the hydrosol extract of *P. europea* produced a considerable synergistic effect against *C. albicans* (IP444), with a FIC value of 0.375 and a significant decrease of the MIC values of amphotericin B against *C. albicans* (ATCC 26790) and *C. albicans* (ATCC 10 231). This association can diminish the minimum effective dose of amphotericin B, consequently minimizing its side effects, which led us to propose this association for treating infections caused by *C. albicans*, the most common human pathogenic fungus that causes a range of disorders from superficial mucosal diseases to deep mycoses.

III.4.2.5 Evaluation of the synergistic effect of ethanolic extract with Amphotericin B:

In the current research, the synergistic effect of ethanolic extract from roots of *P. europeae* with Amphotericin B was studied and the obtained MICs and FIC index values are listed in tables 22. In the current study, an indifferent effect was obtained between the ethanolic extract and Amphotericin B blend against *C. albicans (ATCC 26790), C. albicans (ATCC 10231)* and *C. albicans (IP444)* with FIC index of 0.635, 1 and 0.75 respectively.

Despite the absence of a synergistic effect, the combination of ethanolic extract with Amphotericin B enhanced its antimicrobial activity and growth inhibition of the tested yeast, which was confirmed by the significant decrease in the MIC values of the antimicrobial drug. The MIC values of Amphotericin B (4, 8, 8 μg/mL) against *C. albicans (ATCC 26790), C.*

albicans (ATCC 10231) and *C. albicans (IP444)* respectively were lowered to 1/2, when used in combination with ethanolic extract at MIC of 19, 39 and 39 μg/mL, respectively.

Table 22: Ethanolic extract and amphotericin B- Fractional Inhibitory Concentration (FIC) and FIC Indices

	MICo	MICc	FIC	FICI
Candida albicans (ATCC 26790)				
Ethanolic extract	19	2.375	0.125	0.635
Amphotericin B	4	2	0.5	
Candida albicans (ATCC 10 231)				
Ethanolic extract	39	19.5	0.5	1
Amphotericin B	8	4	0.5	
Candida albicans (IP444).				
Ethanolic extract	39	9.75	0.25	0.75
Amphotericin B	8	4	0.5	
MICo, =MIC of an individual sample, MICc,= MIC of an individual sample at the most effective combination; FIC= Fractional Inhibitory Concentration (see text); FICI = FIC of Ethanolic extract + FIC of Amphotericin B				

As we discussed before, there is a need to find alternative strategies to deal with infections resulting from drug resistant. The incidence of systemic fungal infections has increased dramatically and now affects millions of people throughout the world. Yeasts of the genus Candida are thought the cause of 80% of fungal infections, which are considered to be among the most common nosocomial infections, leading to significant morbidity and mortality rates, especially in immunocompromised patients [307]. Currently, the treatment of these candidiasis infections continues to be based primarily on the use of common polyenes (e.g., amphotericin B) and azole antifungals (e.g., fluconazole), which mainly target ergosterol in the fungal cell membrane or its biosynthetic pathway. However, many of these antifungals have fungistatic properties, which, along with higher therapy costs and the development of clinical drug resistance, restrict their effectiveness in clinical practice [308, 309].

Due to the eukaryotic nature of Candida cells, the development of novel antifungal drugs has become increasingly difficult and challenging, resulting in limited drug targets. The desired antifungal agents should be specific for pathogen targets that are not shared with the human host; otherwise, these antifungal agents should show selective toxicity to fungal cells while being safe to human host cells [310]. Furthermore, synergy between conventional drugs and natural antimicrobial compounds has been described as a promising new strategy to reduce the effective doses of standard antifungals, minimize their side effects and associated toxicity, while improving their biological efficacy [311].

In the present study, the tested combination of amphotericin B and ethanolic extract failed to give a synergistic effect but it reduced significantly the minimum inhibitory concentration of the antifungal of reference against the three tested yeast of *C. albicans*. Consequently, this association may be used as a base for the development of potential therapeutic option for the treatment of candidiasis infections by Amphotericin B while reducing its effective dose and minimizing its adverse effects. Thus, it may be a way to overcome the toxicity problems associated with prolonged use of this gold standard for the treatment of disseminated fungal infections.

III.4.3 Hemolytic activity:

Herbal drugs were commonly used in the form of dried powder, gums, extracts or formulations of more than one plant products. Advanced scientific techniques brought a revaluation in herbal medicine industry and all focus is concentrate on active principles (bioactive molecule). However, a lot of processing is required to develop a drug from the natural sources. Toxicity of the active molecule is a key factor during drug designing, and hemolytic activity represents a useful starting point in this regard, it provides the primary information on the interaction between molecules and biological entities at cellular level. Hemolytic activity of any compounds is an indicator of general cytotoxicity towards normal healthy cells [312].

Haemolysis represents the most commonly employed initial toxicity assessment. It is an easy and effective spectrophotometric assay that provides an evaluation of the effect of different concentrations of biomolecules on the human erythrocytes. The ease associated with isolating erythrocytes and the similarities of its membrane with other cell membranes makes this assay a versatile tool for rapid initial toxicity assessment.

In vitro hemolytic activity on human erythrocytes of different concentrations of ethanolic extract from roots *of plumbago europaea* was performed and obtained results are shown in figure 25. Each concentration shows the mean of hemolysis percentage repeated in three experiments. Phosphate buffer saline and distilled water were used to obtain minimal and maximal hemolytic controls, respectively. All the samples exhibited very low hemolytic effect toward human erythrocytes. However, these extracts showed dose dependent increase in hemolytic activity.

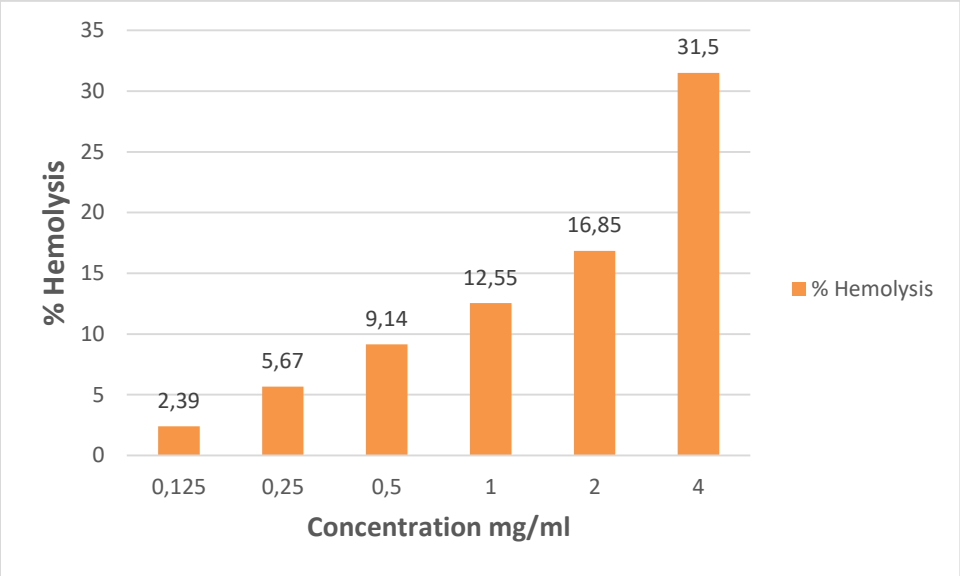


Figure 25: Hemolytic activity of ethanolic extract from roots of *Plumbago europaea* against human erythrocytes

Red blood cells are the main cells in circulation, and they are responsible for transporting oxygen; in fact, any alterations of this process could be lethal. The plasma membrane of red blood cells is a multi-component structure such as to confer to these cells their characteristic biconcave shape, high flexibility, elasticity and deformability. However, there are clear signs of cellular suffering if there are any alterations to this structure[313]. For this reason, the erythrocytes of mammals represent a good model to evaluate the cytotoxicity of molecules, organic and inorganic, natural or synthetic, by cellular damage measure. Indeed, before any investigation on the mechanism of action of different molecules, it is important to perform a cytotoxicity assay.

Among the different cytotoxicity assays that assess a possible toxicity in the red blood cells is the rate of hemolysis, which has proved to be an alternative screening method for simple toxicity. It is fast, reproducible and inexpensive to evaluate erythrocyte haemolytic activity against many compound [314]. This essay is based on the evaluation of the alterations of red cell membranes and release of hemoglobin which will be free in plasma and can causes damage to various vital organs such as the liver, kidney and heart. Furthermore, the lysis of red blood cells prevents direct intravenous administration of desired agents and often increases the toxicity of these agents when administered by other routes[315].

The membrane of the erythrocyte is composed of proteins and lipids, bound by non-covalent interactions, and glycoproteins, in compositions that vary between species. Transmembrane proteins regulate the ion flow, mechanical properties and cell support and this

can have a big effect on the cellular sensitivity to toxic compounds[316]. Generally, the biological activity of plant extract is strongly related to their concentration, potency and chemical composition. In the current study the ethanolic extract from roots of *plumbago europaea* showed low hemolytic effect at high concentration (4 mg/ml) an it had not affected the stability of the erythrocyte membrane. Which allowed us to suggest the non–toxic effect of the extract thus making it suitable for the preparation of drugs involved in the treatment of various diseases.

III.4.4 *In vitro* anti-inflammatory activity:

Inflammation is a natural immune response of the body to various aggressions, which can be of physical, chemical, biological or infectious origin. Inflammation generally occurs in response to infectious microorganisms like bacteria, viruses or fungi invading the body, residing in certain tissues and/or circulating in the blood. Inflammation can also occur in response to other processes such as tissue damage, cell death, cancer, ischemia and degeneration [317]. It is considered as one of the oldest defense mechanisms of the body. This protective immune response can sometimes be harmful due to the aggressiveness of the pathogen, its persistence and the abnormal regulation and production of the cells involved in the inflammation [318].

Inflammation is generally a beneficial process for the body. Nevertheless, repeated stimulation of toxic substances or inefficient regulation of acute inflammation may lead eventually to chronic inflammation [319]. The acute inflammation is characterized by increased vascular permeability, capillary infiltration and leukocyte emigration. In the chronic inflammation, there is an infiltration of mononuclear immune cells, macrophages, monocytes, neutrophils, activation of fibroblasts, proliferation (angiogenesis) and fibrosis [320]. The persistent inflammatory responses can lead to the development of chronic inflammation-related diseases like neurodegenerative, cardiovascular and inflammatory bowel disease [321]. Furthermore, chronic inflammation is also implicated in the physiological and pathological development of various diseases, including: Alzheimer's disease, depression, atherosclerosis (AS), rheumatoid arthritis (RA), obesity, gout and cancer [322].

The inflammation is a complex process, frequently associated with pain that involves phenomena such as increase in vascular permeability, alteration of membranes and loss of function in the affected area. Electrostatic bonds, hydrogen bonds, hydrophobic bonds and disulfide bonds in the protein structure are broken. In addition, a complex series of enzymatic

activation, mediator release, cell migration, tissue degradation, and repair occurs, resulting in proteins losing their molecular conformation and function or its denaturation [323]. Denaturation of protein is the process during which proteins lose both their tertiary and secondary structure through the application of external stress or chemical compounds, such as concentrated inorganic salt, a strong acid or base, organic solvent or heat. When denatured, most biological proteins lose their biological function. The denaturation of proteins is a well-known and proven cause of inflammation [324, 325]. Therefore, it is deduced that compounds capable of preventing these changes and inhibiting heat-induced protein denaturation have the potential therapeutic values of anti-inflammatory agents [326].

Nowadays, steroidal and non-steroidal anti-inflammatory drugs (NSAIDs), with anti-inflammatory, analgesic and other curative effects are the most widely used conventional treatments for inflammation in clinical practice. Nevertheless, long-term use of these medications can lead to undesirable effects, such as gastrointestinal lesions (stomach ulcers, bleeding, etc.), kidney and liver disorders, and skin disease. Nowadays, steroidal and non-steroidal anti-inflammatory drugs (NSAIDs), with anti-inflammatory, analgesic are the most widely used conventional treatments of inflammation in clinical practice. But long-term use of these medications can lead to undesirable effects, such as gastrointestinal lesions (stomach ulcers, bleeding, etc.), liver and kidney disorders and skin diseases [322].

In the rational use of drugs, safer drugs should be used as much as possible. Therefore, it is an urgent problem to be solved in the clinical treatment of inflammatory diseases to find natural plant compounds with no toxic side effects and good curative effect as substitutes for traditional anti-inflammatory drugs. In recent years, great strides have been made in developing and resolving the mechanisms of chronic inflammation-related diseases and in the use of natural products to mitigate inflammatory diseases [327]. Current studies have found that natural products with anti-inflammatory activity include polysaccharides, flavonoids, polyphenols, alkaloids, terpenes, natural pigments, plant volatile oils, quinones, and other compounds [328].

In the current study, heat induced denaturation of egg albumin (EA) was selected for the in vitro assessment of anti-inflammatory properties of ethanolic extract prepared from roots of *Plumbago europaea.* The percentage inhibition of egg albumin was calculated and the obtained results are summarized in table 23.

Table 23: The % inhibition of protein denaturation

Concentration (mg/ml)	The % inhibition of protein denaturation	
	Ethanolic extract	Diclofenac sodium
0.125	33.67	52.45
0.25	52.15	71.38
0.5	70.15	82.54
1	84.43	98.66

The adoption of a protein denaturation assay to assess the anti-inflammatory potential of plant extracts *in-vitro* avoids the ethical problems related to the use of animals, particularly in the early stages of screening plants that have potential as lead anti-inflammatory compounds [329]. The reason for the implementation of this test is that the denaturation of the albumin protein causes the formation of antigens that initiate a hypersensitive type III reaction leading to inflammation. Consequently, the inhibition of its denaturation process by an agent is an indication of its anti-inflammatory properties; the higher the degree of inhibition, the greater its anti-inflammatory potential [330].

Furthermore, protein denaturation has been described as a pathological process associated with loss of conformation, resulting in loss of function. Protein denaturation refers to the loss of biological properties of protein molecules, which is responsible for causing inflammation in certain disorders such as rheumatoid arthritis, diabetes, cancer, etc. Therefore, preventing protein denaturation can also help prevent inflammatory conditions. This makes the reduction in protein denaturation, and by extension the albumin denaturation assay, ideal for the determination of anti-inflammatory potential [331].

According to the above-mentioned results (Table 23), the ethanolic extract of *P. europaea* exhibited concentration-dependent inhibition of denaturation of protein. Similar observations were made for the reference drug Diclofenac sodium. The tested extract exhibited a marked inhibition of protein denaturation in comparison with Diclofenac sodium as standard drug. Maximum inhibition of 84.43% was observed in root extract of *P. europaea* at the concentration of 1 mg/ml. The minimum inhibition of 33.67% was obtained at the concentration of 0.125 mg/ml. In the other hands, the reference drug Diclofenac sodium exhibited a maximum inhibition of egg albumin denaturation at the concentration of 1 mg/ml with a percentage of 98.66% and a minimum of inhibition was obtained with a percentage of 52.45%.

Numerous studies in this field have evaluated the inhibitory effect of different plant extracts on anti-inflammatory activity in vitro by the protein denaturation method and have linked this biological activity to their phytochemical composition [332]. Protein denaturation is among the causes of inflammation. The production of auto-antigens in inflammatory diseases may be due to protein denaturation *in-vivo*. The possible mechanism of denaturation consists in the alteration of electrostatic, hydrogen, hydrophobic and disulfide bonds that maintain the three-dimensional structure of proteins [219, 333].

From the obtained results it can be suggested that, the phytochemicals found in the ethanolic extract contribute to the anti-inflammatory activity of this plant and the capacity of this extract to inhibit albumin denaturation can be attributed to the presence of different bioactive compounds such as: mono(2-ethylhexyl) ester of 1,2-benzenedicarboxylic acid, plumbagin, and phytosterols. However, more detailed studies are needed to determine the mechanisms and constituents behind the anti-inflammatory activity.

III.5 Free Alcohol mouthwash formulation against oral candidiasis:

The most common human fungal infection caused by Candida species is oral candidiasis (OC), commonly known as "thrush". *C albicans* exists as a normal commensal in the oral cavity in 30-60% of all healthy people and is the most commonly isolated strain of stomatitis found in denture wearers. It has been shown that maintaining good oral hygiene in combination with antifungal medication is essential for the prevention and effective treatment of OC. Cleaning of teeth, mouth, tongue and dentures is part of oral hygiene. Anti-Candida rinses are also recommended. antimicrobial-containing mouthwashes exert beneficial clinical effects when used as an adjunct in the treatment of oral candidiasis [189]. In an attempt to treat oral infections caused by *Candida* species, the search for natural products effective against *Candida* spp. has significantly increased with the investigation of many plant species.

Herbal mouthwashes have not been found to cause tooth discoloration or unpleasant taste. Compared to chlorhexidine mouthwash, herbal mouthwash also has the benefit of reducing plaque buildup and gingivitis. Additionally, herbal mouthwashes can be used on a long-term basis due to minimal side effects. [334]. A wide range of products based on natural substances intended for the prevention and treatment of oral diseases such as traditional mouthwashes contain a fairly high percentage of ethanol ranging from 10% to 30% by weight of the total composition. Indeed, more than 95% of the mouthwashes marketed contain more than 15% by

weight of alcohol. This alcohol is used both as a disinfectant and as a solvent in which other additives can be dissolved and then dispersed in the aqueous solution. High concentrations of ethyl alcohol are mainly used to destroy oral bacteria, as lower concentrations are usually sufficient to disperse the additives in the aqueous solution. [335]. However, ethanol's inclusion in oral care products has many adverse effects that can be harmful such as keratosis, mucosal ulcerations, gingivitis, xerostomia and oral pain [336].

Nevertheless, there are several contraindications to the use of alcohol-based mouthwashes, such as in children, pregnant women, alcoholics, and people with mucosal lesions. In addition, there are adverse consequences, such as burning or soreness or pain in patients who already have soft tissue damage, or a dry mouth sensation. McCullough and Farah suggested in their study that when the ethanol concentration exceeds 10%, significant pain may be felt in the oral cavity [337]. Moreover, many studies have been conducted on the relationship between the use of alcohol-based mouthwashes and the risk of oropharyngeal cancer [338]. These mouthwashes seem to increase the risk of developing cancer, regardless of smoking and alcohol consumption [337]. This may be due to the oxidation of ethanol into its toxic metabolite, acetaldehyde, inside the oral cavity [339].

To address these problems while providing the same cleaning quality, alcohol-free mouthwashes were developed. Alcohol-free mouthwashes are gaining popularity in comparison to alcohol-containing mouthwashes they cause little or no irritation or dryness when applied. This is beneficial for people suffering from sensitive gums, dry mouth or other medical disorders [340]. Hence, eliminating ethanol from mouthwash formulation may be a smart strategy. As a result, the ultimate purpose of this study was to formulate a mouthwash without ethanol with *Plumbago europaea* roots extract as main ingredient and to evaluate the *in- vitro* its antifungal activity against three yeast of *Candida albicans.*

It is critical that the solution of the mouthwashes formulated provide a comfortable feeling in the mouth during using, and it must have a pleasant flavor. Base on the needs, there are some basic excipients that must be used in order to formulate the mouthwash such as flavoring agent, humectants, preservatives, colorant and solubilizers.

III.5.1 Organoleptic properties of the prepared mouthwash:

In the present study, Mouthwash formulations were made in five variations to determine the most optimal formula in inhibiting the growth of *Candida albicans.* Concentration variations were made in 5 MIC, 10 MIC, 15 MIC, 20 MIC and 30 MIC of EE as active ingredient while other additives are made permanent, to determine the optimum concentration of Ethanolic extract. Table 24 shows the results of organoleptic test and physical properties of all prepared formulations.

Table 24: Organoleptic test and physical properties of prepared formulations

	F1	F2	F3	F4	F5
Homogeneity	Good	Good	Good	Good	Good
Clarity	Clear	Clear	Clear	Clear	Clear
Color	Clear yellow	Clear yellow	Yellow	Yellow	Yellow
Taste	Sweet	Sweet	Sweet	Sweet	Sweet
Flavor	Fresh, Mint	Fresh, Mint	Fresh, Mint	Fresh, Mint	Fresh, Mint
pH	4.60	4.80	5.09	5.39	5.65

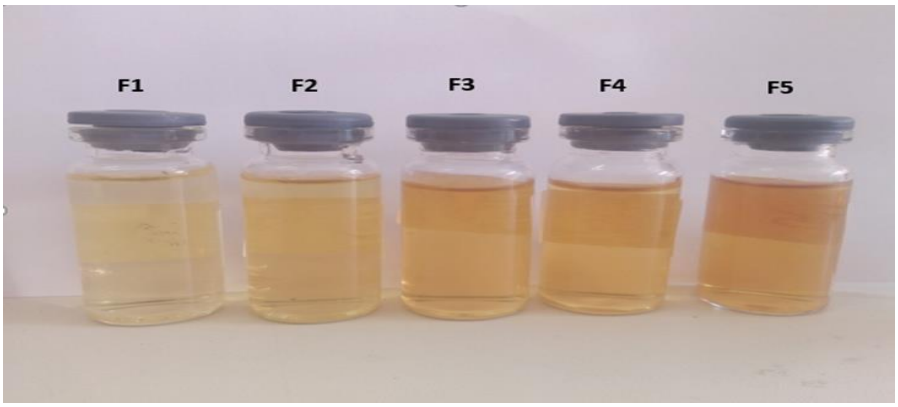


Figure 26: The prepared mouthwash formulations

Organoleptic tests involving homogeneity, color, taste, and flavor. According to previous researches, the aspect of a phytocosmetic, in terms of homogeneity and color is important from a commercial standpoint, because these factors may affect purchase by consumers who are drawn to the product's appearance. They also stated that changes in odor can be linked to microbial contamination even when there is no visible change, underlining the significance of developing a protocol for studying phytocosmetics' physical-chemical stability [341].

The obtained results showed that all obtained mouthwash formulation were yellow in color, homogeneous, clear, had a sweet flavor, smelled of distinctive fresh aroma of Menthol, had the pH value between 4.60 and 5.65. The yellow color of obtained solutions is mainly due to the ethanolic extract of *Plumbago europaea* as active component which has a shiny brown color.

Taste and flavor of the prepared formulations are mainly due to the saccharin, which was used as sweetening agent, and menthol, which was used as flavoring agents. Saccharin is a white powder or crystal, odorless or has weak aromatic smell. It has a very sweet flavor in the form of an aqueous solution. In oral formulations, saccharin is used at the concentrations of 0.02-0.5%. It is about 300–400 times as sweet as sucrose but has a bitter or metallic aftertaste, especially at high concentrations [342]. Flavoring agents have a great importance in the industry of drugs, especially in camouflaging with the medicines by their indispensable flavors. Thus, they are also called as "masking agents" or "bitter blockers. Menthol which confers the mint smell is commonly used in oral hygiene products and bad breath remedies as mint flavoring and cooling agent in mouth washes, toothpaste, tongue spray, chewing gum and candy. Menthol is a naturally occurring plant compound that gives the Mentha genus its distinctive minty aroma and flavor. (-)- Menthol is the isomer that occurs most frequently in nature and is the one assumed by the name menthol. It is characterized by a peppermint odor and provides a cooling sensation when applied to the skin and mucosal surface [343].

The pH value is an important parameter because it affects the type and capacity of a microorganisms to grow. For that reason, the pH of a mouth rinse solution should be in the range of the oral cavity, which is between 5.5 to 7.9, so that the oral mucosa is not irritated when the preparation is consumed [344]. Herbal mouth rinses must have a pH value of 5-7.20, according to the Herbal Medicines Quality Standards. The pH value of the mouth rinse solution in this investigation met the quality standards. The bacteria will multiply if the fluid is extremely acidic, whereas the fungus will grow more easily if the solution is very alkaline.

III.5.2 Antifungal activity of the prepared mouthwash:

Antifungal activity of the prepared mouthwashes was conducted against three yeasts of *Candida albicans: Candida albicans (ATCC 26790), Candida albicans (ATCC 10 231) and Candida albicans (IP444)* and the obtained inhibition zones diameters are shown in table 25.

Table 25: Zones of inhibition of prepared mouthwash

Yeasts	Inhibition zones (mm)					
	F1	**F2**	**F3**	**F4**	**F5**	**F6**
C. albicans (ATCC 26790),	13.5±0.5	14.5±0.5	18±0.0	19.5±0.5	23.5±0.5	-
C.albicans (ATCC 10 231)	15.5± 0.5	16±1	17.5±0.5	20±1.0	23.5±0.5	-
C.albicans (IP444)	10±0.33	12±0.33	14±0.33	16±0.01	19.5±0.01	-

The results of this *in- vitro* study indicated that all prepared mouth rinses formulations exhibited a range of inhibitory effects on the test yeasts with varying magnitude. All prepared formulations effectively inhibited the growth of the tree yeast of *Candida albicans* with an important inhibition zones diameter.

Formulations F4 and F5 were the most active in inhibiting the growth of the three strains of *Candida albicans* with an inhibition zone diameter varying between 16 and 23.5 mm and this can be explained by the high concentration of ethanolic extract of *Plumbago europaea* the active ingredient in these two formulations in comparison with the formulations F1, F2 and F3.

Based on these results, the formulations F4 and F5 were chosen for comparison with two commercialized mouthwashes, one imported (Listerine®) and the other manufactured in Algeria (Xethol®). The results of the comparison of the antifungal activity of these two mouthwashes and the selected formulations are presented in table 26.

Table 26: Diameter of inhibition zones obtained for prepared mouthwashes, Listerine® and Xethol®against candida strains

Yeasts	Inhibition zones (mm)			
	F4	**F5**	**Listerine®**	**Xethol®**
C. albicans (ATCC 26790),	19.5±0.5	23.5±0.5	-	-
C.albicans (ATCC 10 231)	20±1	23.5±0.5	-	-
C.albicans (IP444)	16±0.01	19.5±0.01	-	-

According to the results obtained, no zone of inhibition was detected against the three yeast strains using the commercialized mouthwashes. It can be concluded that the free alcohol mouthwashes prepared in this work based on ethanolic extract from roots of *Plumbago europaea* as natural active principle had a very interesting antifungal activity in comparison with two commercialized mouthwashes that contain a high percentage of ethanol that can go up to 30% for the Xethol®. An optimum mouthwash must have some qualities, such as having antiseptic effects on the mouth, washing the food residue on the gingival (gum) medium and teeth, reducing the mouth microorganisms, masking and neutralizing halitosis, and introducing a good taste and sense of freshness in the mouth. The evaluation of the antimicrobial activity of mouthwash preparations is important to ensure their efficacy in eliminating harmful periodontal bacteria and yeasts, which leads to prevention of future dental carries, gingivitis, and periodontitis.

In our study, the antifungal effectiveness of the prepared mouthwashes was tested against three strain of *Candida albicans* yeasts. The choice of these fungal strains is based on the previous results of the antimicrobial activity of ethanolic extract used as active ingredient in mouthwash preparations and also there are very few mouthwashes on the market with antifungal activity. Despite *Candida albicans* is the most frequently yeast group isolated from the oral cavity and primarily associated with the mucosal infections (oral candidiasis) and denture-related stomatitis. Furthermore, *C. albicans* strains have been shown to be involved in the formation of cariogenic biofilms and many researches revealed that if a toothpaste or mouth rinse has good inhibition properties against *C. albicans,* then it can be recommended for the patients who is susceptible to oral fungal infections [345].

Oral fungal infections and especially oral candidiasis is the one of the most common and severe opportunistic fungal oral infections caused by Candida yeast species. These infections frequently diffuse in immunosuppressed patients and affect internal organs. Antifungal drugs, in different formulations, are used topically (such as nystatin and clotrimazole) and systemically (like amphotericin B and azoles) for the treatment of candidiasis. However, multiple studies in recent years have indicated therapeutic failure in patients with various clinical forms of candidiasis. The long-term usage of antifungal drugs caused adverse side effects and drug resistance which limit their therapeutics' use. Therefore, recent researches have focused on finding more effective antifungal agents with natural sources and fewer side effects[346].This is why it is important to develop a pharmaceutical form based on natural products to treat oral candidiasis, especially in immunocompromised patients.

III.5.3 Stability studies:

In the stability tests carried out in this work, the different mouthwash formulations were stored at three different temperatures: in the refrigerator at 3-5 °C, at room temperature at around 25 °C and in the oven at round 40°C. The three different temperatures were chosen to determine the optimum storage conditions for the mouthwash formulation in which they were able to maintain their activity for the longest time possible. The visual appearance, phase separation, and homogeneity of each formulation were monitored by ocular examination each month. The pH was monitoring using a calibrated pH meter. The obtained results are illustrated in Figure 27.

All the tested formulation stored under different temperature conditions did not experience any change in their visual appearance and formulation maintain their original yellow color which was due to the influence of *Plumbago europaea* ethanolic extract. The results of the stability study for the formulation F5 that was the most active formulation in this study are shown in table 27 and 28.

No phase separation and no sedimentation were observed in the tested formulation throughout the 3-month period. The tested formulation remained well-merged with similar consistency and maintained the same homogenous state it originally had at the time of preparation. There was little variation in the pH values.

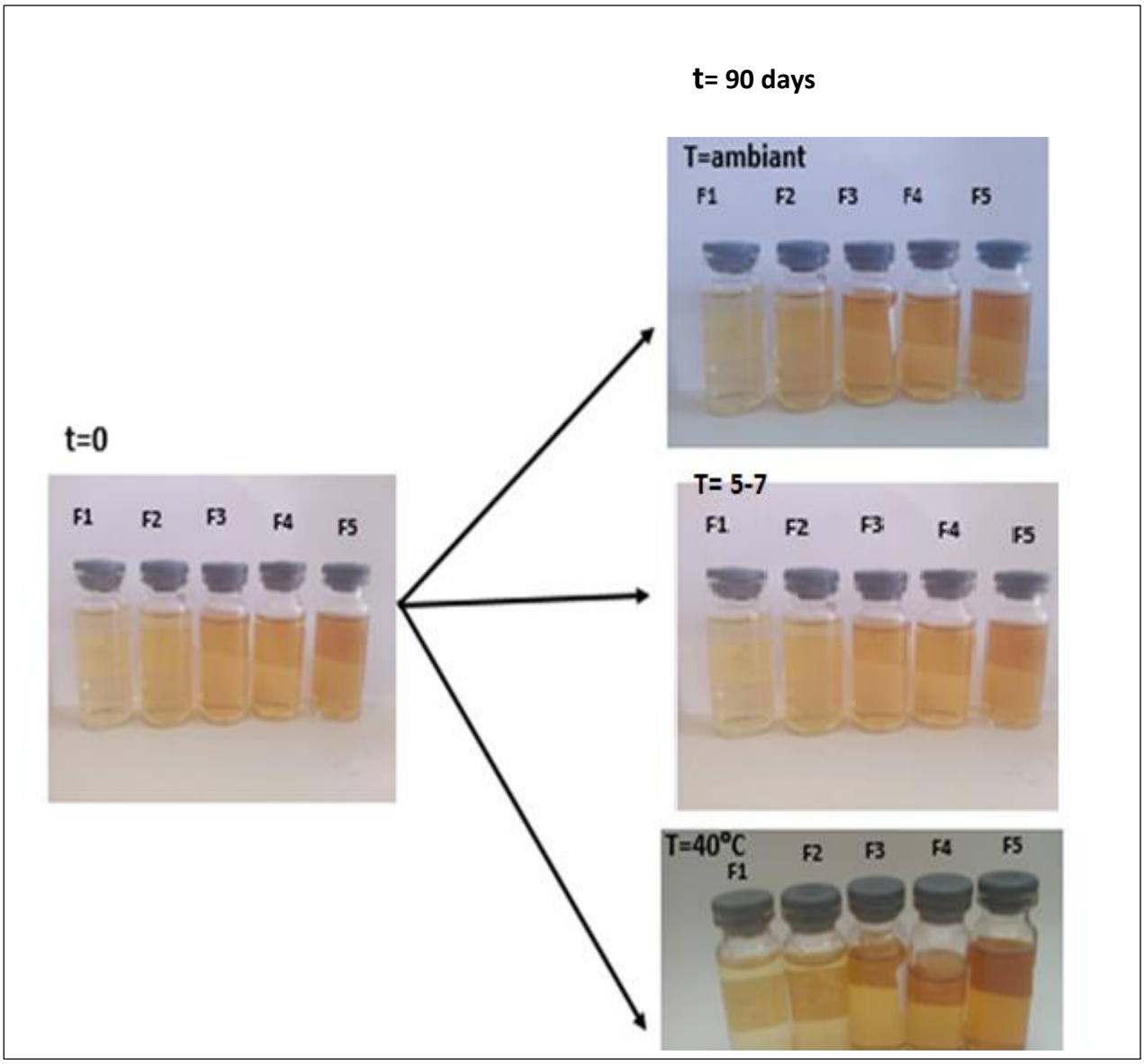


Figure 27: Stability study of prepared mouthwashes.

Tableau 27: Stability study results

Storage temperature	Evaluation parameter	Observations (Month)		
		1	**2**	**3**
3-5º C	**Visual appearance**	Yellow	Yellow	Yellow
	Phase separation	Nil	Nil	Nil
	Homogeneity	Good	Good	Good
	pH	5.66	5.67	5.70
25º C	**Visual appearance**	Yellow	Yellow	Yellow
	Phase separation	Nil	Nil	Nil
	Homogeneity	Good	Good	Good
	pH	5.65	5.66	5.66
40º C	**Visual appearance**	Yellow	Yellow	Yellow
	Phase separation	Nil	Nil	Nil
	Homogeneity	Good	Good	Good
	pH	5.61	5.60	5.60

Table 28: Antifungal activity and microbial stability of F5 the end of the third month of stability studies

Antifungal activity and microbial stability of F5				
Storage temperature	**Colony-forming units**	**Inhibition zones (mm)**		
		C. albicans (ATCC 26790)	*C. albicans (ATCC 10 231)*	*C. albicans (IP444)*
3-5º C	**-**	24±0.5	22±0.5	19±0.0
25º C	**-**	24±0.5	21.5±0.5	18±0.5
40º C	**-**	21±0.5	22±0.5	18.5± 0.0

According to the results mentioned in the table 28 it was observed that the formulation F5 has maintained its antifungal activity under the three storage conditions and has significantly inhibited the growth of the three strains of *Candida albicans.* The studied mouthwash was found to inhibit the growth of tested yeast with an interesting inhibition zone diameter ranging from 18 to 24 mm.

Also, in this part, the aim of the microbiological stability tests was to investigate the growth of microorganisms (bacteria, fungi, yeasts, and total coliforms) in tested formulations. Under the three-storage condition there was no indication of contamination by bacteria, fungi, yeast. It has been reported that fungal contamination of herbal drugs can lead to alteration and/or destruction of the active substances and loss of safety and efficacy, in addition, toxic substances (mycotoxins) may be produced that make these products unfit for consumption, regardless of the degree of contamination [223]. The lack of microbial growth may also be attributed to the use of the preservative sodium benzoate. Sodium benzoate is responsible for inhibiting the growth and survival of microorganisms in food products by decreasing the anaerobic fermentation of glucose. The undissociated benzoic acid, the active component of benzoate, is highly lipophilic and can rapidly enter cells by disrupting the permeability of many microbial cell membranes [347].

In conclusion, the obtained stability study results show that the formulations were stable under the three-storage condition over the period of study and this implies that the prepared product will maintain its basic activity and integrity for a long period. A change in a pharmaceutical product's stability can lead to a shift in the product's physicochemical and pharmacokinetic properties, such as drug penetration, bioavailability, concentration, production of new compounds, and so on, which can completely alter the change the action of the pharmaceutical formulation.

III.5.4 Oral Cavity residence factor (OCRF)

The OCRF of the prepared mouthwash was determined along with that of two commercial mouthwashes and the obtained results values are given in table 29. It revealed that the product of the present invention can be retained in the oral cavity for periods significantly longer than that of the Listerine® and Xethol®. The OCRF value of the prepared mouthwash was 5 times greater than that of Listerine® and 30 times greater than that of Xethol®.

Tableau 29: OCRF of different mouthwashes (Listerine® , Xethol® and prepared mouthwash)

Mouthwash	OCRF
Xethol®.	2.24 ±0.56
Listerine®	11 ±1.56
Prepared Mouthwash	60.88±1.28

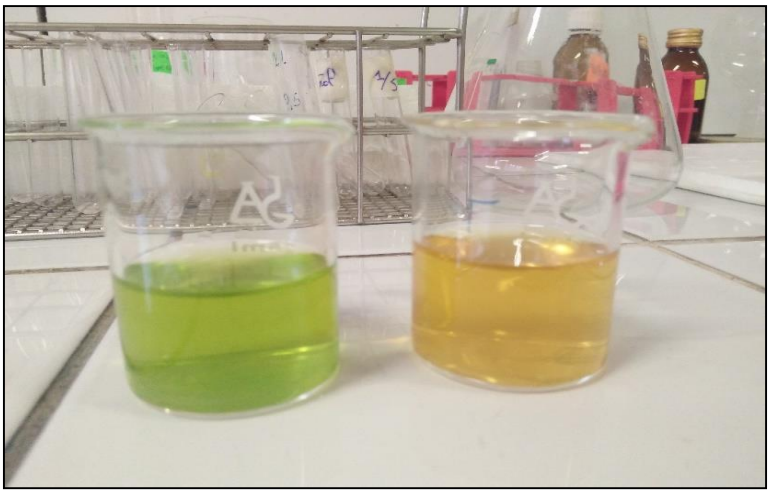
The retention time of our mouthwash in the buccal cavity was clearly greater than that of the Listerine® and Xethol®. Xethol® causes an intense burning sensation with a pronounced bitterness taste with the urge to rinse off urgently after the uses. While Listerine® causes a feeling of burning and freshness at the same time. Our prepared mouthwash can be retained in the mouth for more than a minute with a very slight burning sensation appears late compared to the commercialized mouthwash tested ant this is mainly due to the Menthol used as flavoring agent.

Moreover, it was revealed that the amount of pain induced during mouth rinsing was directly related to the percentage of alcohol contained in the mouthwash and when the ethanol concentration exceeds 10% substantial painful sensation will be produced in the oral cavity. Although other agents present in the product such as flavoring, sweeteners and colorings could also contribute to this side effect but with a lower degree than that of alcohol [336]. Xethol® being the least engaged mouthwash contains a high proportion of alcohol, two essential oil and pure menthol, which explains the intense burning sensation. Listerine® although containing four powerful active substances (eucalyptol, thymol, methyl salicylate, and menthol) had a less pronounced irritant taste than that of Xethol® because containing less alcohol than the latter. Our mouthwash was mild and very well accepted because it is free alcohol product with low percentage of menthol.

III.5.5 Mouthwash color

In the field of oral hygiene products, several colors are used to attract the attention of customers. Blue and green are the most commonly used, they tend to produce strong feelings of freshness and often indicate the presence of mint. In our work, all the people who take part in the color choice trial chose the green color a bright and eye-catching color that caught their

attention by unconsciously giving them an impression of freshness. Figure 28 shows the color of the prepared mouthwash before and after addition of the coloring agent.



A B

Figure 28: Mouthwash with coloring agent(A) and without coloring agent (B)

The appearance of products has been reported to have a significant impact on attracting and retaining consumers' attention on a particular product. Color being the first visual element that people perceive, it plays a crucial role in the design and the appearance of any product. From birth, nature trains us to make environmental judgments based in great part on color. Color influences many of our daily decisions and is related with every part of our existence. Moreover, consumers have the ability to visually check food and drink before selecting whether or not to buy or taste it in most common situations. Indeed, it has long been acknowledged that color is one of the most important visual cues for predicting the sensory qualities (such as taste/flavor) of what we are about to eat or drink [348].

A considerable body of scientific research now indicates that our experience of flavor and taste is greatly influenced by the expectations that we form (often automatically) prior to tasting. Such expectations can be impacted by branding, labeling, packaging, and other contextual factors (a range of product-extrinsic cues) as well as a number of product-intrinsic cues. The smell and aroma are generally considered as the sound of product on the super supermarket shelf. These olfactory cues can be often masked by the product packaging which private product's sound when inspected visually. As a result, the brain uses vision, and most often color, as a cue to help identify food sources and make predictions about their potential taste and flavor. We can said that color creates a psychological expectation for a certain flavor that is often impossible to remove [349].

Conclusion

Due to the increasing prevalence of life-threatening bacterial, fungal and viral infections and the ability of these human pathogens to develop resistance to current treatment strategies, there is a great need to find and develop new compounds to combat them. These molecules must have low toxicity, specific activity and high bioavailability. The most suitable compounds for this task are usually derived from natural sources (animal, plant or even microbial).

Phytomedicines and active substances extracted from plants continue to be an important source of pharmaceuticals. They have an exceptional capacity to produce cytotoxic agents, most likely because the antimicrobial products must be present or synthesized de novo in plants after microbial attack to protect producer from pathogenic microorganisms in their environment. The major advantage of natural agents possessing antibacterial properties is that they rarely enhance "antibiotic resistance," a common phenomenon associated with long-term use of chemical synthetic antibiotics [350].

The work presented in this thesis aims to valorize a medicinal plant used in local traditional medicine which is *Plumbago europaea* and the search for new antimicrobial agents that can be used as active ingredient in pharmaceutical formulations. The obtained results showed concordance with traditional use of this plant and strongly support antimicrobial properties of many plants used traditionally in the form of formulations/individual extracts to treat infectious diseases or related symptoms.

The obtained results revealed that the plumbagin was the main component of both essential oil and hydrosol extract isolated from roots of *Plumbago europaea*. Antioxidant activity revealed that both essential oil and hydrosol extract exhibited a good to moderate scavenging and reducing capacity which was in accordance with previous study in literatures. On the other hand, the hydrosol extract showed a very interesting antimicrobial activity against a large panel of microorganisms. It was also found that the *in-vitro* association of the hydrosol extract with Gentamicin and Amphotericin B led to a considerable decrease of the MIC against all tested microorganisms. This association may contribute to reducing the minimum effective dose of the antimicrobial drugs used, which may help to minimize their side effects, and to deliver these drugs with similar potency.

The fractionation of the ethanolic extract prepared from roots of *Plumbago europaea* resulted in three main fractions named F1, F2 and F3. The GC-MS analysis of fractions F1 and F2 showed that the two analyzed fractions contain numerous phytochemicals belonging to different classes with the presence of plumbagin which confirms the chemotaxonomic role of plumbagin for this genus and family and the existence of a similar chemical profile in species belonging to the same tribe.

The results of the antioxidant activity revealed that the ethanolic extract was effective as antioxidant and showed an interesting free radical-scavenging with IC_{50} of 146 µg/ml and an important reducing capacity. The investigation of the antifungal activity of ethanolic extract and isolated fraction showed that the ethanolic extract exhibited remarkable inhibitory effect on the growth of tested yeast with MIC of 19 µg/ml against *C. albicans ATCC 26790.* Furthermore, the evaluation of the *in-vitro* combination of the ethanolic extract with Amphotericin B revealed that combining Amphotericin B with the ethanolic extract of *P. europea* give an indifference effect against the three-tested yeast *Candida albicans* with the FIC index value between 0.635 and 1. Despite the fact that this association did not give a synergistic effect but it reduced significantly the minimum inhibitory concentration of the antifungal of reference against the three-tested yeast of *Candida albicans.* These finding confirms the interest of the association between antimicrobial drugs and plants extract in combating to deal with microbial infections especially fungal infection as a major public health problem.

The hemolytic activity of ethanolic extract against human erythrocytes was studied and results showed low hemolytic effect at high concentration (4 mg/ml). The studied extract had not affected the stability of the erythrocyte membrane, which allowed us to suggest the non–toxic effect of the extract thus making it suitable for the preparation of drugs involved in the treatment of various diseases. Finally, the ethanolic extract prepared from roots of *Plumbago europaea* produced a marked *in- vitro* anti-inflammatory activity that justifies its use in local traditional medicine.

Finally, the objective of a pharmaceutical formulation based on *Plumbago europaea* extract is achieved and an alcohol free mouthwash was prepared. The prepared free alcohol mouthwash was mild and very well accepted because it is free alcohol product and it was effective in inhibiting the growth of *Candida albicans* yeast *in vitro*, thereby demonstrating the efficacy and the potential usefulness of ethanolic extract of *Plumbago europaea* in controlling candidiasis. Furthermore, it may serve as natural anticandidal mouthwash alternative for patients who suffer from oral candidiasis especially immunocompromised individuals and wish to avoid alcohol in maintaining and controlling their oral hygiene. It was also revealed that the prepared herbal mouthwash could serve as a good compliment/substitution to standard marketed mouthwash in maintaining oral hygiene and prevention of oral diseases caused by *Candida albicans* strains

In conclusion, all the obtained results constitute a scientific justification of the traditional use of the studied plant and confirm once again the relevance of traditional remedies in the treatment of many disorders. The originality of this work is the complete study of a plant extract from the extraction to the pharmaceutical formulation step and the development of a new free alcohol mouthwash against oral candidiasis. The biological and chemical study carried out on the essential oil, the hydrolat and the ethanolic extract remains preliminary it would be much more interesting in the future to complete, widen and deepen this research. We hope for the following:

- Study other biological properties such as, anti-cancer, anti-diabetic, antiviral, insecticidal and larvicidal activity of extracts from roots of *Plumbago europaea*
- Conduct a rigorous toxicity study on other cell lines in vitro and in models using living beings *(In-Vivo*) to determine therapeutic doses.
- Carry out a similar chemical and biological study on the aerial part of this plant.

In respect of formulation, the results of the antifungal activity of the mouthwash prepared against the three candida strains are very promising and encourage us to extend the study to other fungal strains and even to clinical strains of *Candida albicans* and to proceed to *in -vivo* tests on patients suffering from oral candidiasis. In addition to this, the preparation of a 100% natural mouthwash using a vegetable sweetener instead of saccharin (e.g. stevia) and a coloring agent extracted from seaweed instead of the brilliant blue and the transposition of the scale should be considered.

ANNEXES

Annex 1:

Various in vitro assays commonly employed to assess the antioxidant activity

	Underlying mechanism	Antioxidant assay
1.	Hydrogen atom transfer (HAT) methods	Total radical trapping antioxidant parameter (TRAP) assay
		Oxygen radical absorbance capacity (ORAC) assay
		Beta-carotene/crocin bleaching assay
		Inhibited oxygen uptake (IOU) assay
		Inhibition of induced low-density lipoprotein peroxidation assay
2.	Single electron transfer (SET) methods	N,N-Dimethyl-p-phenylenediamine (DMPD) assay
		Ferric reducing antioxidant power (FRAP) assay
		Cupric reducing antioxidant capacity (CUPRAC) assay
		Potassium ferricyanide reducing power (PFRAP) assay
		Total phenolic content assay by Folin-Ciocalteu assay
		Total antioxidant capacity (TAC)/ phosphomolybdenum assay
3.	Mixed methods (have both HAT and SET mechanism)	2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay
		2,20-Azinobis-(3-ethyl-benzothiazoline)-6-sulfonic acid (ABTS) assay Trolox equivalent antioxidant capacity (TEAC)
4.	Chelation methods	Ferrozine/metal chelating assay
		Tetramethylmurexide (TMM) assay
5.	Lipid oxidation methods	Thiobarbituric acid (TBA) assay
		Anisidine assay
		Conjugated diene assay
		Peroxide value assessment assay

6.	Miscellaneous methods	Hydrogen peroxide scavenging (H_2O_2) assay
		Peroxynitrite (ONOO) radical scavenging assay
		Nitric oxide (NO) scavenging assay
		Hydroxyl radical scavenging assay
		Hydroxyl radical averting capacity (HORAC) assay
		Superoxide radical scavenging (SOD) assay
		Xanthine oxidase assay

Annex 2:

Exicipients:

Glycerin [221]:

Nonproprietary Names:

BP: Glycerol

JP: Concentrated glycerin

PhEur: Glycerolum

USP: Glycerin

Chemical name:

Propane-1,2,3-triol

Empirical Formula and Molecular Weight:

$C_3H_8O_3$ 92.09

Description:

Glycerin is a clear, colorless, odorless, viscous, hygroscopic liquid; it has a sweet taste, approximately 0.6 times as sweet as sucrose

Solubility: miscible with water and alcohol, sparingly soluble in acetone, practically insoluble in fatty oils and essential oils.

Applications in Pharmaceutical Formulation or Technology:

Glycerin is used in a wide variety of pharmaceutical formulations including oral, otic, ophthalmic, topical, and parenteral preparations. In topical pharmaceutical formulations and cosmetics, glycerin is used primarily for its humectant and emollient properties.

Menthol [221]:

Nonproprietary Names

BP: Racementhol

JP: dl-Menthol

PhEur: Mentholum racemicum

USP: Menthol

Synonyms

Hexahydrothymol; 2-isopropyl-5-methylcyclohexanol; 4-isopropyl-1-methylcyclohexan-3-ol; 3-p-menthanol; p-menthan3-ol; dl-menthol; peppermint camphor; racemic menthol.

Chemical Name and CAS Registry Number

(1RS,2RS,5RS)-()-5-Methyl-2-(1-methylethyl)cyclohexanol [15356-70-4]

Empirical Formula and Molecular Weight

$C_{10}H_{20}O$ 156.27

Chemical Structure:

Description

It is a free-flowing or agglomerated crystalline powder, or colorless, prismatic, or acicular shiny crystals, or hexagonal or fused masses with a strong characteristic odor and taste.

Applications in Pharmaceutical Formulation or Technology :

Menthol is widely used in pharmaceuticals, confectionery, and toiletry products as a flavoring agent or odor enhancer. In addition to its characteristic peppermint flavor, l-menthol, which occurs naturally, also exerts a cooling or refreshing sensation that is exploited in many topical preparations.

Saccharin [221]:

Nonproprietary Names

BP: Saccharin

PhEur: Saccharinum

USPNF: Saccharin

Synonyms

1,2-Benzisothiazolin-3-one 1,1-dioxide; benzoic sulfimide; benzosulfimide; 1,2-dihydro-2-ketobenzisosulfonazole; 2,3- dihydro-3-oxobenzisosulfonazole; E954; Garantose; gluside; Hermesetas; sacarina; saccharin insoluble

Chemical Name and CAS Registry Number

1,2-Benzisothiazol-3(2H)-one 1,1-dioxide [81-07-2]

Empirical Formula and Molecular Weight

C7H5NO3S 183.18

Description

Saccharin occurs as odorless white crystals or a white crystalline powder. It has an intensely sweet taste, with a metallic aftertaste that at normal levels of use can be detected by approximately 25% of the population.

Applications in Pharmaceutical Formulation or Technology

Saccharin is an intense sweetening agent used in beverages, food products, table-top sweeteners, and oral hygiene products such as toothpastes and mouthwashes. In oral pharmaceutical formulations, it is used at a concentration of 0.02–0.5% w/w

Sodium Benzoate:

Nonproprietary Names:

BP: Sodium benzoate

JP: Sodium benzoate

PhEur: Natrii benzoas

USPNF : Sodium benzoate

Synonyms:

Benzoic acid sodium salt; benzoate of soda; E211; natrium benzoicum; sobenate; sodii benzoas; sodium benzoic acid.

Chemical Name and CAS Registry Number:

Sodium benzoate [532-32-1]

Empirical Formula and Molecular Weight:

$C_7H_5NaO_2$ 144.11

Description:

Sodium benzoate occurs as a white granular or crystalline, slightly hygroscopic powder. It is odorless, or with faint odor of benzoin and has an unpleasant sweet and saline taste.

Applications in Pharmaceutical Formulation or Technology;

Sodium benzoate is used primarily as an antimicrobial preservative in cosmetics, foods, and pharmaceuticals. It is used in concentrations of 0.02–0.5% in oral medicines, 0.5% in parenteral products, and 0.1–0.5% in cosmetics.

Annex 3:

Nutrient agar

Yeast extract……..2g

Peptone……..5g

Sodium chlorid (NaCl)……..5g

Agar…….15g

pH =6,8-7,4

Nutrient broth

Yeast extract……..2g

Peptone……..5g

Sodium chlorid (NaCl)…….…….5g

Beef extract……1g

pH=7.5

Mueller-Hinton agar

Beef extract ……...300g
Acide hydrolysate of caseine……....17,5g
Starch…….....1,5g
Agar ……17g

pH =7,4

Sabouraud Dextrose Agar

Dextrose …….....40g
Peptone …….....10g
Agar ……15g

pH =5.6

Sabouraud Dextrose Agar

Dextrose …….....40g
Peptone …….....10g

pH =5.6

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الملخص

في مجال علم الصيدلة ، يتمثل الهدف الرئيسي لأحدث البحوث على المستخلصات النباتية الطبيعية واستخدامها في الطب في تطوير وإدخال مواد فعالة جديدة مقبولة علميا يمكن أن تحل م العقاقير المضادة للميكروبات في علاج الأمراض المعدية ، تتمثل أهداف هذا العمل في البحث عن جزيئات فعالة جديدة مضادة للميكروبات من مستخلصات جذور نبات يستخدم في الطب التقليدي في منطقة تلمسان (*Plumbago europaea*)، ووضع تطبيق لهذه المستخلصات في صيغ صيدلانية.

تم تحليل الزيت العطري والهيدروسول المستخرج من الجذور باستخدامGC / MS ، حيث كان plumbagine هو المكون الرئيسي. أظهرت دراسة النشاط المضاد للاكسدة للزيت العطري والهيدروسول فعالية مقبولة وهذا ماتبين في قيم IC_{50} والتي قدرت 2.72 ملغ / مل 2.98 ملغ/ مل على التوالي. . أظهرت نتائج دراسة النشاط المضاد للميكروبات ان مستخلص الهيدروسول كان الاكثر فعالية في تثبيط العديد من البكتيريا و الخمائر التي قمنا بتجربتها. حيث تم تسجيل قيم CMIمنخفضةجدًا 19 µ غ/ مل. نتائج دراسة النشاط المضاد للميكروبات لخليط الهيدرسول مع Amphotéricine B أظهرت تاثيرا تازريا ضد (C. albicans (PI 444

تم فصل المستخلص الإيثانولي من جذور *Plumbago europaea* بواسطة كروماتوجرافيا وتم جمع ثلاثة أجزاء رئيسية وأطلق عليها اسم F1. F2 و F3أظهرت دراسة النشاط المضاد للاكسدة و النشاط المضاد الخمائر أن المستخلص الإيثانوليو ثلاثة أجزاء رئيسية لها خصائص مضادة للأكسدة ومضادة للفطريات مثيرة للاهتمامو أخيرًا تم تقييم القوة الانحلالية والمضادة للالتهابات للمستخلص الإيثانولي في المختبر وأظهرت النتائج التي تم الحصول عليها تأثير انحلالي ضعيف ونشاط جيد مضاد للالتهابات.

تم تحضير و صياغة غاسول للفم خالي من الكحول يحتوي على تراكيز مختلفة من للمستخلص الإيثانولي كمادة فعالة ودراسة خصائصه الكيميائية الفيزيائية ، واستقراره، وفعاليته ضد الفطريات اظهرت نتائج الدراسة ان غاسول الفم المحضر كان فعالا في تثبيط نموالخمائرو الفطريات ومما يدل على فعالية المستخلص الإيثانولي وفائدته المحتملة في السيطرة علىفطريات الفم او داء candidoses.

الكلمات المفتاحية : *Plumbago europaea*, plumbagine, الزيت العطري, الهيدروسول, المستخلص الإيثانولي,نشاط مضاد للخمائر والفطريات ,غاسول للفم خالي من الكحول.

Résumé :

Dans le domaine de la science pharmaceutique, l'objectif principal des recherches les plus actuelles sur les extraits naturels de plantes et leur utilisation en médecine est de développer et d'introduire des agents scientifiquement acceptables qui peuvent remplacer les médicaments antimicrobiens dans le traitement des maladies infectieuses. Le présent travail vise à rechercher de nouveaux agents antimicrobiens à partir des racines de *Plumbago europaea*, une plante médicinale originaire de la région de Tlemcen, ainsi qu'à développer une application de ces extraits de plantes dans une formulation pharmaceutique.

L'huile essentielle et l'extrait d'hydrolat obtenu à partir des racines de *plumbago europaea* ont été analysés par CPG/SM et la plumbagine était le composé majoritaire. L'huile essentielle et l'extrait d'hydrolat ont tous deux montré une capacité de piégeage et de réduction des radicaux libres modérée avec des IC_{50} de 2.72 et 2.98 mg/ml respectivement. Les résultats du criblage antimicrobien ont révélé que l'extrait hydrolat était plus actif que l'huile essentielle contre tous les micro-organismes testés avec des valeurs de CMI intéressantes (19 µg/mL). L'efficacité *in-v*itro de l'association de l'extrait hydrolat avec l'amphotéricine B a produit une synergie contre *Candida albicans* (IP444)

L'extrait éthanolique des racines de *Plumbago europaea* a été soumis à une chromatographie sur colonne de gel de silice pour la purification des composés actifs. Trois fractions principales ont été recueillies et nommées F1, F2 et F3. l'extrait brut et les fractions purifiées présentent des propriétés antioxydantes et antifongique très intéressantes. Le pouvoir hémolytique et anti inflammatoire de l'extrait éthanolique a été évalué *in-vitro* et les résultats obtenus ont montré un faible effet hémolytique et une bonne activité anti-inflammatoire. Un bain de bouche sans alcool contenant différentes concentrations de l'extrait éthanolique comme ingrédient actif a été préparé et évalué pour ses propriétés physicochimiques, sa stabilité et son efficacité antifongique *in- vitro*. Le bain de bouche sans alcool préparé a été efficace pour inhiber la croissance de la levure *Candida albicans* in vitro, démontrant ainsi l'efficacité et l'utilité potentielle de l'extrait éthanolique de *Plumbago europaea* dans le contrôle de la candidose. Les résultats de l'étude de stabilité montrent que le produit conservera son activité de base et son intégrité pendant une longue période.

Mots clés *: Plumbago europaea*, plumbagine, huile essentielle, extrait d'hydrolat, extrait éthanolique, antimicrobien, antifongique, bain de bouche sans alcool.

Abstract : In the field of pharmaceutical science, the main objective of the most current research on natural plant extracts and their use in medicine is to develop and introduce scientifically acceptable agents that can replace antimicrobial drugs in the treatment of infectious diseases. The present work aims to investigate new antimicrobial agents from the roots of Plumbago europaea, a medicinal plant native to the Tlemcen region, as well as to develop an application of these plant extracts in a pharmaceutical formulation.

The essential oil and hydrolate extract obtained from the roots of *Plumbago europaea* were analyzed by GC/MS and plumbagin was the majority compound. Both the essential oil and hydrolate extract showed moderate free radical scavenging and reducing ability with IC50s of 2.72 and 2.98 mg/ml, respectively. The antimicrobial screening results revealed that the hydrolat extract was more active than the essential oil against all tested microorganisms with interesting MIC values (19 µg/mL). The in-vitro efficacy of the combination of the hydrolat extract with amphotericin B produced a synergy against Candida albicans (IP444)

The ethanolic extract of *Plumbago europaea* roots was subjected to silica gel column chromatography for purification of active compound. Three main fractions were collected and named F1, F2 and F3. The crude extract and the purified fractions show very interesting antioxidant and antifungal properties. The hemolytic and anti-inflammatory power of the ethanolic extract was evaluated in vitro and the results obtained showed a weak hemolytic effect and a good anti-inflammatory activity. An alcohol-free mouthwash containing different concentrations of the ethanolic extract as active ingredient was prepared and evaluated for its physicochemical properties, stability and in vitro antifungal efficacy. The prepared alcohol-free mouthwash was effective in inhibiting the growth of Candida albicans yeast in vitro, thus demonstrating the efficacy and potential usefulness of the ethanolic extract of Plumbago europaea in the control of candidiasis. The results of the stability study show that the product will maintain its basic activity and integrity for a long period of time.

Keywords: *Plumbago europaea*, plumbagin, essential oil, hydrolate extract, ethanolic extract, antimicrobial, antifungal, alcohol-free mouthwash,

Research article

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RESEARCH ARTICLE

Chemical Composition and Antimicrobial Activity of Essential Oil and Hydrosol Extract From Roots of *Plumbago europaea* and *in-vitro* Combinatory Antimicrobial Effect of Hydrosol Extract with Gentamicin and Amphotericin B

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Abstract: ***Background:*** Infectious diseases are responsible for millions of deaths a year worldwide. Antibiotics, which have saved so many lives and improved life expectancy, may become ineffective due to a worrying increase in bacterial resistance. Some of the appropriate actions that could be initiated to address this problem are to develop and search for new antimicrobial substances from medicinal plants, and combine antibiotics with antimicrobials agents isolated from a reservoir of bioactive natural products.

Objectives: The purpose of this work was to study the chemical composition of the essential oil and hydrosol extract of *Plumbago europaea*, to evaluate their *in-vitro* antimicrobial activities and evaluate *in-vitro* combinatory antimicrobial effect of hydrosol extract with Gentamicin and Amphotericin B against a large panel of microorganisms in an effort to reduce their minimum effective dose and minimizing their side effects.

Methods: The essential oil and hydrosol extract obtained from the roots of *Plumbago europaea* were analyzed by GC/MS and tested for their antibacterial and antifungal activities against twelve different strains of microorganisms. The effectiveness, *in-vitro*, of the association between the hydrosol extract and both Gentamicin and Amphotericin B was also investigated using the checkerboard method.

Results: The obtained results revealed that nine and four components, representing for 92.4% and 97.4% of the total essential oil and hydrosol extract composition were identified, respectively and hydrosol extract was more active than the essential oil against all screened microorganisms, with interesting MIC values (19 µg/mL). An important effect of hydrosol extract was obtained in decreasing the MIC of Gentamicin and Amphotericin B in all tested combinations.

Conclusion: The *in-vitro* combination of the hydrosol extract with Gentamicin and Amphotericin B led to substantial MIC reduction against all tested microorganisms. This combination can help to reduce the minimum effective dose of antimicrobial drugs used, which may help to decrease their side effects; and deliver these medicines with similar potency.

Keywords: *Plumbago europaea*, plumbagin, hydrosol extract, antimicrobial substances, bioactive natural product, antimicrobial drugs.

1. INTRODUCTION

Traditional medicinal practices still play an important role in the primary health care delivery system throughout the world [1].

In most developing countries, traditional medicine and medicinal plants have been widely used as a normative basis for maintaining good health. According to a report released by the World Health Organization (WHO), about 80% of the world's population uses medicinal herbs or derived products for their health care [2]. In addition, several industrialized countries rely significantly on the use of medicinal plants; a large number of their medicines and chemotherapeutic agents are mainly formulated and manufactured using ex-

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tracts from these plants [3]. In the pharmaceutical industry, a wide spectrum of secondary metabolites, which are released by plants, are used either directly as precursors or as lead compounds for the development of novel drugs [4]. Among these compounds, essential oils and extracts of many plant species have attracted significant scientific interest [5-7]. Generally, the process of distillation of any aromatic plant gives two products, *i.e.*, the essential oil (primary oil) as the main product and the hydrosol (distilled water) as a co-product [8].

A review of recent literature has revealed that essential oils possess potential antibacterial and antifungal activity, making them a promising source of new natural drugs [9-11]. Only very few investigations have focused on the chemical composition and biological properties of hydrosols: biological activities of hydrosols from the Australian *Lavandula* species were investigated and an antimicrobial effect was reported [12]. In other works, antifungal potency and antioxidant activity of hydrosols extracts were assessed, and interesting results were obtained [13-15].

With the appearance and spreading of multidrug-resistant strains, microbial infections are becoming a major health care problem. Bacterial and fungal resistance is a phenomenon in constant evolution that continues to defy and confront the healthcare sector almost all over the world, including the developing and developed countries. The leading reason for this rapid increase in antibiotic resistance is the inappropriate usage of antimicrobial agents, which limits their effectiveness significantly and subsequently leads to treatment failure [16-18].

To meet this challenge, today's scientific community and public health authorities are urged to develop efficient strategies and take action for the purpose of solving this problem. The suitable approaches that could be applied are to control and rationalize the antibiotic use, identify and understand the genetic mechanism of drug resistance, discover new antimicrobial substances from medicinal plants, and combine antibiotics with other non-antibiotic drugs, and with natural antimicrobial compounds. It is assumed that some plant extracts, which have target sites different from those of conventional antibiotics, will show better activity against microbial pathogens that are usually resistant to antibiotics without showing the same side effects [4, 11, 17, 19]. According to previous research, the combination of plant metabolites and antibiotics engenders a synergetic effect; moreover, promising antibiotic adjuvants may be provided by phytochemicals [20]. The *in-vitro* association of Gentamicin and different essential oils was found to produce a substantial Gentamicin MIC reduction against Gram-negative bacteria whose pharmacological treatment is very difficult nowadays [21]. Moreover, many studies are conducted towards reducing the amphotericin B dose by combining it with essential oils that have antifungal action in an attempt to produce an effective synergetic action as well as reduce the adverse effects of amphotericin B [10, 22].

The genus *Plumbago* includes 10-20 species of flowering plants that are native to warm temperate and tropical climate regions around the world. *P. europaea* is a perennial plant that is native to Central Asia and the Mediterranean regions. This plant is widely used in traditional folk medicine to treat respiratory disorders, hepatitis, edema, leprosy, inflammations, scabies, toothache, warts, blisters, injury, calluses and skin hardness [23, 24].

The chemical composition of essential oil from *P. europaea* roots has been reported solely in one work [25]. However, our search through the literature indicated that there is no work on the chemical composition of the hydrosol extract, and on the antimicrobial activities of the essential oils and hydrosol extracts from this plant's roots. Consequently, the present research study aims to determine the chemical composition of essential oil and hydrosol extract from roots of *P. europaea*, investigate the *in-vitro* antimicrobial activities of these essential oil and hydrosol extracts, and finally evaluate *in-vitro* combinatory antimicrobial effect of hydrosol extract with Gentamicin and Amphotericin B against a large panel of microorganisms in order to verify the possible synergetic effect between the hydrosol extract and the selected antimicrobial drugs in an effort to reduce their minimum effective dose and minimizing their side effects.

2. MATERIALS AND METHODS

2.1. Collection and Identification of Plant Material

The roots of *P. europaea* were collected from Tlemcen (Algeria) during the period extending from September to October 2017. The plant material was botanically identified by Pr. Noury Benabadji (Laboratory of Ecology and Ecosystem Management of University of Tlemcen Algeria). Voucher specimens were deposited in the herbarium of the University of Tlemcen (P.E. 09.18). The collected plant material was thoroughly washed with water and dried in the shade at room temperature.

2.2. Essential Oil and Hydrosol Extract Isolation

The dried roots of the plant under study were subjected to hydro-distillation using a Clevenger-type apparatus, for a period of 5 h in order to produce a yellow essential oil at 0.016% yield. The essential oil obtained was dried over anhydrous magnesium sulphate and then stored in a dark glass test tube at 4°C until testing and analysis were performed. The hydrosol extract was obtained by Liquid-Liquid Extraction (LLE). Afterwards, the first 500 mL of distilled water was recovered and extracted three times with 200 mL of diethyl ether at room temperature in order to obtain the corresponding hydrosol extract. The organic layer was evaporated and dried with $MgSO_4$, giving yellowish needle-like crystals at an average yield of 0.024%.

2.3. Chromatographic Analysis

2.3.1. Gas Chromatography

Analyses were carried out using a Perkin Elmer Clarus 600 GC apparatus equipped with a dual-flame ionization detection system and two fused-silica capillary columns (60 m x 0.22 mm I.D., film thickness 0.25 µm), Rtx-1 (polydimethylsiloxane) and Rtx-Wax (polyethyleneglycol). The oven temperature was programmed from 60 °C to 230 °C at 2 °C/min and then held isothermally at 230 °C for 35 min. The injector and detector temperatures were maintained at 280 °C. The essential oil and hydrosol extract were injected in the split mode (1/50), using hydrogen as the carrier gas

(0.7 mL min^{-1}); the injection volume was 0.2 µL. The Retention Indices (RI) of the compounds were determined from Perkin-Elmer software.

2.3.2. Gas Chromatography-Mass Spectrometry (GC-MS)

Essential oil and hydrosol extract were analyzed with a Perkin–Elmer TurboMass quadrupole analyzer, coupled to a Perkin-Elmer Autosystem XL, equipped with 2 fused-silica capillary columns and operated with the same GC conditions described above, except for a split of 1/80. In addition, the Electron Impact (EI) mass spectra were acquired under the following conditions: Ion source temperature of 150 °C, energy ionization of 70 eV, and mass range of 35-350 Da (scan time: 1 s).

2.3.3. Component Identification and Quantification

Identification of individual components was accomplished by comparing their GC Retention Indices (RIs) on nonpolar and polar columns, determined relative to the retention time of a series of n-alkanes with linear interpolation, with those of authentic compounds or literature data [26, 27] and through computer matching with commercial mass spectral libraries [28, 29] and also by comparing the spectra obtained with those of the in-house laboratory library. The quantification of Eos and hydrosol extract components was performed using peak normalization (%) abundances calculated by integrating FID response factors relative to tridecane (0.7 g/100 g), used as an internal standard.

2.4. Antimicrobial Activity

2.4.1. Microbial Strains

The microorganisms used in this study belong to the American Typed Culture Collections (ATCC). They were brought from the stock culture of the Laboratory of Food, Biomedical and Environmental Microbiology (LAMAABE) University of Tlemcen, Algeria. These microorganisms included nine (9) bacteria: *Staphylococcus aureus* (ATCC 25923), *Micrococcus luteus* (ATCC 9341), *Listeria monocytogenes* (ATCC 15313), *Bacillus cereus* (ATCC 10876) *Escherichia coli* (ATCC 25912), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 700603), *Salmonella typhimurium* (ATCC 13311), *Enterococcus faecalis* ATCC 49452 and tree yeast *Candida albicans* (ATCC 26790), *Candida albicans* (ATCC 10 231), and *Candida albicans* (IP444) obtained from the Pasteur Institute (Algiers, Algeria).

The antimicrobial activity was determined using the disc diffusion and the micro-well dilution methods. Concentrations of these microorganisms were prepared to contain approximately 1 to 2 × 10^8 CFU/ml of bacteria and 1x10^6 to 5x10^6 CFU/ml of yeast. The Eos was dissolved in tween 80 and hydrosol extract was solubilized in isopropanol/water (50/50) in order to facilitate their dispersion in the aqueous nutrient medium.

2.4.2. Disc Diffusion Assay

The disc diffusion method was performed according to the recommendations of the Clinical and Laboratory Standards Institute CLSI [30]. Mueller-Hinton agar was used for bacteria and Sabouraud supplemented with glucose (2%) for yeasts, the medium of choice was seeded onto the surface with the appropriate microorganisms using a cotton swab. Afterwards, the inoculums were allowed to dry. Then, sterile filter paper disks (6 mm diameter) soaked with essential oil or hydrosol extracts (15µl/disk) were placed on the plates. Gentamicin (10µg/disc) and amphotericin B (0.2 mg/ disc) were used as positive controls for antibacterial and antifungal tests, respectively. The plates were incubated at 37°C for bacteria and 28°C for yeasts for 24 h. Each assay was carried out in triplicates. Then, the inhibition zones were measured.

2.4.3. Micro-Well Dilution Assay

Minimum Inhibitory Concentrations (MICs) of the essential oil or hydrosol extract were performed using the method of the microplate (96 wells) [31]. The test microorganisms mentioned above were inoculated into Mueller Hinton broth for bacteria and Sabouraud broth supplemented with glucose (2%) for yeasts. After 24 h, 100 µl of each inoculum (5 × 10^5 CFU/ml for bacteria and 1 to 5 ×10^4 CFU/ml for yeast strains respectively) was prepared. Then, 100 µl of each sterile broth was placed in each line of the microplate. Afterwards, 100 µl of the extract was introduced into the first well. After thoroughly mixing the contents, 100 µl was removed from the first well and placed in the second well, until reaching the 10th well; the remaining100 µl was then eliminated. As a result, ½ dilution was obtained. In the end, 100 µl of each inoculum was added. The last two wells represented negative controls; the 11th well contained the medium and inoculum, and the 12th one contained only the medium. The microplates were sealed and incubated at 37 °C for bacteria and at 28 °C for yeasts, during 20 h. Each assay was carried out in triplicate incubation. The MIC was defined as the lowest concentration of essential oil or hydrosol extract that inhibits the visible growth.

2.4.4. Determination of the Synergistic Activity

For the purpose of assessing the synergetic effect, a combination of hydrosol extract and some selected antimicrobial agents was investigated using the checkerboard method, as described by White *et al.* [32]. In this method, appropriate hydrosol extract concentrations were mixed with each antimicrobial drug dilution in order to obtain a series of combinations between different concentrations of the hydrosol extract with each dilution of Gentamicin and amphotericin B. The dilutions of hydrosol extract and antimicrobial drugs were prepared using the same method, and then the MIC was evaluated. The concentrations prepared accounted for 1/8 times the MIC to 4 MIC for both hydrosol extract and antimicrobial drugs.

To evaluate the effect of the combinations, the Fractional Inhibitory Concentrations (FICs) and the Fractional Inhibitory Concentration Indices (FICIs) were calculated for the Hydrosol Extract (HE) and Antimicrobial Drugs (AD) in each combination, using the following formulas:

$$\text{FIC of HE} = \frac{\text{MIC HE combinaition}}{\text{MIC HE alone}}$$

$$\text{FIC of AD} = \frac{\text{MIC AD combinaition}}{\text{MIC AD alone}}$$

The MIC values for the HE-AD combinations were defined as the lowest concentrations at which no visible growth of microbial strains could be detected as compared to their growth in the control well.

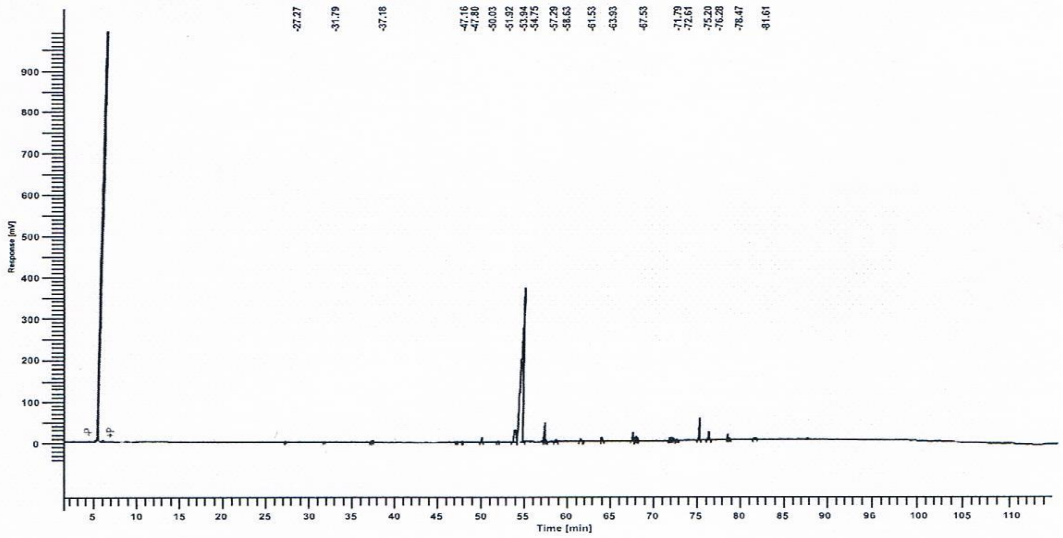

Fig. (1). GC Chromatogram of Hydrosol extract.

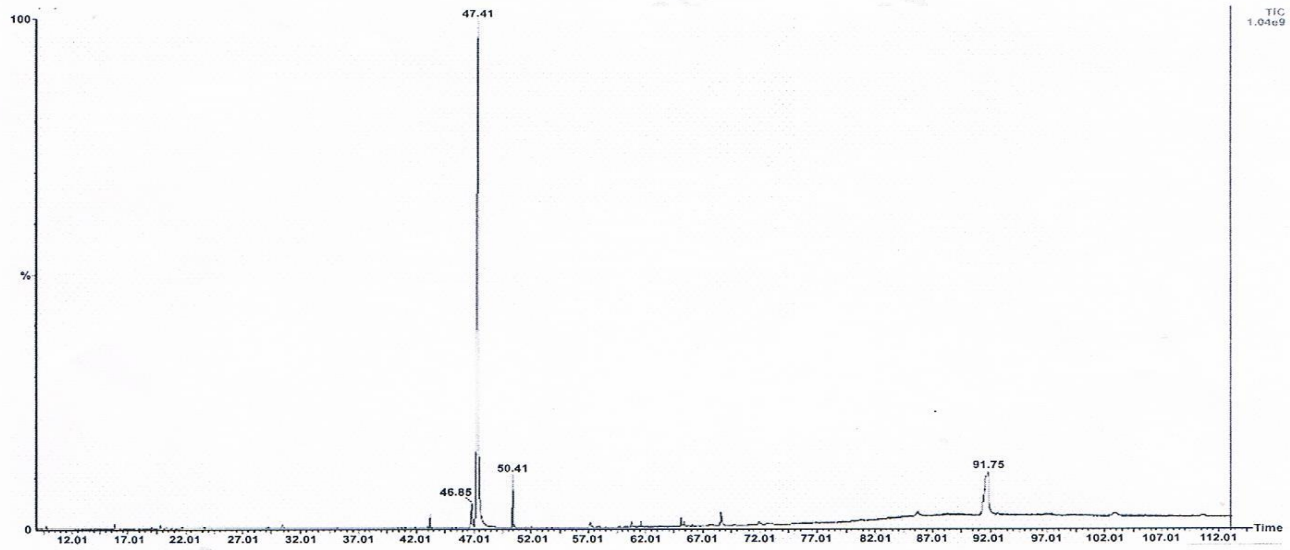

Fig. (2). GC-MS Chromatogram of Hydrosol extract.

$$\text{FIC index} = \text{FIC of HE} + \text{FIC of AD}$$

Synergy was defined as a FIC index value ≤ 0.5. Indifference was defined as a FIC index value > 0.5 but ≤ 4. In addition, antagonism was defined as a FIC index value > 4.

2.5. Statistical Analysis

Statistical analyses were performed using the PAST program version 3.22.

3. RESULTS

3.1. Chemical Composition of Essential Oil and Hydrosol Extract

The essential oil and hydrosol extract obtained from dried *P. europea* roots by hydrodistillation and liquid-liquid extraction methods, respectively, were analyzed using gas chromatography (GC, Fig. **1**) and gas chromatography-mass

Table 1. Chemical composition of essential oil and hydrosol extract of *P. europea*.

No [a]	Compounds	*Lit*/RI [b]	RIa [c]	RI_p [d]	*Essential oil*	*Hydrosol extract*	Identification [e]
1	α-Pinene	931	931	1022	0.5	-	RI, MS
2	Myrcene	980	979	1159	0.5	-	RI, MS
3	p-Cymene	1011	1013	1270	0.1	-	RI, MS
4	Limonene	1020	1023	1196	3.5	-	RI, MS
5	Linalol	1081	1094	1544	2.8	-	RI, MS
6	Nonanal	1083	1090	1394	16.2	tr	RI, MS
7	Thymol	1266	1278	2189	0.2	4.8	RI, MS
8	Carvacrol	1281	1278	2289	tr	12.4	RI, MS
9	Plumbagin	1580	1600	2822	68.6	80,2	RI, MS, ref
% Identification					**92.4**	**97.4**	
% Oxygenated monoterpenes					2.8	-	
% Monoterpene hydrocarbons					4.5	-	
% Phenolic compounds					68.9	97.4	
% Non-terpenic compounds					16.2	-	

[a]:Order of elution is given on apolar column (Rtx-1), [b]: Retention Indices of literature on the apolar column (IRIa) reported from könig *et al.* 2001, [c]: Retention indices on the apolar column Rtx-1 column RIa, [d]: Retention Indices on the polar Rtx-wax column (RIp)

Table 2. Inhibition Zones of Essential Oil (EO) and Hydrosol Extract (HE).

-	Microorganims	Zones of Inhibition (mm)			
		EO (0.5mg/disc)	HE (0.83mg/disc)	Gent (10 µg/disc)	Ampho B
Gram-positive bacteria	*S. aureus*	15±1	40.33±0.577	33±0,577	-
	M. luteus	23±1	45±0.577	19±0.333	-
	L. monocytogenes	7±1	25±1	12±0,577	-
	E. faecalis	-	10.5±0,5	18,5±0,5	-
	B. cereus	10±0	31.66±0.577	22±0.70	-
Gram-negative bacteria	*E. coli*	0±0	11.66±0.577	23±0,5	-
	P. aeruginosa	6±1	10±1	25±0.0	-
	K. pneumoniae	0±0	11±1	15±0.70	-
	S. typhimurium	10±0.577	22.5±0.577	26.5±0.333	-
Yeast	*C. albicans ATCC 26790*	43±0.333	51±0.0	-	30±0.333
	C. albicans ATCC 10 231	39±0.666	51±0.333	-	32±0.333
	C. albicans IP444	0	15 ± 1	-	30±0.333

spectrometry (GC-MS, Fig. **2**) techniques. The chemical compositions of the essential oil and hydrosol extract are given in Table **1**. Nine components accounting for 92.4% were identified in the essential oil (Table **1**).

The major classes of components were phenolic compounds with a percentage of 68.9% followed by non-terpenic compounds (16.2%), monoterpene hydrocarbons (4.5%) and oxygenated monoterpenes (2.8%). Phenolic compounds identified were plumbagin with a percentage of 68.6%. The class of monoterpene hydrocarbons was presented by limonene (3.5%), α-pinene (0.5%) and myrcene (0.5%). The linalool (2.8%) was the single constituent of oxygenated

Table 3. Minimum inhibitory concentration of Essential Oil (EO) and Hydrosol Extract (HE).

	Microorganims	MIC (µg/mL)			
		Eo	HE	Gent	Ampho B
Gram-positive bacteria	*S. aureus*	> 1000±0.0	19±0.0	0.5±0.0	-
	M. luteus	>2000 ±0.0	19±0.0	8±0.0	-
	L. monocytogenes	>4000±0.0	19±0.0	8±0.0	-
	E. faecalis	-	78±0.0	16±0.0	-
	B. cereus	>3120±0.0	19±0.0	0.5±0.0	-
Gram-negative bacteria	*E. coli*	-	78±0.0	0.5±0.0	-
	P. aeruginosa	-	78±0.0	0.5±0.0	-
	K. pneumoniae	-	78±0.0	8±0.0	-
	S. typhimurium	>4000±0.0	156±0.0	0.25±0.0	-
Yeast	*C. albicans ATCC 26790*	>4000 ±0.0	19±0.0	-	4±0.0
	C. albicans ATCC 10 231	>4000±0.0	19±0.0	-	8±0.0
	C. albicans IP444	-	19±0.0	-	8±0.0

monoterpenes class identified in *P. europeae* essential oil. A study of the chemical composition of the essential oil of *P. europaea* roots from Iran revealed the presence of 15 components.

The major components turned out to be Plumbagin (69.1%), 1-octen-3-yl acetate (9%), limonene (5.7%), nonanal (2.5%) and β-bisabolene (2.1%) (34). With regard to the results of our study, the same main component (plumbagin) was found with almost the same percentage. However, some differences were found regarding the other components, which may be attributed to the geographical region, species, and age of the plant. This should certainly give a different chemical composition of essential oils. Therefore, it may be stated that the same species/variety can produce essential oils with different compositions [33, 34]. The hydrosol extract was obtained using the liquid-liquid extraction technique and was then analyzed by GC-RI and GC-MS methods. Four components, accounting for 97.4 % of the total extract composition, were identified in the hydrosol extract of *P. europeae* (Table **1**). Hydrosol extract was represented only by phenolic compounds with a percentage of 97.4%. The major components were plumbagin (80.2%), carvacrol (12.4%) and thymol (4.8%). To the best of our knowledge, no work has been reported on the chemical composition of hydrosol extract of *P. europea* roots. For this, the present study seeks to address this issue.

3.2. Antimicrobial Activity of Essentials Oil and Hydrosol Extract

The antimicrobial activity of *P. europeae* essential oil and hydrosol extract was evaluated against a panel of 12 microorganisms (9 bacteria and 3 yeast); their potencies were qualitatively and quantitatively assessed by the presence or absence of the inhibition zones; moreover, their inhibition zone diameters and MIC values were determined. The results obtained are summarized in Tables **2** and **3**.

The results obtained revealed that both essential oil and hydrosol extract exhibited antibacterial activity of varying magnitude. The hydrosol extract turned out to be more active than the essential oil against all tested bacteria. Furthermore, the data obtained from the disc diffusion method showed the highest activity of the essential oil was obtained against *M. luteus* (ATCC 9341) and *S. aureus* (ATCC 25923) with inhibition zones of 15 and 23 mm respectively.

However, no activity against the three microorganisms *K. pneumoniae*, *E. coli* and *P. aeruginosa* was observed (Table **2**). Moreover, it was revealed that the hydrosol extract inhibited the growth of bacterial strains, producing an inhibition zone diameter between 10 and 45mm, depending on the susceptibility of the tested species. In addition, the Gram-positive strains showed more susceptibility towards the tested hydrosol extract than the Gram-negative ones. The most sensitive microorganisms towards the hydrosol extract were *Micrococcus luteus* (ATCC 9341), *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 10876), with inhibition zones of 45, 40 and 31 mm, respectively. A very interesting activity against the tested yeast *Candida albicans* was observed. According to the inhibition zones diameter obtained, essential oil and hydrosol extract showed activity against the tested yeasts, significant to that of positive control (Amphotericin B) (Table **2**). To obtain more precise data about the antibacterial properties of the tested essential oil and hydrosol extract, the Minimum Inhibitory Concentration (MIC) was determined (Table **3**). The hydrosol extract of *P. europeae* exhibited a remarkable inhibitory effect on the growth of tested micro-organisms as compared to Gentamicin. The most prominent inhibitory action of hydrosol extract was observed against *S. aureus, L. monocytogenes, M. luteus, B. cereus* and *C. albicans* with MIC values of 19 µg/ml. The lowest antibacterial activity of hydrosol extract was obtained against *S. typhimurium* (156 µg/ml). While essential oil exhibited weak activity against all microorganisms with MICs exceeding 312 µg/ml.

3.3. Synergistic Activity

Recently, the evaluation of *in vitro* synergistic activity between different antibiotics has been mainly based on the interpretations of stated data from FIC index experiments. According to many specialist journals in the antimicrobial domain, synergy is defined as a FIC index value ≤ 0.5. Moreover, indifference is defined as a FIC index value > 0.5 but ≤ 4. (0.5 < FICI value ≤ 4), and antagonism is defined as a FIC index value > 4 [32, 35]. The synergistic effect of hydrosol extract from roots of *P. europeae* with Gentamicin and Amphotericin B was studied and the obtained MICs and FIC index values are listed in Tables **4** and **5**. In the current study, an indifferent effect was produced between the hydrosol extract and Gentamicin against all tested microorganisms, with a FIC index value between 1 and 1.5. An indifferent effect was also obtained between the hydrosol extract and Amphotericin B blend against *C. albicans* (ATCC 26790) and *C. albicans* (ATCC 10231), while a synergic effect was observed against *C. albicans* (IP444) with FIC index of 0.375 (Table **5**). Despite the absence of a synergistic effect, the combination of hydrosol extract with Gentamicin and Amphotericin B enhanced their antimicrobial activity and the inhibition of the growth of all tested bacteria and yeast, which was confirmed by the significant decrease in the MIC values of the antimicrobial drugs. The MIC value of Gentamicin (0.5 µg ml) against *S. aureus* (ATCC 25923), *M. luteus* (ATCC 9341), *E. faecalis* (ATCC 49452) *B. cereus* (ATCC 10876) and *P. aeruginosa* (ATCC 27853) was lowered to 1/2 (combined MIC 0.25µg/mL), when it was used in combination with the hydrosol extract of *P. europea* at MIC of 19, 19, 39, 9.5 and 39 µg/mL, respectively. With regard to *L. monocytogenes* (ATCC 15313), *E. coli* (ATCC 25912), *K. pneumoniae* (ATCC 700603) and *S. typhimurium* (ATCC 13311), this combination was more efficient since it reduced the MIC of Gentamicin to 1/8 of its initial value. Indeed, the combined MIC of Gentamicin was reduced from 0.5 to 0.0625 µg/mL for *E. coli*, from 8 to 1 µg/mL for *K. pneumoniae* and *L. monocytogenes*, and from 0.25 to 0.0312 µg/mL for *S. typhimurium*) (Table **4**). Furthermore, the MIC values of amphotericin B against *C. albicans* (ATCC 26790) and *C. albicans* (ATCC 10 231), *i.e.*, 4 and 8 µg/mL respectively, were lowered to 1/2 and to 1/4 when combined with the hydrosol extract of *P. europea* at the MIC values of 2.375 µg/mL and 9.5 µg/mL, respectively (Table **5**).

4. DISCUSSION

Hydrosol extract and essential oil of *P. europea* roots expressed the best antibacterial activity in the MIC test on Gram-positive tested bacteria. The investigation and analysis of the above-mentioned results, together with the observation of the chemical composition of both essential oil and hydrosol extract, lead us to conclude that the effective antimicrobial activity of hydrosol extract is probably mainly due to the presence of a high concentration of plumbagin (80.2%). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a secondary metabolite found in *Plumbagenaeace, Droseraceae*, and *Ebenceae* families. The pharmacological screening of different extracts of *Plumbago* species revealed that this compound is a bio-active marker that is endowed with a number of pharmacological activities [36-38]. The results of the present study suggest that the hydrosol extract can act as an antimicrobial agent against *S. aureus, L. monocytogenes, M. luteus* and *B. cereus* with the MIC value of 19 µg/ml that is close to that obtained with the isolated plumbagin from *Aristea ecklonii* (16µg/ml) [39]; however, this value is higher than that obtained from the plumbagin isolated from other *Plumbago* species [40-42]. In this study, the hydrosol extract was found to contain plumbagin in combination with thymol and carvacrol which can have an antagonistic effect on the antimicrobial activity of the plumbagin. On the other hand, the essential oil displayed weak antibacterial and antifungal effects on the micro-organisms tested. This decrease in activity can be due to the association of non-active compounds such as nonanal. Furthermore, the limited water solubility of the essential oil is believed to limit the diffusion of its components through the agar medium, which consequently reduces its antimicrobial activity [43]. Several mechanisms of antimicrobial activity of plumbagin have been described in the literature. Indeed, Asche *et al.* reported that plumbagin intercalates into the DNA, inducing single- or double-strand breaks [44]. As for Padhye *et al.*, they established the capacity of plumbagin to chelate multiple trace metals, which can enhance its antimicrobial activities [38].

The widespread dissemination of microbial multidrug resistance has become a significant global concern such that in 2016, about 600000 incident cases of tuberculosis from a total of 10.4 million were rifampicin-resistant and 490 000 were multidrug-resistant. Moreover, drug resistance has started to complicate the fight against HIV and malaria, as well [45]. The research and development of innovative antibiotics have decreased to such an extent that today there are practically no new and more effective antibiotics developed in the market [46]. In May 2017, it was reported that a total of 51 antibiotics (including combinations) and 11 biologicals were in the clinical pipeline with 42 new therapeutic entities that target priority pathogens. The qualitative analysis shows a lack of potential treatment options for multidrug- and extensively drug-resistant Gram-negative pathogens [47]. Since the development of new antibiotics involves a great deal of time, effort, scientific research, and expense and in order to deal with infections caused by multidrug-resistant bacteria, alternative strategies must be found. Indeed, one scheme that can be adopted to improve the efficacy of existing antibiotics is their combination with phytochemicals [46, 48]. The combinations of antimicrobial compounds are crucial as they can reduce or prevent the emergence of resistant strains, diminish dose-related toxicity, and achieve a broad-spectrum antimicrobial activity [49]. Gentamicin is an aminoglycoside antibiotic that is broadly used to treat and prevent life-threatening Gram-negative bacterial infections. However, this antibiotic drug has several side effects that limit its use. Nephrotoxicity, psychiatric disorders (depression, confusion, anorexia, visual hallucinations and disorientation) as well as the toxicity to the ear sensory cells, are the most pertinent side effects worth mentioning [21].

In this study, a growth-inhibitory effect of Gentamicin was observed when combined with the hydrosol extract against a different strain of gram-negative tested bacteria; *Escherichia coli* (ATCC 25912), *Klebsiella pneumoniae* (ATCC 700603), and *Salmonella typhimurium* (ATCC

Table 4. Hydrosol extract and Gentamicin - Fractional Inhibitory Concentration (FIC) and FIC Indices.

	MICo	MICc	FIC	FIC index
Staphylococcus aureus (ATCC 25923)				
Hydrosol extract	19	19	1	1.5
Gentamicin	0.5	0.25	0.5	
Micrococcus luteus (ATCC 9341)				
Hydrosol extract	19	19	1	1.5
Gentamicin	8	4	0.5	
Listeria monocytogenes (ATCC 15313)				
Hydrosol extract	19	19	1	1.125
Gentamicin	8	1	0.125	
Enterococcus faecalis (ATCC 49452)				
Hydrosol extract	78	39	0.5	1
Gentamicin	16	8	0.5	
Bacillus cereus (ATCC 10876)				
Hydrosol extract	19	9.5	0.5	1
Gentamicin	0.5	0.25	0.5	
Escherichia coli (ATCC 25912)				
Hydrosol extract	78	78	1	1.125
Gentamicin	0.5	0.0625	0.125	
Pseudomonas aeruginosa (ATCC 27853)				
Hydrosol extract	78	39	0.5	1
Gentamicin	0.5	0.25	0.5	
Klebsiella pneumoniae (ATCC 700603)				
Hydrosol extract	78	78	1	1.125
Gentamicin	8	1	0.125	
Salmonella typhimurium (ATCC 13311)				
Hydrosol extract	1.56	1.56	1	1.125
Gentamicin	0.25	0.0312	0.125	

MICo,= MIC of an individual sample, MICc,= MIC of an individual sample at the most effective combination; FIC= Fractional Inhibitory Concentration (see text); FICI = FIC of Hydrosol extract + FIC of Gentamicin.

13311) which confirmed by the significant decrease in the MIC values. These combinations can reduce Gentamicin minimum effective dose and minimize its secondary effects.

Different hypotheses could be taken into account in order to shed light on the mechanism responsible for this interaction. Concerning the chemical composition, it was found that plumbagin is the main component of the hydrosol extract with a percentage of 80.2 %, which prompted us to suppose that the high content of this component favors and potentializes the mechanism of action of Gentamicin in interrupting the protein synthesis by binding the 30S subunit of the bacterial ribosome.

Since the 1950s, the polyene antibiotic Amphotericin B has been widely used in the treatment of fungal infections. It can bind to the fungal cell membrane causing permeability alteration. The most common adverse effects of Amphotericin B are nausea, loss of appetite, stomach pain, weight loss, headache, fever and chills, muscle and joint pain, hepato- and nephrotoxicity [50,51]. The increasing number of immunodeficient and immunocompromised patients over the last few years has led to a raised incidence of fungal infections [52]. *C. albicans* is one of the major opportunistic fungal pathogens generally located in the oral cavity, digestive tract and genital region, just like the commensal flora encountered

Table 5. Hydrosol extract and amphotericin B- Fractional Inhibitory Concentration (FIC) and FIC Indices.

-	MICo	MICc	FIC	FICI
Candida albicans (ATCC 26790)				
Hydrosol extract	19	2.375	0.125	0.635
Amphotericin B	4	2	0.5	
Candida albicans (ATCC 10 231)				
Hydrosol extract	19	9.5	0.5	0.75
Amphotericin B	8	2	0.25	
Candida albicans (IP444).				
Hydrosol extract	19	2.375	0.125	0.375
Amphotericin B	8	2	0.25	

MICo. =MIC of an individual sample, MICc,= MIC of an individual sample at the most effective combination; FIC= Fractional Inhibitory Concentration (see text); FICI = FIC of Hydrosol extract + FIC of Amphotericin B

in more than half of the healthy population. When given the pathogenic opportunity, *C. albicans* can be responsible for more than 50% of human candidiasis and may cause severe infections [53, 54]. This study revealed that combining Amphotericin B with the hydrosol extract of *P. europea* caused a significant synergetic effect against *C. albicans* (IP444), with the FIC index value of 0.375 and induced a significant decrease in the MIC values of Amphotericin B against *C. albicans* (ATCC 26790) and *C. albicans* (ATCC 10 231). This combination can reduce the minimum efficacious dose of amphotericin B, thus minimizing its adverse effects, which motivated us to suggest this association for the treatment of infections caused by *C. albicans* which is the most common human pathogenic fungus that causes a variety of disorders ranging from superficial mucosal diseases to deep-seated mycoses. Actually, this *Candida* species is the major cause of invasive candidiasis characterized by a high rate of morbidity and mortality in critically immunodeficient patients [55, 56].

CONCLUSION

The present research aimed to determine the chemical composition of the essential oil and hydrosol extract from *Plumbago europea* roots and to discover that the plumbagin is their main component. On the other hand, the hydrosol extract exhibited a very interesting antimicrobial activity against a large panel of microorganisms. It was also revealed that the *in-vitro* combination of the hydrosol extract with Gentamicin and Amphotericin B led to substantial MIC reduction against all tested microorganisms. This combination can help to reduce the minimum effective dose of antimicrobial drugs used, which may help to decrease their side effects; and deliver these medicines with similar potency. Thus, these important results may help the formulation of new antimicrobial agents for the cure of infections caused by the Gram-negative bacteria and by *Candida albicans*. Finally, these promising findings encouraged us to carry out *in vivo* testing in further research for the purpose of assessing the therapeutic effectiveness of these drug combinations.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are basis of this research.

REASEARCH INVOLVING PLANTS

The plant used are not on the IUCN endangered list.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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