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THEME

Exploration into the Antibacterial Attributes of Medicinal Plants: *In Vitro* Examinations, ADMET Prediction, Molecular Docking Analysis and Molecular Dynamics simulation.

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With deep appreciation,

Amira Berretima

Dedications

Dedications:

I dedicate this noble work ..

To my family, who taught me the true meaning of resilience and unconditional love.

For [Name], whose unwavering support and belief in my story gave me the courage to share it with the world.

To the friends who became family, thank you for standing by my side through the highs and lows of life. This memoir is as much yours as it is mine.

In honor of the countless individuals whose stories have intertwined with mine, shaping the person I am today.

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For [KARIM], whose presence continues to inspire me every day. whose absence is felt but whose spirit lives on in the pages of this memoir.

Summary

The graduation thesis “Exploring the antibacterial properties of medicinal plants” aims to study the antibacterial potential of compounds extracted from medicinal plants. Laboratory assays have been performed to evaluate antibacterial activity against important species such as Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, and Bacillus subtilis. The Reviews of Drugs and Bioscenarios (ADMET) included analysis of the distribution, absorption and druggability of plant compounds. Molecular binding analysis and molecular dynamics simulations have also been used to understand the molecular interactions between compounds and bacteria. The results showed the strong potential of plant compounds to combat bacteria, suggesting they could be used as a natural alternative to traditional antibiotics. Further research is recommended to determine the exact mechanisms of this effectiveness and to develop new treatments to combat bacterial infections.

Keyswords: Medicinal Plants, Vitro Examinations, ADMET Prediction, Molecular Docking Analysis, Molecular Dynamics simulation.

Resume

Le mémoire de fin d'études « Exploration des propriétés antibactériennes des plantes médicinales » vise à étudier le potentiel antibactérien des composés extraits des plantes médicinales. Des tests en laboratoire ont été effectués pour évaluer l'activité antibactérienne contre des espèces importantes telles que Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli et Bacillus subtilis. Les examens des médicaments et des bioscénarios (ADMET) comprenaient une analyse de la distribution, de l’absorption et de la pharmacobilité des composés végétaux. L'analyse des liaisons moléculaires et les simulations de dynamique moléculaire ont également été utilisées pour comprendre les interactions moléculaires entre les composés et les bactéries. Les résultats ont montré le fort potentiel des composés végétaux

pour lutter contre les bactéries, suggérant qu'ils pourraient être utilisés comme alternative naturelle aux antibiotiques traditionnels. Des recherches plus approfondies sont recommandées pour déterminer les mécanismes exacts de cette efficacité et développer de nouveaux traitements pour lutter contre les infections bactériennes.

Mots de clés: plantes médicinales, examens vitro, prédiction ADMET, analyse d'amarrage moléculaire, simulation de dynamique moléculaire.

ملخص

تهدف الأطروحة النهائية بعنوان "استكشاف الخصائص المضادة للبكتيريا للنباتات الطبية" إلى دراسة الإمكانات المضادة للبكتيريا للمركبات المستخرجة من النباتات الطبية. تم إجراء الاختبارات المعملية لتقييم النشاط المضاد للبكتيريا ضد أنواع مهمة مثل المكورات العنقودية الذهبية، والكلبسيلا الرئوية، والإشريكية القولونية، والعصيات الرقيقة. تضمنت مراجعات الأدوية والسيناريو الحيوي (ADMET) تحليلًا لتوزيع المركبات النباتية وامتصاصها وقابلية تعاطيها للعقاقير. كما تم استخدام تحليل الروابط الجزيئية ومحاكاة الديناميكيات الجزيئية لفهم التفاعلات الجزيئية بين المركبات والبكتيريا. وأظهرت النتائج الإمكانات القوية للمركبات النباتية في مكافحة البكتيريا، مما يشير إلى إمكانية استخدامها كبديل طبيعي للمضادات الحيوية التقليدية. يوصى بإجراء مزيد من الأبحاث لتحديد الآليات الدقيقة لهذه الفعالية وتطوير علاجات جديدة لمكافحة الالتهابات البكتيرية.

الكلمات المفتاحية: النباتات الطبية ، فحوصات المختبر ، التنبؤ ADMET ، تحليل الإرساء الجزيئي ، محاكاة الديناميات الجزيئية.

List of abbreviations:

MDR: multiple drug resistance

WHO: world health organization

ATP: adenosine triphosphate

MWHD: Microwave assisted hydro distillation.

GC: Gas Chromatography

MS: Mass Spectrometry

HPLC: Hight Performance Liquid Chromatography

TLC: Thin Layer Chromatography

FTIR: Fourier Transform Infrared Spectroscopy

13C-NMR: Carbon-13 Nuclear Magnetic Resonance

FID: flame ionization detector

CN: Chemical Negative

CP: Chemical Positive

EI: Electron Impact

TR: times retention

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INTRODUCTION:

Medicinal plants, additionally called medicinal herbs, have been found and utilized in conventional medication rehearses since ancient times. Plants combine many substance compounds for different capabilities, including safeguard and insurance against bugs, parasites, infections, and herbivorous well evolved creatures (Gershenzon, 2022)

In recent years ,The failure of the regular treatment was due to the habitual usage of traditional herbal medicine (M.Tech, 2022) the global healthcare landscape has been increasingly challenged by the rise of antibiotic-resistant bacteria ,However !emergence and dissemination of multidrug resistant (MDR) strain in pathogenic bacteria have become a significant public health threat as there are fewer, or even sometimes no, effective antimicrobial agents available for the infection caused by pathogenic bacteria.

Thus, in the light of the evidence of the rapid global spread of resistant clinical isolates, the need to find new antimicrobial agents is of paramount importance, A vast number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds as an alternative that can potentially be effective in the treatment of these problematic bacterial infections. According to the World Health Organization (WHO), medicinal plants would be the best source to obtain a variety of drugs.

Many plants have been used because of their antimicrobial traits, which are due to phytochemicals synthesized in the secondary metabolism of the plant. Plants are rich in a wide variety of secondary metabolites such as tannins, alkaloids, phenolic compounds,

and flavonoids, which have been found *in vitro* to have antimicrobial properties. A few phytotherapy manuals have mentioned various medicinal plants for treating infectious

diseases such as urinary tract infections, gastrointestinal disorders, respiratory disease, and cutaneous infections. (Manandhar, 2019)

Although medicinal plants have been used for a long time and there is anecdotal evidence of their effectiveness, their antibacterial properties have not yet been thoroughly investigated and scientifically confirmed. Phytochemical analysis, *in vitro* and in vivo studies, computational modeling, and clinical trials are examples of modern research approaches that provide effective instruments for deciphering the intricate mechanisms behind the antibacterial activity of medicinal plants.

By using a multidisciplinary approach, this study aims to contribute to the growing body of knowledge on the antibacterial properties of medicinal plants by employing a multidisciplinary approach. By integrating computational and experimental methodologies, we want to clarify the bioactive compounds that display antibacterial activity, characterize their mechanisms of action, and explore their potential applications in the treatment of bacterial infections.

CHAPTER 1

General Overview of Medicinal

I-. General Overview of Medicinal Plants:

I-1.1. Introduction:

One of the great blessings bestowed upon humanity is the rich diversity of nature adorned with various colours of plants and crops, endowed with beauty and utility by the Creator. This has been mentioned in the Book of Allah, Almighty, in multiple instances. Since the existence of humankind on the surface of the Earth, the method of treating ailments with medicinal plants and natural herbs has been known instinctively through nature and personal experiments. In ancient times, all diseases and pains were treated with herbs. As days passed and civilizations evolved, chemically manufactured drugs emerged to compete with herbs.

Thanks to practical and technological advancement, humanity gradually began to rely on drugs and chemical medications, gradually replacing herbs in treatment. However, despite this, herbs have managed to capture attention once again in the present time, becoming a subject of discussion among scientists, doctors, and patients alike, sparking debates between endorsement and rejection. (Omar, 2018-2019)

I-1.2. Historical Insight into Medicinal Plants:

I-1.2.1. Primitive Human Perception of Medicinal Herbs:

Humans want to revive their health and enhance their tools led them to their presence in the ear list to provide media herbs and their uses in treatment.

The beginnings of folk medicine or home medicine in prehistoric times depended on the system of experience and error; Where some plants were used as a food to find out any toxic and any of them carrying a medical value and helping to recover from diseases. Some diseases were treated using herbal remedies such as constipation and colds. It should be noted that magic and religion had a great role at that time, as it was accompanied by giving vegetarian or talisman, dance, and some magicians' tricks, so the first doctors in ancient times

were magicians and sorcerers. Primitive doctors have shown their wisdom by treating the soul and body of the patient, where the patient feels better when both the doctor and the patient believe in the effectiveness of the drug, and this is called modern medicine, the imaginary treatment that has become used today. (altibbi, 2008)

I-1.2.2. Healing with Herbs in Ancient Egypt and Babylonians:

The Egyptians were an organized society with tools such as written language and mathematics, which enabled them to record and develop ideas, and they believed in prayer as a solution to health problems, but they also have natural or practical treatments. (medicalnewstoday, 2018)

such as herbs and them:

- Abies Cilicica (PINACEAE) plant It is extracted from it Resin and Oils used in antiseptic and an emulting material prootes Hair Growth
- Senegal Akassia plant is used to treat gums and diabetes
- Aloe vera plant used to treat Expels catarrh from the nose, relieves headaches, soothes chest pains, for burns, ulcers, skin disease, and allergies. (National library of medcin, 2021)

The Babylonians' knowledge of the benefits of herbs was not inferior to that of the Egyptians. Historical records mention that Babylonian herbs were familiar with the properties of over 250 medicinal herbs, which they tested and experimented with, as well as 180 animal drugs and 180 mineral drugs. History indicates that the Babylonian king “Marduk kaldu” (710-772 BC) established a garden with over 64 types of plants, including apples, pomegranates, and poppies (Omar, 2018-2019)

I-1.2.3. Healing in India and China:

This system has arose in China more than 2000 years ago, and it is based on a theory that the disease results in an inappropriate flow of life (Qi Qi) through the body. Chi is restored by budget the opposite forces of Yin and Yang, which appears in the body. The heat and cold, externally, and internally, and a deficiency and surplus. Therefore, different practices are used to maintain and restore Chi, such as medicinal herbs.

Traditional Chinese medicine uses preparations that contain a mixture of herbs to treat various diseases such as irritable bowel syndrome (colon cramping syndrome), Torite syndrome, and many other disorders. (proof, 2021) , Indian civilization also contributed to herbal treatments, especially considering the climatic conditions that facilitated the growth of spices and other natural herbs unique to India, such as cinnamon, black pepper, and ginger. Herbal healing in ancient India was not exempt from magic, similar to ancient and antique treatments in general. However, despite this, Indian medicine introduced vaccination and cosmetic surgery, providing many benefits to Greek and Arab medicine that followed.)Omar(2019-2018 ،

I-1.2.4. Healing Among the Greeks:

Herbs in ancient Greek medicine were of great importance, as they are full of vitamins and beneficial minerals that the body needs to stay in good condition. And from these herbs:

- The dill was used as the dill was used to treat burns and wounds
- Fennel is another herb that the ancient Greeks used, because fennel contains large amounts of anti -inflammatory that can help relieve any problems in the digestive system.
- Greek mountain tea, colds, sore throat, or any disease in this regard) Constitution(2021 ،

I-1.2.5. Herbal Healing in the Islamic Era:

During the Islamic Renaissance, which emerged a century after the death of the Prophet Muhammad, Muslims translated, researched, compiled, and established large libraries. Among those who contributed to herbal medicine were Al-Biruni, Al-Idrisi, Al-Ghafiqi, Al-Antaki, Al-Razi, and Ibn Sina. However, the most prominent herbalist was Ibn al-Baitar, known for his book "Al-Jami' li-Mufradat al-Adwiya wa al-Aghdhiya," in which he described over 1400 herbs, including 300 species with illustrations, many of which were mentioned for the first time.

The credit goes to the Arabs for opening the first pharmacy in Baghdad in the early 8th century CE. They were also credited with establishing the science of pharmacy, which was initially just a trade in drugs. They contributed to setting the rules for medicines, such as Al-Razi's "Al-Hawi," Ibn Sina's "Al-Qanun," Al-Antaki's "Al-Tadhkirah," and Ibn al-Baitar's "Al-Jami'." Additionally, Arabs separated medicine from pharmacy and separated pharmacy from herbalism.

Muslims introduced reforms, organization, and explanations to the ancient heritage, which often contained mystery and errors. They also contributed to the development and advancement of sciences with their new discoveries and inventions in various fields. For example:

1. They invented the anesthesia sponge, which was soaked in opium or belladonna during surgeries.

2. They distinguished between acids and alkalis, and they knew distillation, sublimation, filtration, crystallization, and vaporization.

3. They extracted alcohol from sugar and starch materials and produced new drugs through their chemical processes.

4. They discovered numerous medicinal plants and added them to the medical herbal dictionary. They also discovered drugs that were previously unknown and realized that herbs from hot regions were more effective than those from cold regions.

5. They emphasized preventive measures before treatment and advocated for wisdom)Omar(2019-2018 ،

I-1.2.6. Herbal Healing in Europe:

Europeans began to enter the vast world of medicine, especially in the early 19th century, when researchers, herbalists, botanists, and chemists turned to organic chemistry to discover the secrets of plants and their active elements. They made significant progress in this field, enabling them to understand the secrets of cell composition, isolate elements from each other, reassemble them, and simulate nature. This led to the superiority of synthetic chemical drugs, and treatment with synthetic chemical drugs dominated herbal medicine (Phytotherapy) to the point where herbal healing almost disappeared.)Omar(2019-2018 ،

I-3.1. Definition of Medicinal Plant:

Medicinal plants are defined as plants that have medicinal advantages and properties that can heal the human and animal body and treat it from diseases. Medicinal plants were used as a basic treatment for various diseases in various cultures of the world, especially in Africa and developing countries, where there are 80% of the world's population who still use them as traditional medical treatment Many diseases. Medicinal plants have many biological properties that must be discovered, identified and documented for use safely and directing others on how to use them, as some strong medicinal plants have harmful side effects on

humans and animals and sometimes, they may be toxic that destroys the body parts. (Medical R. A.-S., 2021)

I-1. 4. Principles of Classifying Medicinal Plants:

To study medicinal plants and their effective materials, there must be a division of these plants in groups to facilitate their studies and there are many ways to divide medicinal plants, including the following: (amer, 2014)

1. **Alphabetical Classification:**

Where the plants are arranged in a newly arrangement based on the first letter of the scientific name of the plant, and this helps in the speed and ease of finding the plant to be known from the index pages and then see it and study it.

2. **Taxonomical Classification:**

Here the division is based on the genetic characteristics and the related morphological, anatomical, and physiological characteristics, and the degree of kinship between plants appears. The flower organs are the basis for the division and the distinction between plants and some Pest resistance.

3. **Morphology Classification:**

Plants are divided based on the medical used, as follows: Leaves, seeds, Barks, Its roots and weights, Entire Herbs, flowers, Its fruits.

4. **Pharmacological Classification:**

It is divided into groups depending on the nature of their uses that are similar in their medical or physiological affairs, regardless of the parts used in the plant or effective ingredients, so the plants are divided into:

- The group of strengthening plants, and this contains materials suitable for general strengthening and repair of digestion and nerves.
- The group of digestive plants is the most important uses of opening appetite and strengthening digestion by alerting them to the oral and stomach secretions.
- This spice group is an appetite and auxiliary to digest.
- A group of smiles is a disinfectant and stimulating plants of mucous membranes and urinary tract, which are a resinate plant.
- A group of plants used in the treatment of heart disease used in the treatment of heart disease.
- The group of intestinal stimulating plants are adolescent and bored plants, most of which contain resin and glycosides that alert the bowel movement and stimulate them to expel intestinal content and are used in constipation and in hepatic disorders.
- The group of holding plants is a holding, as it reduces bowel movement and is useful in cases.
- The collection of plants in the worms is against cylindrical worms, against tapeworms, against ritual worms, and against the parasite of the dysentery.
- The spoiled group is plants that increase the secretions of the air canal, increase their liquidity, and help to remove it.
- This causes its effect due to the irritation of the stomach membranes, or its alarm expression of the secretory glands in the respiratory system during its secretion.

5. Commercial Classification:

Plants are divided into groups depending on the requirements of the commercial market and divided into the following:

A- Medical plants:

They are all plants that have a physiological effect and trade these plants, individuals or companies specialized in collecting, preserving and storing them at different levels.

B- Aromatic plants:

It is characterized by its volatile essential oils and is used in the manufacture of perfumes and cosmetics.

C- Flavour plants:

It is the group of spices, as its trade is separate from the medical value it contains, and this group is linked to the trade of food commodities and their production factories.

D- Insecticides:

As the plants or their components are used in the genocide of the research, such as derris and pyreithm.

6. Chemical Classification:

Here, medicinal plants are divided according to the active ingredients in terms of their chemical composition in it, and medical plants usually contain more than one effective substance, but in this division the most effective substance is taken into account the most in the plant than others. This division includes the guest groups:

a- **Plants containing alkaloids: alkaloides:** Like paranonna- Sakran- Al Datoura- Tea-Broke.

b- **Plants containing glycosids = glycosids**: Digitalis- Rwand-Senamaki.

c- **Tabat contains volatile oils = Volatile Oils**: Such as anise- caraway- mint- a loser- mer water- jasmine- chamomile- fennel.

d- **Plants containing tannins = tannins:** Such as tannins- henna- tea.

e- Plants containing resins = Resins: Like hemp hemp - ginger ginger.

f- plants containing carbohydrates = carbohydrates: Al-Kharroub- Winkiya (Aithea).

g- Plants containing saponins: Licorice- Solanim.

7. Seasand Classification:

1- **Winter plants:** Which is agriculturally in winter, such as pacaluna-chamomile and the character.

2- Summer plants: And that it is cultivated in the summer, the mill of castor- henna and the other. This division is useful in terms of planting plants and their times, and there are other groups of accuracy mourners:

3- Durable plants: Red- Licorice- Lemon.

4- Plants- frost: Some types of thyme- digitalis.

5- Plants that need a long summer season: Tobacco.

6- Plants that need heat in a wide range: Hemp- dollars.

(amer, 2014)

1-4.3.1. Classification in Botany:

In this Classification, Plants are Divied Based on their Genets Traits and the Associated Mophological and Anatomical Characteristics. The Degree of Relatedness Between Plants Becomes Apparent, and the Floral Organs Serv as the Basis for Distingeshing Between Plants.)Dr. Fawzi Hussein(1981 ،

The Units of Classification Can Be Expressed in the Following Diagram (1):

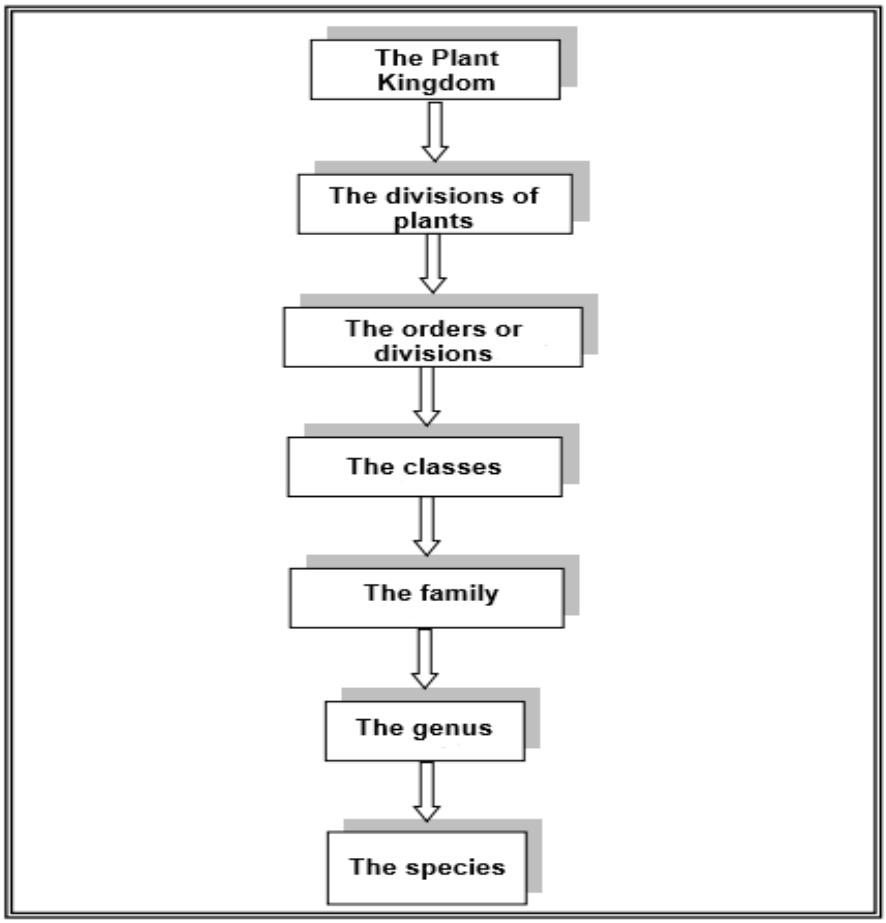


Figure 1Classification Chart in Botany

5.1-1. Source of Medicinal Plants:

Medicinal plants can be obtained from two sources. Firstly, they can be sourced from wild plants, as many species grow in valleys, plains, and forests. This may suffice as a source for some plants, such as the Yohimbe plant, which grows wild in Central African countries. The second source for obtaining medicinal plants is through cultivation. Pharmaceutical companies or investment institutions establish private farms to produce specific varieties or types needed by the local or international market in specific quantities. (M, 2002)

1-1. 6. Factors Influencing the Collection and Harvesting of Medicinal Plants:

The active components in medicinal plants are usually not evenly distributed throughout all parts but are concentrated in specific organs, such as seeds, leaves, or fruits. (Jordan, 2004م) Medicinal plants can be used either as a whole for treatment or only specific parts containing a high percentage of active substances. For example, basil leaves, clove flowers, caraway fruits, fenugreek seeds, and ginger rhizomes are used for their high concentration of active ingredients. (Dr. Fawzi Hussein, 1981)

The process of collecting cultivated or wild medicinal plants is one of the most important stages of production and depends on: (Jordan, 2004م)

1. **Quantity of Active Substances:**

The quantity of active substances obtained from the plant varies according to the plant's growth stage, collection times during day and night, and different seasons. For instance, the alkaloids in Datura plants are highest in the early morning and decrease significantly in the afternoon due to the sun's heat, especially in summer. Similarly, aromatic plants containing volatile oils like jasmine and chamomile are usually harvested early in the morning before losing some of their volatile oils due to atmospheric heat, especially during summer.

2. **Quality of Active Substances:**

The timing of plant collection is determined not only by the quantity but also by the quality of active substances. For instance, the autumn crocus plant contains the alkaloid colchicine in its corms. However, this substance disappears entirely from the corms if they are harvested in autumn. Therefore, plants used for medicinal purposes are collected in spring or early summer when the alkaloid is present, known for its bitter taste, making the plant extremely toxic and unsuitable for consumption.

3. Plant Age:

The quantity, quality, and composition of active substances in plants are greatly influenced by the stages of growth and the age of the plants. In some perennial plants, it has been found that the quantity of active substances varies with the age of the plant. Typically, this quantity increases with the advancement of the plant's age and then gradually decreases after a certain number of years. For example, liquorice plants do not harvest their roots until two or three years after planting. Digitalis plants produce a larger quantity of glycosides in the second year of cultivation compared to the first year. Rhubarb plants exhibit strong medicinal effects when harvested at six years of age.

1.6.1. Collection of Medicinal Plants:

1.1.6.1. Roots and Rhizomes:

Harvesting takes place during the plant's dormant growth period, either in the autumn or in the spring before the start of vegetative growth. Typically, uprooting occurs in the second or third year for perennial plants, and in the autumn of the first year for annual plants. (S.A., (2004)) Before drying, roots and rhizomes are washed and cleaned from soil and sand residues with ordinary water. Peeling the roots is only allowed if harvested in the spring, whereas roots harvested in the autumn have their active substances stored in their own bark. (Amin, 1983)

2.1.6.1. Bulbs:

They have a thick structure consisting of layers of scales, primarily used in folk medicine, such as onions. (plants, (2006))

3.1.6.1. Tubers:

Tubers are swollen, growing underground, with one of the most used being the African potato tuber (Hypoxis sp). (plants, (2006))

4.1.6.1. Bark:

Bark is usually collected in the spring when sap flows within the plant due to its vegetative growth activity. This sap flow in the bark vessels facilitates easy removal of the bark during this period. The collection time is chosen after a period of humidity in the air, which also helps in the separation of the bark layer from the wood, making the collection process easier, such as cinnamon. (Jordan, 2004م)

5.1.6.1. Flowering Tops:

Refers to the leafy stem or aerial part of the plant axis, provided it includes its flowers. They are usually aromatic, such as lavender, rosemary, and mint. (M, 2002)

6.1.6.1. Flowers:

Flowers differ from other parts of the plant in that they have a very short harvesting period and require precision and care in selecting the appropriate time for collection. Generally, (Jordan, 2004م) (médicinales, 2006)flowers are collected before or immediately after blooming, such as chamomile and jasmine. Some flowers are harvested as buds before fully blooming, such as lavender and cloves, because if left to fully bloom, (Jordan, 2004م)they may lose a significant portion of their active components or even lose them entirely. Depending on the desired materials to be collected, the appropriate time for harvesting may be midday when they are fully open and dry. Sometimes, they are picked in the morning after drying from dewdrops to prevent loss of active components due to heat. Harvesting may be limited to specific parts, such as petals for mallow and poppy. Flowers are collected by hand

or using a comb (for chamomile), and they are very sensitive to washing with hot water. Additionally, they should not be stored in sealed plastic bags. (S.A., (2004))

7.1.6.1. Fruits:

The entire fruit may be used, and sometimes only the fruit peels are utilized, such as pomegranate peels. If the flesh is collected, it is usually harvested when ripe or slightly before ripening, like blueberries and raspberries. Dry fruits are collected when they start to turn yellow, such as poppy capsules and cumin seeds. However, if the goal is to obtain the milky substance "morphine, which quickly dries," from poppy fruits, they are incised while still immature. (Abdul, 1993)

8.1.6.1. Seeds:

Seeds are typically used along with fruits, but sometimes they may be used alone. The harvesting process occurs after ripening, but if they are present inside an open fruit, one should not wait for the fruit to open naturally, as in the case of colchicum, flax, and mustard seeds. However, some seeds found in fleshy fruits require removal of the pulp through fermentation, such as cocoa beans. (S.A., (2004))

9.1.6.1. Plant-Derived Raw Materials:

This refers to gums, resins, and plant latex, such as pine resin, which is usually obtained by cutting or slashing the plant. It is preferable for the harvesting process to occur in the morning and during dry times. (S.A., (2004))

2.6.1. Preservation and Drying:

1.2.6.1. Drying:

Some medicinal plants are used fresh after harvesting to prepare active ingredients, such as roses and jasmine flowers, from which essential oils are extracted from the fresh petals.

However, most often, plants are dried under precise and controlled conditions to preserve their active components. (Medical t. e., 2002)

Drying is defined as follows: It is the removal of moisture from the substance, and its objectives include:

- Preserving the substance from rotting by halting bacterial activity.
- Halting chemical reactions.
- Stopping enzymatic activity.
- Facilitating grinding and crushing processes.
- Facilitating storage.

Medicinal plants are dried at temperatures ranging from 40 to 60°C. If the plant is aromatic, it is harvested in the morning and dried at a temperature not exceeding 50°C. (médicinales, 2006)

The drying rate of medicinal plants varies depending on the structure of the organ and the temperature. Medicinal plants contain a significant amount of water, which varies depending on the plant organ. Flowers and fruits contain the highest water content (70-90%), roots and rhizomes contain between (30-50%), leaves contain (50-70%), bark (20%), and seeds and dry fruits have the lowest moisture content (10%). (S.A., (2004))

Leaving the plant to dry in normal air may activate the enzymes present in the cellular sap, leading to the degradation of active ingredients and their conversion into medically useless substances. Therefore, medicinal plants are dried in ovens where hot air flows through, and the temperature is controlled so that it does not exceed 60°C until complete drying. Then, the dried plants are stored in conditions free from moisture, light, and high heat, as these conditions can affect the content of active ingredients in the plants. (Medical t. e., 2002)

2.2.6.1. Storage:

This process is of great importance for preserving the characteristics and quality of the plant material. Storage should be in warehouses with the following characteristics:

- Non-flammable, typically made of reinforced concrete and steel.
- Warehouses should be cool, dark, and well-ventilated.
- Warehouses should be protected from attacks by rodents and pests. (Jordan, 2004م)

CHAPTER 2

Studied Plants

I.2-The studied plants:

"The studied plants" refers to plants that are carefully and thoroughly studied to understand their physical, chemical, and biological characteristics, as well as their medicinal, industrial, or food uses. This includes studying the plant's structure, functions, and its effects on the environment and humans. Studied plants encompass a wide range of plant species, including medicinal, aromatic, agricultural, wild, ornamental, and others.

1.2-1. Cotula cinerea Plant:

1.1.2-1. Definition of Cotula cinerea Plant:

The Cotula cinerea plant, known as "Al-Shaheya," has a strong fragrance, resembling somewhat the scent of rue. It is a perennial herbaceous plant with light green colour and reaches an average height of 30 cm. The entire plant is covered with dense, whitish hairs. The leaves are thick and divided at the upper part into two or three lobes, and the branches terminate in compound yellowish discs. This plant grows in spring and blooms at the end of this season.

The "Al-Shaheya" plant is found in valleys and plateaus, but sporadically. However, in highland areas and meadows near agricultural areas, it flourishes and may form dense communities, especially in the southern hemisphere in general and in Arabian desert regions in particular.

This plant contains several active compounds, including flavonoids, terpenes, and essential oils. The latter gives the strong fragrance to Al-Shaheya. Additionally, this plant possesses significant biological activities due to its content of active compounds as mentioned earlier. (Acetylenes.P.425, 1973) (Univ, 1976)

1.1.2.2. The botanical classification of Cotula cinerea is as follow:

Cotula cinerea Del :Scientific Name

Brocchia cinerea (Del) Vis :Synonym

Shihia, Rubaytah, Bil Shihia *:Common Name*

Table 1:Botanical Classification of Cotula cinerea

Species	Cinerea
Gender	Cotula
Family	Asteraceae (compounds)
Order	Tubiflorals
Class	Dicotyledons
Branch	Angiosperms
reign	Vegetable

1.1.2.3.The botanical description of Cotula cinerea is as follows:

It is a small perennial herbaceous plant, ranging from 10 to 40 cm in height, characterized by a strong distinctive odor. The stems are covered with fine whitish hairs, either erect or slightly creeping, cylindrical and greenish yellow. The leaves are greenish white, densely covered with woolly hairs, thick, trifoliate with serrations at the apex, and divided at their up00per part into 3-5 lobes. The flower heads are small, discoid in shape, with a diameter ranging from 6-10 cm. All flowers are tubular, compressed, with four-toothed florets, and overlapping bracts. Initially, the flower buds are dark, turning golden-yellow upon opening, and the fruits are poor, achene-like, and pale. Photo Figure (2) illustrates this [(N.H., 1987)- (cotula., 1987)].

Figure 2 :Photographic image of Cotula cinerea.

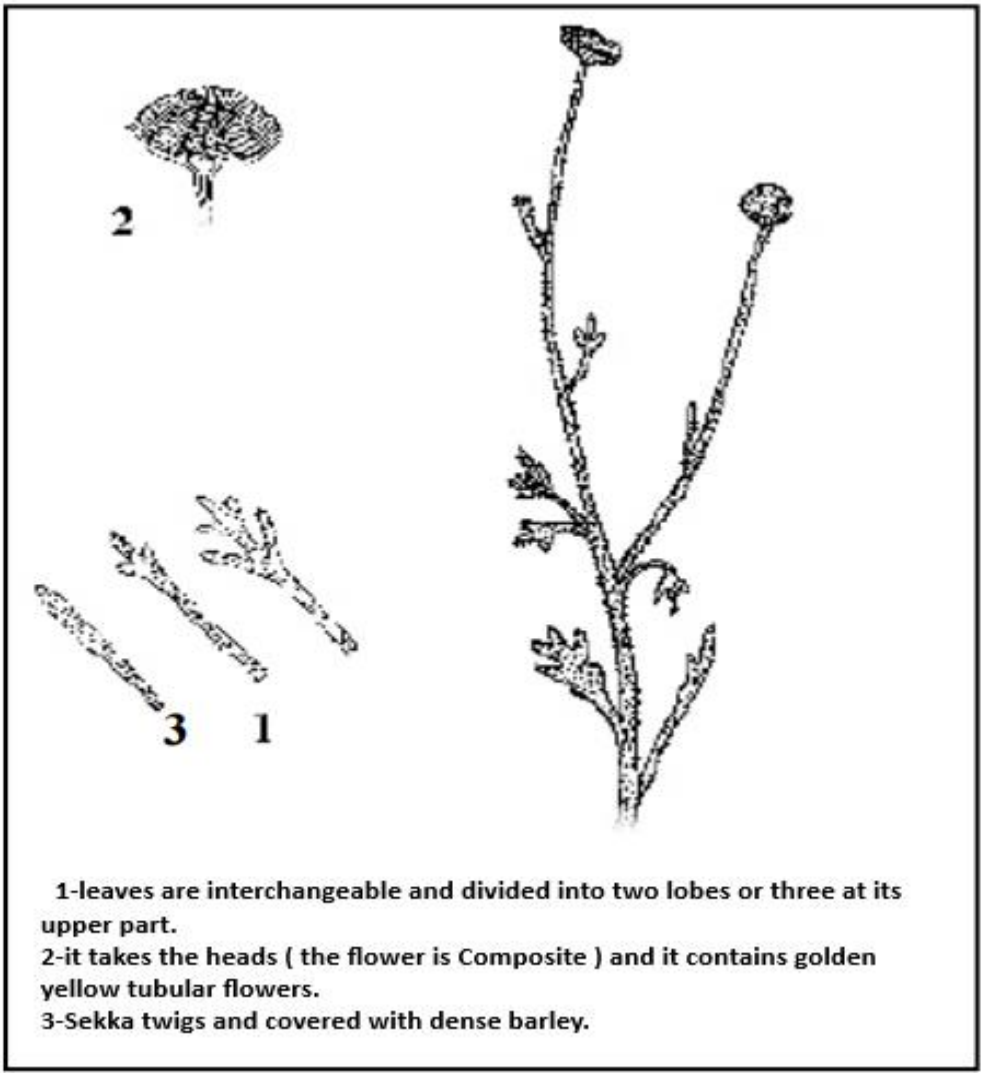


Figure 3:Schematic representation of Cotula cinerea

1.1.2.4. Geographical Distribution of *Cotula cinerea*:

Cotula cinerea is abundant in the southern hemisphere of the Earth and is found in the deserts of the Indian Iranian region as well as in the deserts of the Arabian Peninsula. [(Rezaei M.B.*) (Oil.)]

In Algeria, it grows in desert regions and semi-arid areas, especially in the southeastern part of the country. Figure (4) illustrates the distribution of Cotula cinerea in Algeria.

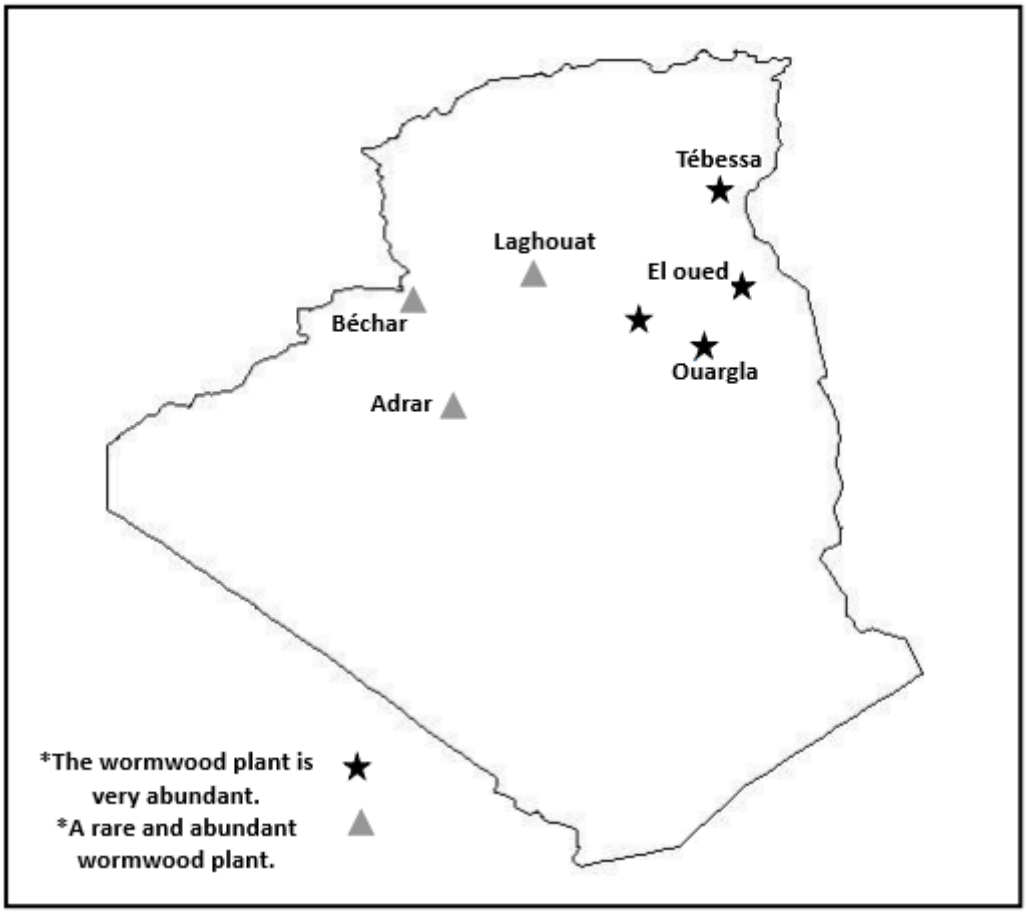


Figure 4: Distribution of the Cotula cinerea Plant in Algeria

1.1.2.5. Economic and Medicinal Uses of *Cotula cinerea*:

The plant Cotula cinerea is used as a spice and is added as a flavoring agent to tea and coffee in some countries. It is also used as an infusion beverage. Additionally, it is utilized in folk medicine for treating abdominal pain, particularly as a digestive aid. It is also used against respiratory tract inflammations due to its properties in relieving cough pains [(Cinerea), (clinique., 2002)]. It has been observed that leaf extracts of Cotula cinerea exhibit antifungal activity [(antifongiques, 1999)]. Moreover, flavonoid compounds extracted from this plant have analgesic, anti-inflammatory, and antiseptic (antimicrobial) effects. On the

other hand, Cotula cinerea is used in some regions for treating stomach and abdominal pain [(antibiotiques., 1989)].

1.2-2. Plant *Origanum Majorana* L:

1-2.2.1. Introduction to *Origanum Majorana* L Plant:

The name "Origanum" originates from two Greek words "oros" meaning mountains and "gonos" meaning brightness or delight, thus it became known as the delight of the mountains for its beauty and abundance in mountainous regions around the Mediterranean basin. Origanum Majorana L, formerly known as Majoranahortensis Moench, is a perennial herb [(VagiE S. B., 2002)] characterized by great morphological and chemical diversity. It has forty-nine classifications divided into locally distributed varieties around the Mediterranean Sea. The genus is commonly known as sweet marjoram and is native to Anatolia (Turkey) and Cyprus, with hybrids found in parts of the Mediterranean region, especially Egypt [(Novak J, 2002)]. Marjoram was initially used by Hippocrates as an antiseptic agent. It is a good home remedy for chest infections, coughs, and sore throats [(Shahriyary, 2008)- (BremnessL, 1994.)].

1-2.2.2. Botanical Classification of *Origanum Majorana* L:

Scientific Name: *Origanum Majorana* L

Synonym: Marjolaine

Common Name: Marjoram.

Table 2:Botanical Classification of *Origanum Majorana* L.

Species	*Majorana* L
Gender	Oreganum
Family	Lamiaceae
Order	Lamiales
Class	Mangnoliopsida
Branch	Mangnoliophyta
reign	Plants

1-2.2.3. Morphological Description L *Origanum Majorana*:

It is a semi-perennial shrub that grows annually, sensitive to cold, aromatic shrub reaching 30-60 cm in height, with reddish square stems, multiple branching, spreading to form a cluster. The stems are straight, weak, hairy, round, green with red spots [(Pimple, 2012)]. The leaves are soft, simple, opposite, ovate to ovate-rectangular, grayish green in color, arranged opposite each other on a square stem. They are very soft due to the presence of numerous hairs. They are 0.5-1.5 cm long and 0.8-0.2 cm wide, with a divergent apex, entire margin, symmetrical but straight base, and reticulate veins. It has small tubular flowers, white or pale pink, with grayish-green bracts blooming in spikes from mid to late summer (June to September). They are less than 0.3 cm long and arranged along 13 cm long spikes. The flowers are hermaphroditic in nature [(Simandi, 2005)]. The seeds are tiny, dark brown to brownish oval, maturing from August to September. They have subcylindrical branches. The roots are longitudinally furrowed with transverse cracks: 0.2-0.6 mm in diameter. The outer surface of the root is dark brown while the inner color is light brown with numerous long rootlets, and root scars are also present. The cracks are long, symmetrical, fibrous with an aromatic, non-pungent scent [(N., 2014)].

Figure 5:Photographic Image of Origanum Majorana

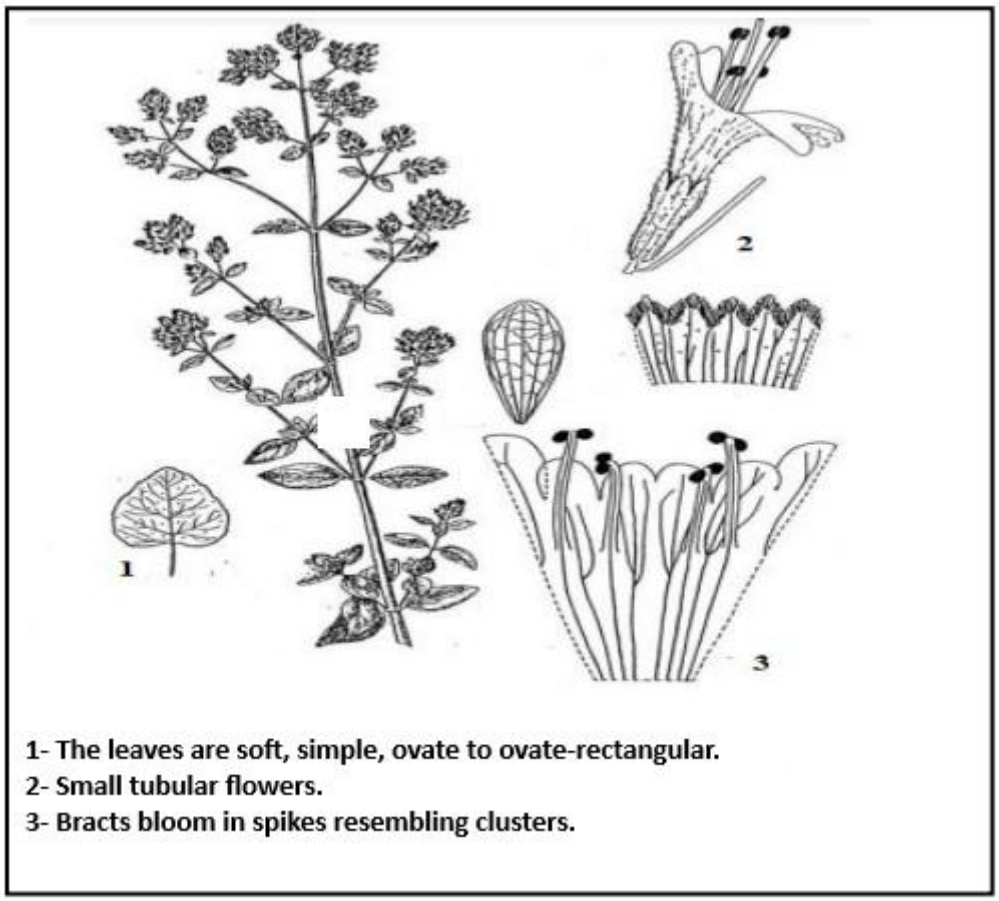


Figure 6:Schematic Diagram of Origanum Majorana

1-2.2.4. Geographic Distribution of Origanum Majorana L:

Its native habitat includes Turkey and Cyprus, and it has spread from there to countries around the Mediterranean basin (such as Lebanon), as well as in Iran, North America, the Arabian Peninsula, and India. It grows on sunny slopes in meadows, fields, and rocky terrain in dry climates. It is extensively cultivated in the southern regions of Saudi Arabia. Figure (7) illustrates the distribution of the Origanum Majorana L plant in Algeria.

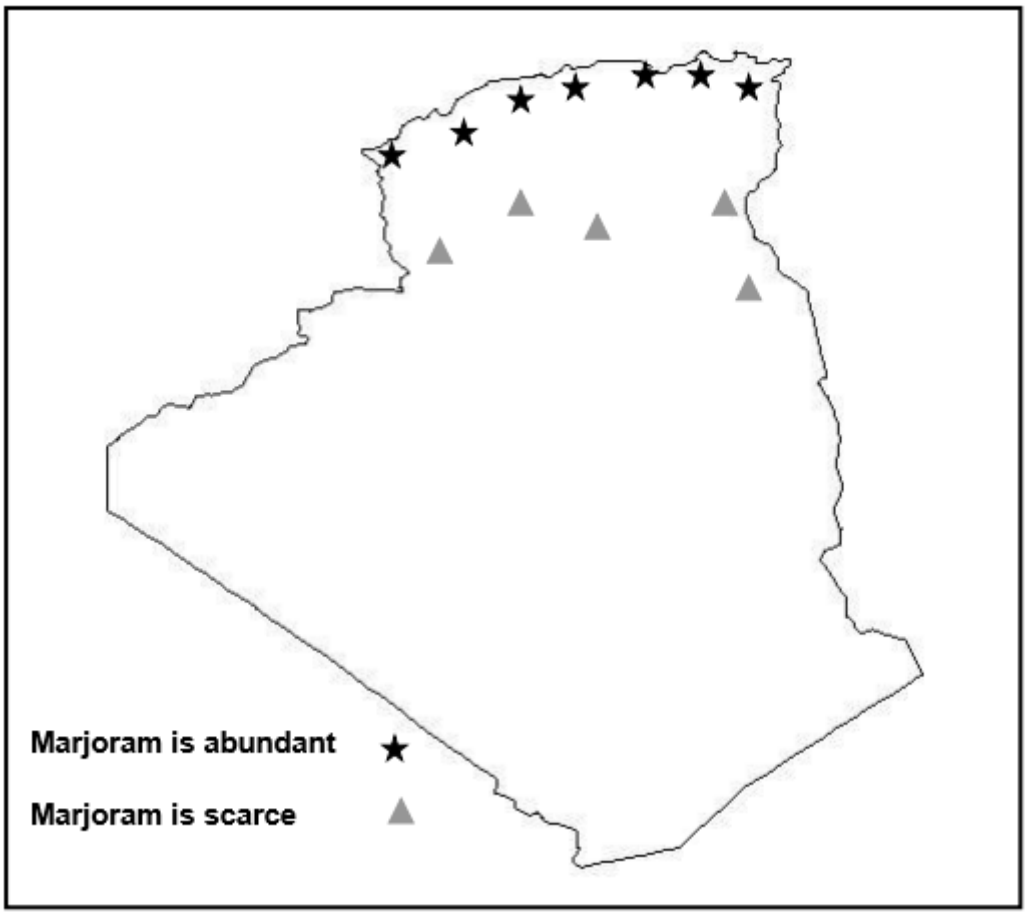


Figure 7:Distribution of Origanum Majorana L Plant in Algeria

1-2.2.5. Economic and Medicinal Uses of Origanum Majorana L:

It is a good home remedy for chest infections, coughs, sore throat, rheumatic pain, neurological disorders, cardiovascular diseases, epilepsy, bloating, skin care, and stomach disorders [(BremnessL, 1994.)- (Shahriyary, 2008)]. Widely used in the food industry as a spice with meats and vegetables. It is advised not to consume during pregnancy due to its uterine stimulant activity.

In traditional medicine, the leaves are used to treat diabetes, insomnia, colds, asthma, and anxiety [(Volpato, 2004)]. Its essential oil is used to flavour various foods, especially soups, broths, meats, fish, canned foods, wines, and beer [(Lavrenov, 1999)- (Farrell K T Spices, 1985)].

CHAPTER 3

Essential Oils

II-3. Essential Oils:

II-3.1 Generalities about Essential Oils:

II -3.1.1. Introduction:

Essential oils are considered one of the organic food products of plants. They are the most important secondary products due to the primary secretions naturally produced or exuded by certain specific plants, known as aromatic plants. Essential oils represent the main substances responsible for the distinctive aroma of plants and their various organs. Essential oils are characterized by their ease of separation from the plant organs carrying them through various distillation and extraction methods. The extraction method using solvents is the most common in the fragrance industry. [(Ghassan Majawi, 1999) (Jeanfils, 1991)]

Often, a significant difference is observed between extracted essential oils, attributed to factors such as the maturity of the plant, harvesting methods, storage, climatic conditions, and soil nature. [(Qubaisi, 2002) (Qader, 1996)]

II -3.1.2. Definition of Essential Oils:

"Essential oils are defined as oils that evaporate or vaporize without decomposing, distinguishing them from fixed oils. The latter do not vaporize, and if exposed to evaporation or heating, they decompose and condense." (Louail, 2016)Essential oils are also referred to as aromatic oils (les huiles aromatiques) due to their aromatic scent, or ether oils for their solubility in ether. They are also called volatile oils.

Volatile oil is defined as a compound or a somewhat complex substance containing volatile elements found in plants. These elements consist of volatile oil-scented elements, colorless or yellowish (jaunatres), non-flammable, easily deteriorating in the air, forming gum. These oils are naturally in liquid form at ambient temperatures, and they lack the fatty and ointment properties of oils upon touch.)Study(2015 ، -)Qadir(2007 ،

All definitions of essential oils are clear and precise. The best and most comprehensive definition is as follows:

- "Essential oils are mixtures of various compounds derived from plant origins. These mixtures pass with proportions of water through distillation carried out by a stream of water vapor."

The Association Française de Normalisation (AFNOR) defines essential oils as follows:

- "Essential oil is a compound obtained from a plant-based raw material, either by steam distillation or mechanically from citrus peel (epicarpe de citrus), which is subsequently separated from the solution by physical methods.")Al -Qadir(1996 ،)Medical d.(1981 ،
- This definition is also recognized by ISO/AEOL organizations and is also widely used in professional circles such as pharmacists.

II -3.1.3. Localization of Essential Oils:

Essential oils are found in more than two thousand (2000) plant species and in over sixty (60) plant families. They may be present in all parts of the plant or concentrated in specific parts, varying in concentration from one plant to another. These oils are located within the living cell cytoplasm and mostly exist in a free liquid form, while a small portion may be non-volatile and solid due to their association with glycosidic or resinous compounds. Essential oils accumulate within plant tissues in structures known as secretory structures.

Aromatic plant oils can be found in various plant parts,)Plants(1994 ،)The gifts(1995 ،)Al -Zahriya(2000 ، such as leaves in the case of mint, flower petals in roses and jasmine, or fruits and their peels in oranges. They may be present in multiple plant parts, with their concentrations varying accordingly.

Examples:

- **Rutaceae Family**: Essential oils are concentrated in leaves, flowers, and seeds, for example, (Citrus aurantium L.).

- **Myrtaceae Family:** Essential oils are concentrated in leaves, as seen in the case of eucalyptus.

- **Umbelliferae Family:** Essential oils are localized in secretory ducts, as in angelica (Angelica archangelica).

- **Labiatae Family:** Essential oils are concentrated in the aerial parts of plants, as in mint and thyme.)Mahmoud Saleh Siraj Ali(2002 ،

II -3.2. Physical Properties of Essential Oils:

Although essential oils differ significantly in their chemical composition, they generally share most of their natural properties when fresh. The general characteristics of essential oils include:) Rubin(2004 ،)Ghassan Hijjawi(2004 ،

1- Colour:

Most essential oils are colorless, but they oxidize and turn dark upon storage. Rarely, they may have a bluish-green color due to the presence of azulene and chamazulene compounds responsible for the green or blue color. Some oils may have a pale-yellow colour. Changes in color may occur due to oxidation, degradation, or exposure to non-natural factors during extraction.)Amin Ruwaih(1983 ،

2- Scent:

Most essential oils are characterized by a pleasant aromatic scent, rarely having an undesirable pungent odor. Essential oils can be distinguished by the presence of certain terpene and key compounds, even before extraction. For instance, while walking among plants and trees, or even in nearby areas, one can notice the distinctive scent due to the diffusion of compounds like citral from lemongrass, menthol from mint, geraniol from geranium, and anethole from anise. Each oil has its own unique and recognizable scent.)Gurib(2006 ،

3- Evaporation:

Essential oils are so named due to their property of evaporation at room temperature, which sets them apart from fixed oils that do not evaporate even with heating, like lemon oil, owing to their non-volatile components such as resinous substances. For example, placing a drop of both a volatile and a fixed oil on filter paper, it's observed that the volatile oil completely disappears after a while due to its evaporation, leaving the paper dry and clear, while the fixed oil remains on the paper.) Gurib(2006 ،

4- Solubility:

Essential oils are sparingly soluble in water but readily dissolve in most organic solvents such as petroleum ether and ether, with high solubility in alcohol reaching up to 95%. However, rose oil, when dissolved in alcohol, becomes cloudy due to the presence of some organic compounds like paraffins. Complete dissolution of essential oils in alcohol is important to verify their purity and absence of adulteration, achieved by using different concentrations ranging from 95% to 35% diluted with water, as the addition of fixed oils reduces the solubility of essential oils in alcohol.) Gurib(2006 ،) Schauenberg(2006 ،

5- Specific Gravity:

The specific gravity of essential oils varies depending on their botanical sources, ranging from 0.1 to 1.7. Most essential oils have a specific gravity less than one, indicating a lower density than water, causing them to float on the water surface, except for clove oil with a specific gravity ranging from 1.02 to 1.07, and cinnamon bark oil ranging from 1.03 to 1.04, where the oil settles below the water surface. Specific gravity provides a significant indicator of the contents of the essential oil; a value less than 0.9 indicates the presence of terpenic and high aliphatic compounds, while a value greater than one suggests the presence of various and chemically different aromatic ring compounds.) Gurib(2006 ،

6- Optical Rotation:

Essential oils are characterized by their optical rotation property. Assessing the optical rotation is one of the most important natural evaluations of essential oils to determine their purity, absence of adulterants, and differentiation between natural and synthetic counterparts. There are other natural properties and characteristics of essential oils such as:

-Consistency: All essential oils are liquid at room temperature, except for rose oil and anise oil, which solidify at normal temperatures.

-Refractive Index: Most essential oils have a high refractive index.

II -3.3. Difference between Essential Oils and Fixed Oils:)Amin Ruwaih(1983 ،

The key differences between volatile and fixed oils can be summarized in Table (8):

Table 3:The key differences between volatile and fixed oils can be summarized

Fixed oils	Allergenic oils
- Cannot be obtained by distillation, but rather by pressing. - Composed of fatty acids. - Emulsify when bases are added to them. - Leave a clear trace when applied to paper. - Oxidize more easily and quickly than essential oils. - Do not evaporate at room temperature.	- Can be obtained from its natural sources by distillation. - Comprised of aromatic compounds (terpenes). - Do not saponify when bases are added to them. - Do not leave a clear trace when applied to paper. - Have a lighter consistency compared to fixed oils. - Evaporate at room temperature.

II -3.4. Storing and Preserving Essential Oils:

It's undeniable that oils with a high terpene content are susceptible to spoilage due to oxidation and resinification. This is because terpenes, unsaturated compounds, absorb oxygen from the air, oxidize, and produce compounds with different aroma and consistency from the original oil.

Similarly, oils containing esters, such as lavender oil, decompose due to improper storage and transform into acids. However, oils containing alcohols are less affected by long-term storage. Essential oils, in their natural state in plants, do not oxidize due to the presence of natural antioxidant compounds, preserving them from oxidation.

It's important to use colored bottles for final packaging, store them at low temperatures, away from light and air, keep the bottles dry, and use aluminum or glass containers. Refrigeration is also recommended.) Bohlmann(1973 ،

Experiments have shown that essential oils, after extraction and during storage, undergo natural and chemical changes in their properties, leading to deterioration and reduced quality. Several factors contribute to the spoilage of essential oils, including:

II -3.4.1. Natural Factors:

- **Temperature:**

Elevated temperatures during storage affect enzyme activity, increase chemical reactions, and promote the growth of microorganisms. Temperatures affecting umbelliferous plants containing volatile oils like chamomile flowers, and fruits of umbelliferous plants like anise and cumin can cause these plants to lose their entire or partial oil content.

- **Humidity:**

Enzymes during storage degrade the active components of plants, causing these medicinal plants to lose their natural value and spoil. The activity of enzymes depends on the presence of water in plant cells. Therefore, it's crucial to eliminate moisture during storage to stop enzyme activity. Moisture can reach medicinal plants during storage either by absorption from the air, especially if the plants are water-loving, or due to insufficient drying.

In the final packaging, it is important to use colored bottles stored at low temperatures, away from light and air. The bottles should be dry and made of aluminum or glass. It's also recommended to store the oil in refrigerators. Experiments have shown that essential oils,

after extraction and during storage, are subjected to factors that lead to natural and chemical changes in their properties, resulting in deterioration and reduced quality. The main reasons for the spoilage of essential oils include the following natural factors:

- **Temperature:**

Elevated temperatures during storage affect enzyme activity, increase chemical reactions, and promote the growth of microorganisms. The heat also affects umbelliferous plants containing volatile oils, such as chamomile flowers, and fruits of umbelliferous plants like anise and cumin, causing them to lose their oil content either partially or entirely.

- **Humidity:**

Enzymes during storage degrade the active components of plants, causing them to lose their natural value and spoil. Enzyme activity depends on the presence of water in plant cells. Therefore, it's essential to eliminate moisture during storage to halt enzyme activity. Moisture can reach medicinal plants during storage either by absorption from the air, especially if the plants are water-loving, or due to insufficient drying.

- **Light:**

The presence of light during the storage process affects many medicinal plants, causing a change in their natural color or the color resulting from the drying process. This color change diminishes the commercial value of the plant, even though it may not affect its active ingredients. Plants such as rose, Karade, and some leafy plants like the sugar cane are affected by this color change. The color change may result from a change in the active ingredients themselves, as in the case of Wormseed, where the yellow-colored santonin substance changes to orange and then black. Therefore, it is important to store these medicinal plants away from light or in dark places. In the case of small quantities, colored or opaque bottles or containers are used.

- **Oxygen:**

Oxygen present in the air affects the oxidation of some components of medicinal plants during storage, especially plants containing volatile oils like lemon oil. Consequently, the natural and chemical properties of these plants change, reducing their medicinal and commercial value. Therefore, many of these oils are stored isolated from the air or in the presence of an inert gas.

II -3.4.1.Biological Factors:

Essential oils stored may be affected by fungi, bacteria, or insects. To minimize this infection, storage should be done at low temperatures and with a humidity level of about 5-10% of the weight of the dry plant. Insect infestation can be fatal to plants, even those packed in tightly sealed containers. Infestation usually occurs during handling in storage facilities in the field. If necessary arrangements to eliminate these eggs are not made, they hatch inside the storage container, and the insects emerge to destroy the stored product.) Gallily(1962 ، To eliminate the growth of these insects in their various stages, it is advisable to fumigate the storage facilities once or more at close intervals with chemicals, provided that these substances do not leave any toxic residues on the stored material.)Mohamed El -Sayed Haykal(1993 ،

II -3.5. Composition of Essential Oils:

All essential oils are complex mixtures of several compounds. They primarily consist of hydrocarbons, which are the main part of the aromatic oil. The other part consists of oxygen-containing compounds, belonging to an organic group of acids, alcohols, esters, aldehydes, ketones, and ethers. These components may also contain sulfuric or nitrogenous compounds in small proportions. The hydrocarbon part of the aromatic oil is mainly derived from terpenes.)Amin Ruwaih(1983 ،) Bohlmann(1973 ،

II -3.5.1.Terpenes:

These are natural products found in various parts of plants and represent a large proportion of secondary metabolic products. They are characterized by the distinctive pleasant smell of many plants. The name "terpenes" is derived from the compounds that could be separated from turpentine oil. Most terpenes are volatile oils separated from plants like the method used to separate essential oils. Terpenes play a significant role in our daily lives. For example, Vitamin A1, which is derived from terpenes, leads to impaired vision in humans if deficient.)Gurib(2006 ،Studies conducted by Owllach of the University of Göttingen in Germany in 1877 demonstrated that the diversity of terpenes is attributed to the number of isoprene units, and Table (9) illustrates the types of terpenes.) Gallily(1962 ،)Amin Ruwaih(1983 ،

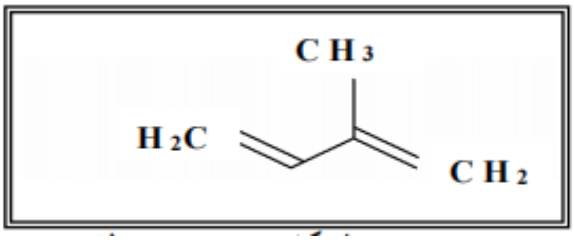


Figure 8:The Isoberne Unit

1. **Monoterpenes:**

Monoterpenes are among the most common types found in the plant kingdom [ )Schauenberg(2006 ،]. They consist of two isoprene units and are divided into monocyclic and bicyclic compounds.

- Monocyclic compounds: The most important compounds include limonene and Q-phyllandrene.

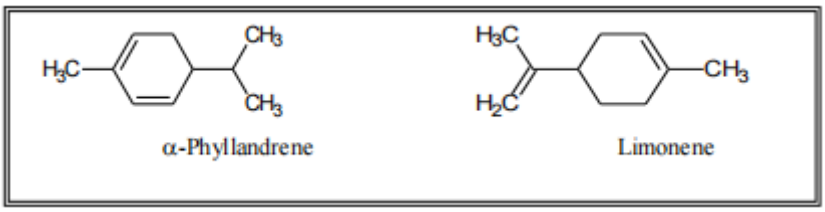


Figure 9:Monocyclic Monoterpenes

- Bicyclic compounds: The main compounds include B-pinene, α-pinene, and sabinene [ )Amin Ruwaih(1983 ،,) N.H.(1987 ،].

Monoterpenes with a bicyclic structure exist in large numbers and are classified into four groups based on their carbon structure:

- Thujene group
- Carene group
- Pinene group
- Camphene group

Derivatives of thujene and carene are found in cedarwood oil, while the main source of the pinene group is crude turpentine oil, which can be obtained from the bark of pine trees.

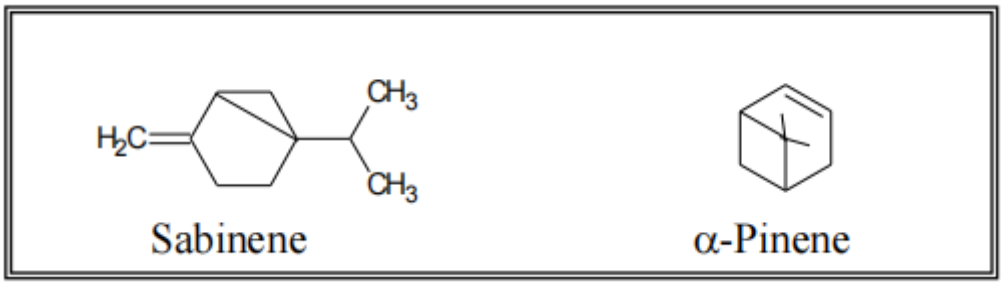


Figure 10: Bicyclic Monoterpenes

2. Diterpenes:

These terpenes contain 20 carbon atoms, which corresponds to four isoprene units. Examples include Phytol and Vitamin A1.

3. Sesquiterpenes:

Their number exceeds 2500 molecules, each composed of three isoprene units, meaning each molecule contains 15 carbon atoms. They are usually divided into two types based on their carbon structure. Examples of the first type include Cadinenes, while examples of the second type include Cillinenes, found in celery oil.

4. Triterpenes:

An example of triterpenes is the compound known as Squalene, which is considered the precursor of cholesterol.

5. Tetraterpenes:

Each of these terpenes consists of eight isoprene units and is also known as carotenoids. Three isomers of carotenoids have been isolated from carrots: α-carotene, β-carotene, and carotene-8. (N. Sadaoui, 2018)

6. Polyterpenes:

One of the most important examples of polyterpenes is natural rubber, which is found in nature as a milky latex obtained from rubber trees in tropical regions. Its spatial configuration around the double bonds forms a cis configuration. (Lotfi Baameur Abd-el-Kader Benmenine)

Table 4:Types of Terpenes

Type of Terpene	Number of Carbon Atoms	Number of Isoprene Units
Monoterpene	10	2
Sesquiterpene	15	3
Diterpene	20	4
Triterpene	30	6
Tetraterpene	40	8
Polyterpene	More than 40	More than 80

II-3.6 Benefits and Uses of Essential Oils:

For thousands of years, essential oils have been used in China and in Egyptian civilization. They were also commonly used in our Arab and Islamic civilization, and over the years, their use has evolved to become widespread in beauty institutes and centers. They were used in treating diseases as a medical system aimed at maintaining human health.

The benefit of essential oils for plants is that they attract insects that carry out pollination, thus increasing fruit production. Additionally, some oils repel insects or are toxic to animals, thus protecting the plant from these insects that can harm it. (Amin Ruwaih, 1983)

Essential oils also remove the byproducts of biological processes and excrete them outside the plant. There are several other benefits and uses that essential oils serve for all living organisms. They strengthen most body systems, including the immune system, and help activate blood circulation and invigorate the body.

Some essential oils could aid in hair growth, a property found in lavender, for example. They also increase oxygen absorption and ATP (Adenosine Triphosphate) production. (Bohlmann, 1973)

Table 5:summarizes the most important benefits and uses of essential oils.

Medical Uses:	Other Uses:
- Soothing and Antiseptic (Calmant, Sedative).	- Adds acceptable taste and aroma to some medications.
- Hypotensive (Blood pressure-lowering).	- Used as flavoring or seasoning in food.
- Antiviral.	- Used in perfumes and cosmetics.
- Anti-inflammatory.	- Used in pharmaceutical and soap manufacturing.
- Restores balance to the internal nervous system.	- Repellent for worms and parasites.
- Muscle relaxant.	- Insect repellent, such as mosquitoes.

II-7.3. Methods of Essential Oil Extraction:

7.3.1 Introduction:

There are several techniques for extracting essential oils known today, but several factors influence the choice of the extraction technique. The chemical composition of the essential oil requires a method of extraction that ensures obtaining it in its natural state, without any degradation or alteration in its chemical properties, thus preserving its aroma and taste. It is essential to consider the differences between the parts of the plant containing essential oil, the location of the oil cells, and the sensitivity and thickness of the walls of these cells. The method of extracting oil from flower petals differs from the method of extracting it from fruits or roots. Additionally, the economic factor plays a significant role in the extraction method, as it is necessary to obtain the maximum amount of oil present in the plant at the lowest possible cost. (Plants, 1994) (Al -Qadir, 1996) (Mahran, 1976)

Other factors include the quantity of volatile oil present in the plant. For example, if its proportion is small, it should be extracted using organic solvents to prevent loss of this quantity if the water distillation method is used. Extraction methods adapt according to the most important characteristics of the essential oil to be extracted, such as volatility in the air and steam, and solubility in organic solvents. (Rezaei M.B.*)

7.3.2 Distillation:

It is worth noting that the lower the distillation temperature, the higher the quality of the oil obtained, meeting natural, chemical, and quality specifications. There are three distillation methods:

1. Hydrodistillation:

This method is one of the most used techniques. The plants to be distilled are placed in a distillation vessel, either directly immersed in distillation water or placed in a basket container submerged entirely in water, ensuring that the plant material does not touch the walls of the distillation vessel. Typically, the distillation vessel is made of non-corrosive materials like copper or glass, and heating is achieved either by fire or an electric heater. (-Qadir, 2007) (Medical t. e., 2002)

The hydrodistillation method is used for dried plants capable of withstanding boiling, which contain high oil content in some parts such as roots, leaves, fruits, and certain flowers. Table (11) outlines the advantages and disadvantages of this technique.

Table 6:Advantages and Disadvantages of Hydrodistillation

Advantages of Hydrodistillation:	Disadvantages of Hydrodistillation:
- Easy to use. - Low cost of setup. - Simple construction and assembly of the apparatus. - Does not require a dedicated space or special conditions.	- Complete extraction is impossible. - Loss of some compounds that are soluble in water, such as oxygenated compounds. - It requires a long time to complete the extraction process.

2. Steam Distillation:

In this type of distillation, the plant material does not come into contact with water, and steam is essential for extraction. The plant parts to be distilled (such as leaves, flowers, seeds, fruits, stems, and roots) are placed in a vessel called an "alambic" or distillation apparatus, (Study, 2015)

which has a base with a clip or mesh allowing the passage of steam, with water designated for boiling at the bottom of the vessel and the mesh placed in the middle.

Steam is generated uniformly through a circular tube with several openings, then condensed in condenser tubes mounted at the end of the alambic. It converts to a liquid state, separating from the water and collected in vessels called "Vase-florentin," allowing for the separation of water and essential oil through decantation. This method is economical, quick to operate, and easy to execute. The oil produced is high in quantity and possesses high chemical qualities due to the absence of aldehydic, ketonic, and other ester compounds that can dissolve in water. However, there are new techniques that have overcome the drawbacks of hydrodistillation and steam distillation, such as the Microwave Hydro-Distillation (MWHD) technique. ((30) G. Fournier'3) ((31) M.Markouk) (127-145, 2002)

3. Hydrodistillation by Microwave:

This method is one of the newest techniques used, and it is very fast with minimal energy consumption. Its most important advantage is that it provides high-quality essential oil in considerable quantities compared to simple hydrodistillation. (105-121., 1999)

This extraction method relies on efficient heating from microwave radiation, which is selective because of the strong dielectric constants and high dipole moments of the media, capable of absorbing microwave energy and converting it into heat. The plant material is placed inside the device under low pressure, where it is selectively heated by microwave rays,

causing the essential oil to be drawn into the mixture of pure water vapor produced from the studied plant.

There are several types of microwave-assisted extraction, including:

1.MAP: Microwave-assisted extraction under atmospheric pressure.

2.PMAE: Microwave-assisted extraction under pressure.

3.FMAE: Microwave-assisted extraction under atmospheric pressure with focused microwaves.

4.EMHD: Microwave-assisted hydrodistillation under vacuum.

5.ESAM: Microwave-assisted extraction with solvent assistance.

Types (1), (2), and (3) can provide better yields than classical methods. Type (4) is more beneficial than the first three types as it can provide high yields in a short time, and type (5) allows for the use of small amounts of solvents and very short extraction times compared to classical methods like Soxhlet. However, the main drawback of types (2), (3), (4), and (5) is their very high cost.

It's worth noting that all these types reduce extraction time and preserve heat-sensitive compounds from degradation. (J.J., 1989)

7.3.3 Extraction under Cold and High Pressure (Ex - pression a froid):

This technique is specific to extracting essential oils from citrus fruits only, as they are easily oxidizable and cannot withstand high temperatures. The extraction process requires mechanical or manual action, and citrus peels are typically extracted under a stream of water with high pressure. The obtained essential oil is subsequently separated from the aqueous phase using centrifugation. (J.J., 1989)

7.3.4 Organic Solvent Extraction:

In this method, the plant material is brought into contact with a solvent (either cold or hot) and usually occurs at room temperature. The principle of this method involves adding the organic solvent to the aromatic plant material (such as jasmine or rose flowers) arranged in thin layers, allowing the solvent to penetrate the cells containing the aromatic oil, dissolve it, and carry it out as a solution of solvent and oil. Then, the two are separated by low-pressure distillation. (N. Sadaoui, 2018)

The substance obtained after evaporation is called "concrete," a term derived from the fact that the resulting material solidifies due to the presence of a fatty substance drawn by the solvent. Subsequently, the concrete is treated at low temperatures with absolute alcohol, which separates the fatty substances, and after evaporation, pure essential oils are obtained. This phase is called the absolute phase of essential oils. (Prena, 2015) (VagiE, 2002)

The main advantage of this method is a considerable reduction in extraction time and an increase in yield, allowing heat-sensitive molecules to be extracted. However, the limited use of organic solvent extraction is justified due to its high cost, safety and security issues, as well as environmental protection regulations. (Mahran, 1976)

II-8.3. Methods for Analysing Essential Oils:

II-8.3.1. Introduction:

Recent technological advancements in the past two decades, in terms of devices and methods used in electrical analysis, have led to the discovery of several broad analytical techniques and highly accurate and sensitive devices such as Gas Chromatography (GC), Gas Chromatography-Mass Spectrometry (GC/MS), High-Performance Liquid Chromatography (HPLC), Thin-Layer Chromatography (TLC), Fourier Transform Infrared Spectroscopy (FTIR), etc. Each technique has its specificity and field of application. However, analysing the chemical components of a natural mixture remains a highly sensitive process and

sometimes requires the use of multiple complementary techniques [(Yazdanparast, 2008)]. Analysing the chemical composition of a complex mixture is done according to well-established methods A and B, as shown in diagram (2).

- **Method A:** This method requires the sequential pairing of one or more chromatographic techniques with one or more spectroscopic techniques, depending on the techniques used (e.g., GC/MS, GC/FTIR). This method is particularly suitable for routine analysis such as monitoring samples of essential oils or plant extracts whose components do not require difficulty in detection. On the other hand, the introduction of pairings such as HPLC/MS and HPLC/NMR has opened up new research avenues and advanced chemical analysis methods.

- **Method B:** This method requires pre-cleaning of the components before detection, and it is advisable for analysing newly studied samples whose components are difficult to detect (complex structures and very close in composition).

Analysis may also lead us to Method C, as shown in diagram (2), where Carbon-13 Nuclear Magnetic Resonance (13C-NMR) is used to detect mixed compounds without prior separation, or fragmentation into smaller compounds. This method can also be used to determine the quantity of components when they are sensitive to conventional methods.

II-8.3.1.Gas Chromatography (GC):

Gas chromatography separates compounds from volatile oil mixtures without altering their composition. Therefore, gas chromatography allows for the separation of mixture components and the simultaneous measurement of the separated compounds [(Mahran, 1976)]. It is used in various fields including chemistry, medicine, biochemistry, environmental science, and more. In the field of essential oils, the effectiveness of analysis was greatly enhanced by the advent of capillary columns [(Blazovics, 2005)]. A good decision was made to link this technique with mass spectrometry, where chromatography separates the components of the

compound mixture, and mass spectrometry acts as a highly sensitive detector [(Singla, 2014)-(P, 2012)].

Selecting the stationary phase for the analysis of essential oils by GC is very important because essential oils are highly complex mixtures consisting of a large portion of terpenes and other organic compounds such as alcohols, acids, esters, aldehydes, ketones, and ethers. Among the commercially available stationary phases, two types are specialized for the analysis of natural compounds such as essential oils [(K, 1999)- (Singla, 2014)].

Non-polar phases such as SE-30, OV-1, BP-1, CP Sil-5 CP, D-1, DB5, HT1.

Polar phases such as CW-20M, DB-Wax, PEG-20M, CP Wax 10cb.

The components of the injected mixture pass through the chromatography column in the gas phase at different speeds, and this difference is due to the retention time for each substance in the stationary phase. The eluted substances are detected, and a chromatogram with different FID spectra is obtained from the column by a detector, such as a flame ionization detector, showing sharp and separate peaks if the mixture components are well separated.

Many have attempted to develop a method for calculating retention coefficients for the components of the analysed compound mixture based on temperature [(piperita, 2014), (Linnaeus),]. The method that has gained user satisfaction and is most commonly used today is the one developed by VAN DEN DOOL and KRATZ for retention coefficients. The relationship is:

$$I_R = 100Z + 100n\frac{t_{R(s)} - t_{R(z)}}{t_{R(z+n)} - t_{R(z)}}$$

Z : Number of carbon atoms in the compound under study.

$t_{R(s)}$: Retention time for the compound under study.

$t_{R(z):}$ Retention time for the alkane with Z carbon atoms.

($t_{R(z+n)}$): Retention time for the alkane with Z+n carbon atoms.

n: The difference in the number of carbon atoms between the alkanes (usually n=1).

II-3.8.3 Gas Chromatography Coupled with Mass Spectrometry (GC/MS):

The coupling of gas chromatography with mass spectrometry allows for simultaneous separation and analysis of the various components of a complex mixture. The significant advancement of mass spectrometry in identifying and discovering the components of fragrances and essential oils was made possible by linking gas chromatography with mass spectrometry. This innovation enabled obtaining a clear mass spectrum for small quantities of material ranging from micrograms to nanograms. Mass spectrometry has become the most sensitive technique for obtaining important data regarding the structure of unknown organic compounds. (Medical t. e., 2002)

Among the most commonly used ionization methods in analysing compound mixtures are Chemical Negative Ionization (CN), Chemical Positive Ionization (CP), and Electron Impact Ionization (EI). The components of the injected mixture pass through the column of the gas chromatograph in their gaseous state at different speeds, being retained at different retention times (TR) depending on their solubility in the stationary phase. Subsequently, the separated components of the compound mixture enter the chromatograph column, inside the ionization chamber, where they are bombarded with a stream or shower of electrons with an energy of about 70 eV. This energy causes the fragmentation of various molecules. Then, the resulting positive ions move towards the analyser (Magnetic or Quadrupole), and the resulting stray

current is converted into an electronic current, which amplifies and expands to provide the mass spectrum. (Huxley, 1992)

II-3.8.4. Chromatographic Linkage CPG/IRFT/MS and CPG/IRFT:

IRFT spectroscopy, like other spectroscopic methods, provides a fingerprint of the compounds that can be compared with reference spectra. Even in the absence of such spectra, this technique informs us about the chemical functionalities present in the particles, as well as compounds with double bonds, and generally allows differentiation between homologues. This technique is particularly important when it enables the detection of particles with insufficient mass spectra. (Blamey, 1989)

IRFT spectroscopy has proven its effectiveness by allowing the discovery of compounds that can undergo positional shape changes upon electronic impact, such as B-germacrene and bicyclogermacrene, which transform into y-elemene and bicycloelemene, respectively. Indeed, this technique has discovered the positional change of B-germacrene and bicyclogermacrene present in orange essential oil, in coordination with GC/MS and GC/IRFT. (McKay, 2006)

Vera and coll. discovered this change in the essential oil of Origanum Majorana L. Similarly, the coupling of GC/IRFT allowed the discovery of the compound B-cedrene, which was not detected by GC/MS. The coordination between IRFT and retention indices allowed the discovery of some components of essential oils. However, the smaller database of IRFT compared to MS formed the limits of coupling between GC/IRFT. Indeed, the spectra recorded in the vapor phase were less clear than those recorded in the dominant phase.

On the other hand, GC can be coupled simultaneously with an infrared spectrophotometer and mass spectrometry GC/IRFT/MS. (Maberly, 1998)

II-3.8.5. Chromatographic Linkage HPLC/MS and HPLC/MS

The GC technique cannot detect compounds with very high vapor pressure. Therefore, we use high-quality liquid chromatography coupled with mass spectrometry (HPLC/MS) to detect this type of compound. However, implementing this technique requires solving some problems resulting from experimental conditions of high pressure and normal temperature for high-quality liquid chromatography, in addition to high temperature and gaseous phase for mass spectrometry. (Hammiche, 2006) The coupling between these two techniques became possible thanks to the development of very low-flow columns used, and this coupling is used in the analysis of terpenes (diterpenes, acids, or glycosylated, triterpenes) and alkaloids or even phenolic compounds. (Ozenda, 1977)

High-quality liquid chromatography coupled with mass spectrometry (HPLC/MS/MS) is often used to detect pesticide residues. Gas chromatography (GC) has been linked to high-quality liquid chromatography (HPLC), where the latter separates components according to chemical families (hydrocarbons, aldehydes, esters, alcohols, etc.), and then GC separates the components individually. This coupling provides better separation between the components and is more accurate than the results obtained from MS mass spectrometry. The coupling between high-quality liquid chromatography and gas chromatography coupled with mass spectrometry (HPLC/GC/MS) has been used to analyse the chemical components of essential oils from the peels of several citrus species such as Citrus. (Velasco, 2006)

CHAPTER 4

Bacteria

1.Definition of bacteria :

Bacteria are microscopic living organisms that have only one cell. The word for just one is "bacterium." Millions (if not billions) of different types of bacteria can be found all over the world, including in your body. They're on your skin and in your airways and mouth. They're also in your digestive system, reproductive system, and urinary tract. Scientists estimate you have 10 times more bacterial cells than human cells in your body.

These bacteria make up your microbiome, which keeps your body healthy. Other bacteria can make you sick. Healthcare providers can treat many bacterial infections with antibiotics. (clevelandclinic, 1921)

2.Classification of Bacteria :

Bacteria can be classified into various categories based on their features and characteristics. The classification of bacteria is mainly based on the following:

- **Shape:** Classification of bacteria based on Shape

Type of Classification	Examples
Bacillus (Rod-shaped)	Escherichia coli (E. coli)
Spirilla or spirochete (Spiral)	Spirillum volutans
Coccus (Sphere)	Streptococcus pneumoniae
Vibrio (Comma-shaped)	Vibrio cholerae

- **Composition of the cell wall:** Classification of bacteria based on the Composition of the Cell Wall

Type of Classification	Examples
Peptidoglycan cell wall	Gram-positive bacteria
Lipopolysaccharide cell wall	Gram-negative bacteria

- **Mode of respiration:** Classification of bacteria based on the Mode of Nutrition

Type of Classification	Examples
Autotrophic Bacteria	Cyanobacteria
Heterotrophic Bacteria	All disease-causing bacteria

- **Mode of nutrition:** Classification of bacteria based on the Mode of Respiration

Type of Classification	Examples
Anaerobic Bacteria	Actinomyces
Aerobic Bacteria	Mycobacterium

We will study four types of bacteria in this memoire

3.1.Escherichia coli :

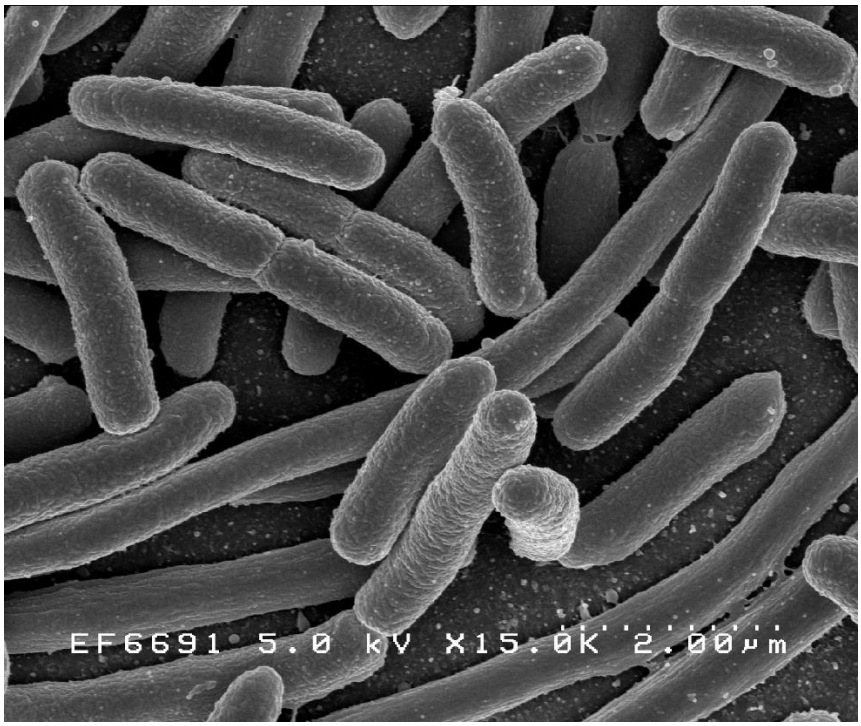


Figure 11:Micrograph of Escherichia coli

Escherichia coli, commonly known as E. coli, is a bacterium found in the human and warm-blooded animal intestines. While most strains of E. coli are harmless, some, like Shiga toxin-producing E. coli, can cause severe foodborne illnesses. This bacterium produces toxins called Shiga-like toxins due to their similarity to those produced by Shigella dysenteriae. E. coli can multiply at temperatures between 7°C and 50°C, with an optimal temperature of 37°C. It can be transmitted to humans primarily through consuming contaminated foods like raw or undercooked ground meat, raw milk, raw vegetables, and contaminated sprouts. Cooking food thoroughly at a temperature of at least 70°C can destroy Shiga toxin-producing E. coli if present[(who, s.d.)][(pasteur, s.d.)][(wikipedia, s.d.)].

3.1.1.Symptoms and Transmission

Infections caused by Shiga toxin-producing E. coli can lead to symptoms such as abdominal cramps, diarrhea, and in severe cases, bloody diarrhea (hemorrhagic colitis). Fever and vomiting may also occur. The duration of illness from this strain of E. coli is typically around a week in adults but can be longer in children. Preventing infection involves taking measures at all stages of the food chain, including production on farms, to reduce the risk of contamination. The transmission of E. coli primarily occurs through consuming contaminated foods, with the main reservoir being the digestive tract of cattle. Contamination can also arise from fecal matter of ruminants in the environment[(who, s.d.)][(pasteur, s.d.)].

3.1.2.Prevention and Treatment

Preventing infections from Shiga toxin-producing E. coli involves hand hygiene practices, thorough cooking of meat, avoiding raw milk and soft cheese made from raw milk, peeling and washing vegetables, and refraining from consuming uncooked flour-based products. Treatment for severe infections caused by this bacterium typically requires hospitalization to manage complications such as anemia and kidney failure. It is important to note that most antibiotics are contraindicated as they can worsen the condition by releasing more toxins into the body[(pasteur, s.d.)].

Escherichia coli, a versatile bacterium with both harmless and pathogenic strains, underscores the importance of food safety and hygiene practices to prevent infections and mitigate the risks associated with this bacterium.

3.2.Bacillus subtilis

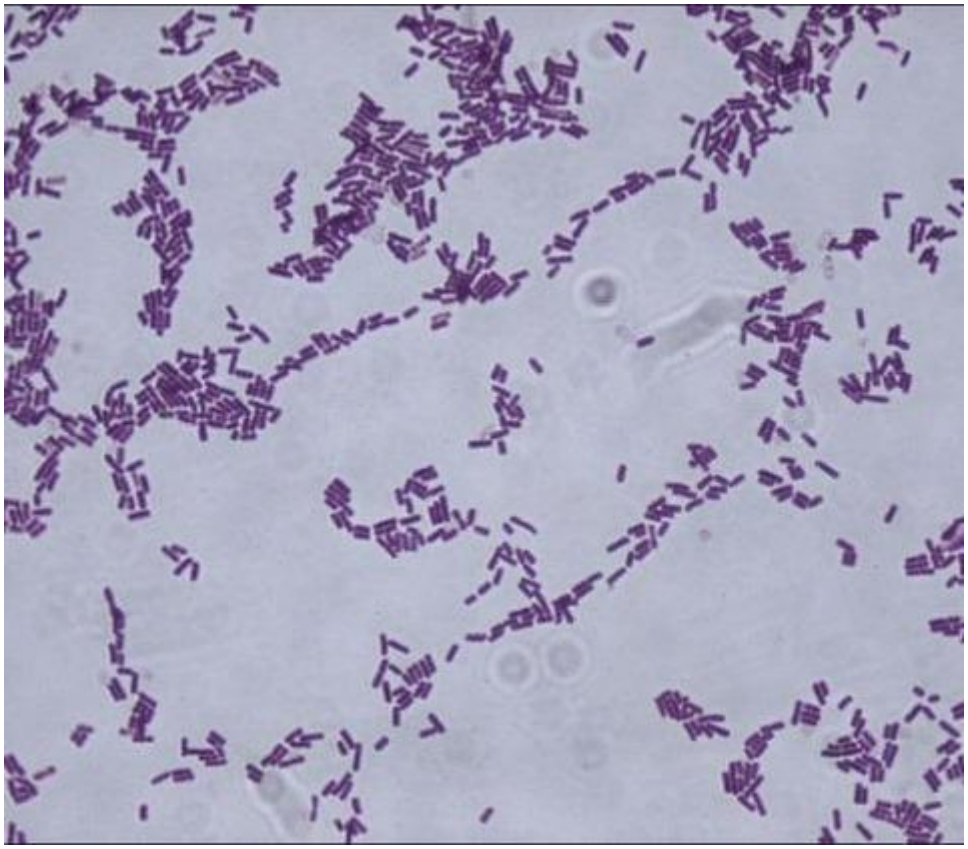


Figure 12:Micrograph of Bacillus subtilis

Bacillus subtilis, also known as the hay bacillus or grass bacillus, is a Gram-positive, catalase-positive bacterium commonly found in soil and the gastrointestinal tract of ruminants, humans, and marine sponges[(wikipedia, n.d.)][(sciencedirect, 2001)]. It is a rod-shaped bacterium, typically 4–10 micrometers (μm) long and 0.25–1.0 μm in diameter, with a cell volume of about 4.6 fL at stationary phase.

As a member of the genus Bacillus, B. subtilis can form tough, protective endospores, allowing it to survive extreme environmental conditions[(wikipedia, n.d.)][(sciencedirect, 2001)]]. It is a facultative anaerobe and has been considered an obligate aerobe until 1998.

3.2.1 Genome and Model Organism

Bacillus subtilis has about 4,100 genes, and its genome has been extensively studied[(futura-sciences, n.d.)]. It is considered the best studied Gram-positive bacterium and a model organism for laboratory studies, especially of sporulation and cellular differentiation[(ncbi.nlm.nih.gov, 2020)].

In terms of popularity as a laboratory model organism, B. subtilis is often considered the Gram-positive equivalent of Escherichia coli, an extensively studied Gram-negative bacterium. It is frequently used as a genetic model, and its study has contributed to establishing cellular metabolism pathways and regulation (wikipedia, n.d.)][(sciencedirect, 2001).

3.2.2 Applications and Characteristics

Bacillus subtilis is an excellent model for studying pathogenic bacteria, such as Staphylococcus aureus, Streptococcus pneumoniae, Bacillus anthracis, Bacillus cereus, and Listeria monocytogenes. It is used to investigate strategies these bacteria use for growth, causing diseases, multiplication, protection, and gene transfer (sciencedirect, 2001).

B. subtilis produces enzymes of interest for industry, including an amylase used in industrial bread production. It is also used in the production of natto, a Japanese fermented soybean dish. (futura-sciences, n.d.)

3.3. Klebsiella Bacteria:

3.3.1. Definition of Klebsiella :

Klebsiella bacteria is a race of negative bacteria, belonging to the family of intestinal bacteria. These bacteria are characterized by its bacillus shape, its lack of movement, and the presence of a prominent capsule consisting of many sugars. It usually lives in the intestine naturally, but if it spreads to other parts of the body, it may cause a serious infection. Klebsiella bacteria are commonly spread in health care environments such as elderly care and intensive care units in hospitals. (altibbi, 2008)

3.3.2.Species :

Klebsiella species are classified based on biochemical reactions and clinical significance:

- Klebsiella pneumoniae: Causes pneumonia, urinary tract infections, septicemia, and liver abscesses.
- Klebsiella oxytoca: Found in the nose, mouth, and intestines.
- Klebsiella rhinoscleromatis: Causes rhinoscleroma.
- Klebsiella ozaenae: Causes ozena.

Other species include K. planticola, K. terrigena, and K. ornithinolytica (Medscape, 2017). (NCBI, 2019)

3.3.3 Characteristics :

- Klebsiella species are encapsulated, which contributes to their virulence.
- They are commonly found in the human gastrointestinal tract but can cause serious infections if they spread to other parts of the body.

- Klebsiella infections are often associated with healthcare settings, such as nursing homes and intensive care units.
- K. pneumoniae is the most clinically significant species, causing pneumonia, urinary tract infections, septicemia, and liver abscesses.

In summary, Klebsiella is an important genus of bacteria that can cause severe infections, particularly in healthcare settings. Understanding its classification and characteristics is crucial for effective prevention and treatment of Klebsiella infections. (Larry M. Bush, 1443) (altibbi, 2008)

3.4. Staphylococcus Bacteria :

3.4.1.Definition :

Staphylococcus is a genus of Gram-positive bacteria in the family Staphylococcaceae from the order Bacillales. These bacteria appear spherical (cocci) under the microscope and form grape-like clusters. They are facultative anaerobic organisms, capable of growth both aerobically and anaerobically. The name "Staphylococcus" was coined in 1880 by Scottish surgeon and bacteriologist Alexander Ogston, combining the prefix "staphylo-" (from Ancient Greek: σταφυλή, meaning 'bunch of grapes') with the New Latin suffix "coccus" (from Ancient Greek: κόκκος, meaning 'spherical bacterium'). (Taxonomy, 2018)

3.4.2. Species and Characteristics:

- Staphylococcus includes at least 44 species, with some having subspecies. Many of these species reside normally on the skin and mucous membranes of humans and animals, while some can cause infections.
- These bacteria have been found to be nectar-inhabiting microbes and are a small component of the soil microbiome.

- Staphylococcus species have been implicated in human infections, with strains like S. lugdunensis, S. schleiferi, and S. caprae being notable.
- Antibiotic resistance is a significant concern with Staphylococcus, and many strains have become resistant to antibiotics, posing challenges in treatment. (Ghebremedhin B & 10.1128/JCM., 2008)

3.4.3. Taxonomy and Genomics:

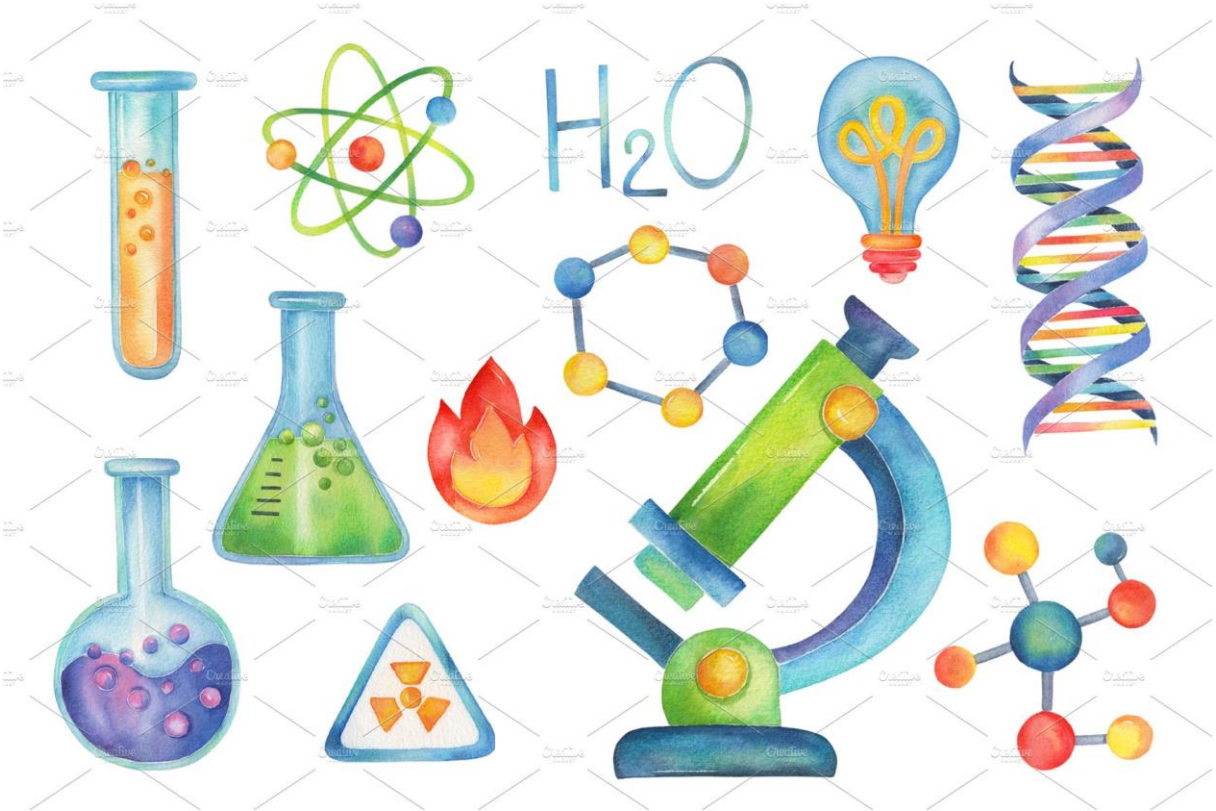
- The taxonomy of Staphylococcus is based on 16S rRNA sequences, and most species fall into distinct clusters.
- Genomic data, including sequences of various genes like gap, 16S rRNA, hsp60, rpoB, sodA, and tuff, have been used to classify and distinguish Staphylococcus species. (Book of Microorganism, n.d.)
- Whole genome technologies have provided valuable insights into the diversity of Staphylococcus strains, aiding in understanding their pathogenic behavior and evolution.

In conclusion, Staphylococcus bacteria are diverse, with some species causing infections and exhibiting antibiotic resistance. Understanding their taxonomy, characteristics, and genomic features is crucial for effective management and control of infections caused by these bacteria. (Shuaib, s.d.)

SECOND PART:

EXPERIMENTAL

CHAPTER ONE: MATERIALS AND METHODS



1. Introduction:

This study was conducted collaboratively between two specialized laboratories: the Valorisation and Technology of the Saharian Resources Laboratory (VTRS) at the Faculty of Exact Sciences, Department of Chemistry, University of El Oued, and the Centre de Recherche Scientifique et Technique en Analyses Physico-Chimique (CRAPC) in Ouargla, Algeria. The VTRS lab facilitated essential oil extraction, conducted *in vitro* and *in-silico*. Meanwhile, CRAPC handled the precise GC/MS analysis of the extracted oils. This collaborative effort ensured thorough and accurate experimentation, combining expertise and resources from both institutions.

2. Plant Material:

2.1. *Cotula cinerea*

The *Cotula cinerea* plant was harvested at various time intervals ranging from December 2023 to January 2024 in a desert forest area in southeastern Algeria, specifically in Hassi Khalifa region in El Oued province. This region is characterized by the following specifications:

- Geographic Coordinates: Longitude 48°10' East, Latitude 23°09' North.
- Elevation above Sea Level: 44 meters.
- Distance from Sea Level: 290 kilometres.
- Bioclimatic Characterization: Desert.

2.2. *Origanum Majorana L:*

The *Origanum Majorana L* plant was harvested at different time intervals extending from March 2023 to April 2023 in a desert forest area in southeastern Algeria, specifically in Akfadou region in El Oued province. This region is characterized by the following specifications:

- Geographic Coordinates: Longitude 67°6' East, Latitude 33° North.
- Elevation above Sea Level: 58 meters.
- Distance from Sea Level: 300 kilometres.
- Bioclimatic Characterization: Desert.

3. Chemicals and reagents:

Microorganisms Used:

All microorganisms used in these studies were provided by the pedagogical laboratory of the faculty of biology.

- *Escherichia coli (ATCC 25922):* Belongs to the Enterobacteriaceae family. It is non-spore forming, facultative anaerobic, and typically motile due to flagella. Its length ranges from 2 to 6 µm and its width from 1.1 to 1.5 µm.
- *Bacillus subtilis (ATCC 6051-U)*: A Gram-positive bacterium with a length ranging from 2 to 3 µm and a width from 0.3 to 0.5 µm.
- *Staphylococcus aureus (ATCC 25923)*: Is a Gram-positive spherical bacterium with a diameter ranging from 0.5 to 1.5 µm, belonging to the genus Staphylococcus.

4. Materials and Methods:

4.1. Essential Oils Extraction:

4.1.1. Apparatus:

The essential oil extraction process required the use of specialized equipment to ensure accuracy and efficiency. In this study, the following apparatus was utilized:

- ✓ Adventurer – Pro AV53 sensitive balance
- ✓ Heating flask
- ✓ Refrigerant
- ✓ Clevenger apparatus
- ✓ Separating funnel
- ✓ Rotary evaporator

The Clevenger apparatus, a key component in the extraction process.

4.1.2. Procedure:

The procedure for essential oil extraction involved a series of meticulous steps to ensure optimal results. The extraction process was conducted as follows:

- ✓ Cleaning of the plant sample to remove any impurities.
- ✓ Weighing the plant sample to a quantity of 100 g using a sensitive balance.
- ✓ Washing the plant sample with water to prevent burning, followed by placement in a heating flask.

- ✓ Addition of small pieces of boiling regulator to the flask.
- ✓ Direct heating of the mixture at a temperature of 100 degrees Celsius for three and a half hours.
- ✓ Conducting the steam distillation process and subsequent separation of oil and water phases using liquid-liquid separation with diethyl ether.
- ✓ Drying of the organic oily phase with anhydrous sodium sulphate.
- ✓ Filtration of the dried oil phase through filter paper to remove diethyl ether particles.
- ✓ Evaporation of the filtered oil phase using a rotary evaporator to remove any remaining organic solvent.
- ✓ Storage of the obtained essential oil in small brown bottles and refrigeration at a temperature of 5°C.

4.2. Yield of Essential Oil Extraction:

Two distinct methodologies are commonly employed to ascertain the yield of essential oil extraction, each offering unique perspectives on the efficiency of the process.

4.2.1. Volumetric Yield - Mass-based: (Naima et al., 2019)

This method entails an assessment of the mass of the utilized plant material intended for essential oil extraction, juxtaposed against the volume of the resultant oil. The yield is then computed utilizing the following Equation 1:

$$R_{EO} = \frac{V_{EO}(ml)}{m_0(g)} \times 100 \quad (1)$$

Where: R_{EO}: Essential oil yield, m_0: Mass of the utilized plant sample, V_{EO}: Volume of the extracted essential oil

4.2.2. Mass-based Yield - Mass-based: (Larbi et al., 2018)

Alternatively, the essential oil extraction yield is defined as the quotient of the mass of the extracted essential oil and the mass of the plant material utilized. This yield is calculated using Equation 2:

$$R_{EO} = \frac{m_{EO}(g)}{m_0(g)} \times 100 \quad (2)$$

Where: R_{EO}: Essential oil yield, m_0: Mass of the utilized plant sample, m_{EO}: Mass of the extracted essential oil

These methodologies have been systematically applied to derive essential oil yields for all examined plant specimens.

4.3. Characterization of Essential Oils:

The characterization of essential oils comprises two fundamental components: physicochemical properties and Gas Chromatography-Mass Spectrometry (GC/MS) analysis.

4.3.1. Physicochemical Properties:

Here, we delve into the fundamental traits of essential oils, including their relative density, acidity, ester content, and refractive index. These properties offer valuable insights into the composition and behavior of essential oils, helping us understand their chemical makeup and how they interact with their environment (Atti-Santos et al., 2005).

➢ *Relative Density (AFNOR NF T75-111 Standard):*

At 20°C, 1 mL of the essential oil is measured using a pipette, and its mass is then determined. The procedure is repeated for distilled water, and density is calculated using the following Equation 3 (Valarezo et al., 2015):

$$d = \frac{m_{EO}}{m_{H_2O}} \quad (3)$$

Where: m_{EO}: Mass of the extracted essential oil, m_{H_2O}: Mass of the distilled water

➢ *Acidity Index (AFNOR NF T75-111 Standard):*

To determine the acid value, representing the concentration of free acids in 1 g of the essential oil, a titration method with potassium hydroxide (KOH) solution is employed (Sahoo et al., 2007).

Initially, a small aliquot (0.5 mL) of the essential oil is mixed with 2 to 3 drops of phenolphthalein indicator in a small vessel. Subsequently, titration is performed with 0.5 N KOH solution until the appearance of a faint pink colour, indicating complete neutralization of the acids. The acid value is then calculated using the following Equation 4.

$$I_a = \frac{56.11 \times V \times C}{m} \quad (4)$$

Where: V: the volume of the KOH solution, C: Concentration of KOH, m: the mass of the essential oil

➢ *Ester Index (AFNOR NF T75-111 Standard):*

The determination of free acids resulting from ester hydrolysis within the essential oil involves a titration process utilizing 0.5 N potassium hydroxide (KOH) solution (Alajtal et al., 2018).

- Begin by placing 0.5 mL of the essential oil into a small vessel.

- Add 1 mL of 0.5 N potassium hydroxide (KOH) solution to the vessel to initiate the titration process.
- Place the mixture in a gas-evacuated water bath for a specified duration to facilitate reaction.
- After cooling, introduce 0.5 mL of distilled water and add 3 drops of phenolphthalein indicator to the mixture.
- Titrate the excess potassium hydroxide (KOH) using 0.5 N hydrochloric acid (HCl) until a colour change is observed, indicating neutralization.
- Quantify the volume of hydrochloric acid (HCl) required to neutralize the excess potassium hydroxide (KOH).
- Calculate the ester content using the following Equation 5:

$$I_e = \frac{2805}{m}(V_0 - V_1) - I_a \quad (5)$$

Where: V_0 (ml): Volume of hydrochloric acid (HCl) without essential oil, V_1 (ml): Volume of hydrochloric acid (HCl) in the presence of essential oil, I_a: Acid value, I_e: Ester value and m: Mass of the essential oil sample.

➢ *Refractive Index (AFNOR NF T75-111 Standard):*

The refractive index of an essential oil is defined as the ratio between the sine of the angle of incidence and the sine of the angle of refraction of a light ray, with a specific wavelength, transitioning from air into the essential oil, while the latter is maintained at a constant temperature (Singh, 2002)

The refractive index of the essential oil is directly measured using a refractometer at a reference temperature 20°C.

4.3.2. Gas Chromatography-Mass Spectrometry (GC/MS) analysis:

The coupling (GC/MS) technique stands as the most frequently employed method within the field of essential oils, facilitating the concurrent separation, identification, and quantitative measurement of the various constituents present in extracted oils.

➢ *Principle:*

The principle is founded on the varying affinities of compounds within the mixture towards two phases: a stationary phase and a mobile phase. This technique relies on the distribution of constituents between a stationary phase and a gas phase. The stationary phase comprises a silicone-based liquid that permeates an inert and granular solid material, housed within a typically coiled steel or glass column measuring 1 to 3 meters in length and 2 to 4 millimetres

in diameter. The mobile phase consists of an inert carrier gas such as nitrogen, helium, or argon.

The column is maintained at a high temperature via a furnace. Under the influence of temperature, constituents vaporize and become separable. The basis of separation lies in the discrepancy of partition coefficients of volatile compounds between the stationery and gas phases. A detection system generates a signal at the exit of each molecule from the column, manifesting as the recording of peaks corresponding to each constituent.

Gas chromatography is coupled with a mass spectrometer (MS); this coupling relies on computerized comparison of the spectrum of an unknown peak with one or more reference libraries, enabling its identification.

➢ *Apparatus:*

The identification of the chemical constituents of our essential oils was performed using a gas chromatographic system (HP 5890-SERIE II) equipped with an HP5 MS capillary column (30 meters in length, 0.25 mm internal diameter, and 0.25 µm film thickness) coupled with a mass spectrometer (HP-MSD 5972).

N_2 was employed as the carrier gas for the analysis of the two essential oils. Spectra were recorded at an emission energy of 70 eV, and spectral analysis of the compounds was conducted by comparison with their counterparts using the WILEY275 (Chiu et al., 1982; Guinaudeau et al., 1975; Kiryakov, 1968; Shamma, 1972) spectral librairies.

➢ *Procedure:*

The carrier gas (N_2) is introduced at a flow rate of 1 mL/min, with the injected volume of the essential oil being 1 µL in split injection. The injector and detector temperatures are set at 250°C and 320°C, respectively. The oven temperature is programmed to initially reach 60°C and held for 8 minutes, then gradually increased to 250°C at a rate of 2°C/min, maintained isothermally at 250°C for 15 minutes, and finally elevated to 300°C at a rate of 10°C/min.

4.4. *In Vitro* Assessment of the Antibacterial Activity:

✓ Procedure:

Antimicrobial tests were conducted to evaluate the biological activity of the studied essential oils against Escherichia coli, Bacillus subtilis and Staphylococcus aureus. The antimicrobial activity was assessed using the disc diffusion method as described in the literature (Klančnik et al., 2010):

- *Preparation of test products:* Dissolve EOs in DMSO to prepare solutions of different concentrations ranging from 2 to 40 mg/ml.

- *Preparation of precultures:* Cultivate the microbial strains to be tested on Petri dishes containing nutrient agar and incubate for 24 hours at 37°C to obtain a young culture of bacteria and isolated colonies.
- *Preparation of discs:* Prepare discs using 6mm diameter Whatman filter paper, followed by sterilization for 30 minutes at 120°C in the autoclave.
- *Preparation of bacterial suspensions:* Take a few well-isolated and identical colonies using a Pasteur pipette and suspend them in 10 ml of sterile physiological saline solution (0.9% NaCl). Standardize the bacterial suspension using a densitometer for seeding into the MH milieu.
- *Preparation of culture milieu:* Utilize various culture milieu including nutrient broth for isolation and maintenance of bacterial strains, nutrient agar for reactivation of the bacterial strain, and Mueller-Hinton agar for studying bacterial sensitivity to different concentrations of EO.
- *Testing antibacterial activity:* Spread the bacterial suspension over the entire surface of Mueller Hinton agar (MHA) three times using a sterile swab, then leave on the bench for 30 minutes. Assess antibacterial activity by measuring the diameter of the inhibition zone using a ruler and comparing it with that of DMSO as a negative control and antibiotics as positive controls (Amoxicillin 25 µg/disc). Measure absorbance using UV-vis spectroscopy.

The inhibitory activity of bacteria was calculated as a percentage of inhibition using the following Equation 6 (Klančnik et al., 2010):

$$\%Inhibition = \left(1 - \frac{Abs_{Essential\ oil}}{Abs_{Controle}}\right) \times 100 \quad (6)$$

4.5. *In-Silico* analysis:

4.5.1. Software

Computational simulations, including Induced Fit Docking, molecular dynamics studies, and MM-GBSA calculations, were conducted employing the Glide module, Induced Fit Docking module, Prime module, and the Desmond module within the Maestro version 11.7 user interface of the Schrödinger suite (Small-Molecule Drug Discovery Suite 2021-4, Schrödinger, LLC, New York, NY, 2021) (Schrödinger, 2015). The simulations were executed on a DELL Intel(R) Core (TM) i9-13900HX CPU @ 2.20 GHz processor, equipped with 32.0 GB RAM, and operated on a 64-bit Linux Ubuntu 18,04.1 LTS operating system.

4.6.2. ADMET and drug-likeness evaluation:

Drug candidates should possess favourable ADMET properties and ideally non-toxic. Therefore, the major identified compounds from the essential oils extract were evaluated of their ADME profile, including physicochemical, lipophilicity, water solubility, pharmacokinetics, drug-like nature, medicinal chemistry, and several other parameters using SwissADME (Daina et al., 2017; Riyadi et al., 2021) module provided in SIB (Swiss Institute of Bioinformatics) webserver (https://www.sib.swiss). Furthermore, the toxicity aspect of designed compound was also predicted using ProTox (Banerjee et al., 2018) webserver (https://comptox.charite.de/protox3/).

4.6.3. Docking setup:

- *Ligands preparation:*

The three-dimensional configurations of the major compounds isolated from *Cotula cinerea* and *Origanum Majorana L e*ssential oils were obtained from the National Library of Medicine (NCBI) database (Kim et al., 2016), accessible through the NCBI website (https://pubchem.ncbi.nlm.nih.gov/).

Ligand preparation involved an energy optimization process to derive the most energetically favourable conformations for each compound. Utilizing LigPrep module within the Schrödinger suite (Schrödinger, 2024), this optimization procedure ensured the attainment of the lowest energy state for the studied drugs, including Montbretin A (a co-crystallized ligand). The ionization states were established at a pH of 7.0 ± 2.00, as computed by the Epik classic module, while maintaining specified chirality and generating relevant tautomeric forms. Furthermore, partial atomic charges were computed using Optimized Potentials for Liquid Simulations OPLS4 force field (Lu et al., 2021).

- *Receptor Preparation:*

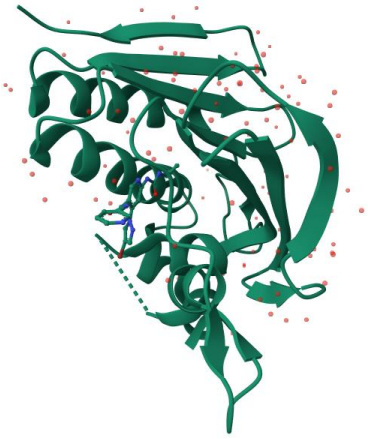
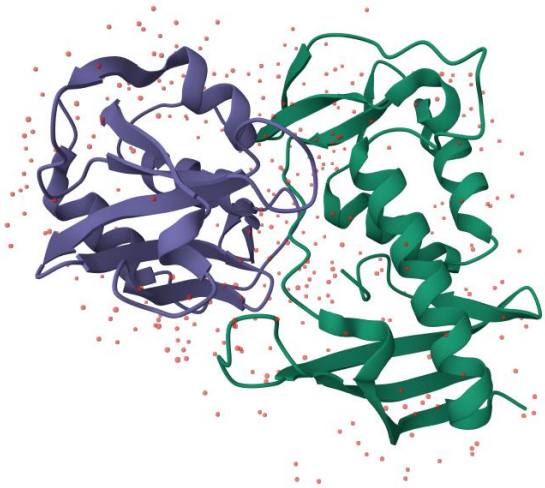
The 3D structures of specifically selected receptors have been scrutinized, including the Escherichia coli (PDB ID: 6F86) (Narramore et al., 2019), Bacillus subtilis (PDB ID: 8I2F) (Tandukar et al., 2023), and Staphylococcus aureus (PDB ID: 6LKI) (M. Wang et al., 2020). Details pertaining to these receptors, encompassing their designations and corresponding PDB IDs, are delineated within Table 1.

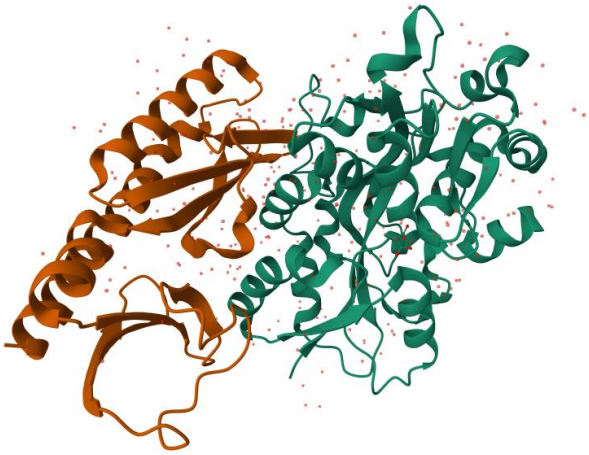
Processing of the proteins and DNA structures was executed through the "protein preparation Workflow" module within the Schrödinger suite (Madhavi Sastry et al., 2013), involving consecutive stages of import and processing, review and modification, and refinement.

In the initial stage, the Prime tool was employed to address missing residues and side chains, maintaining the pH of PROPKA at 7.0 ± 2.00. Subsequent steps included the optimization and assignment of hydrogen bonds, along with the removal of water molecules beyond 8 Å. Restrain minimization utilizing the Optimized Potentials for Liquid Simulations (OPLS4) force field was performed to achieve a low-energy state for the protein (Lu et al., 2021). This phase of protein preparation signifies an energy optimization methodology, presenting the protein in its energetically favourable state for subsequent *in-silico* studies.

The "Receptor grid generation" panel facilitated the creation of a grid encompassing the active site of the protein, delimited by the co-crystallized ligand Montbretin A. Default parameters were maintained, and the grid centre was generated at the coordinates X = -6.65; Y = 6.99; Z = -20.65.

Table 7. Target receptors information chosen for docking studies

	Crystal Structure of E. coli GyraseB 24kDa
	
PDB ID	6F86
Mutation	No
Resolution (Å)	1.90
R-Value Observed	0.204
Organism	Escherichia coli
Space Groupe	P 3_2 2 1
Sequence Length	206
	Crystal structure of Bacillus subtilis LytE catalytic domain in complex with IseA
	
PDB ID	8I2F
Mutation	No
Resolution (Å)	2.03
R-Value Observed	0.175
Organism	Bacillus subtilis
Space Groupe	P 2_1 2_1 2_1

	Sequence Length	282
	Two-component system protein mediate signal transduction	
	PDB ID	6LKI
	Mutation	No
	Resolution (Å)	1.78
	R-Value Observed	0.210
	Organism	Staphylococcus aureus
	Space Groupe	C 2 2 2_1
	Sequence Length	471

➢ *Molecular Docking:*

Computational simulations, including Induced Fit Docking, molecular dynamics studies, and MM-GBSA calculations, were conducted employing the Glide module, Induced Fit Docking module, Prime module, and the Desmond module within the Maestro version 11.7 user interface of the Schrödinger suite (Small-Molecule Drug Discovery Suite 2021-4, Schrödinger, LLC, New York, NY, 2021) (Schrödinger, 2015). The simulations were executed on a DELL Intel(R) Core(TM) i9-13900HX CPU @ 2.20 GHz processor, equipped with 32.0 GB RAM, and operated on a 64-bit Linux Ubuntu 18,04.1 LTS operating system.

The molecular docking tool employed for all docking studies was Glide (Grid-based Ligand docking with Energetics), a module within the Schrödinger suite (Yang et al., 2021). The prepared ligands underwent docking onto the specified protein site utilizing the Glide module, in Standard Precision (SP) modes (Friesner et al., 2006).

➢ *Induced Fit Docking (IFD):*

The Induced Fit docking (IFD) module of the Maestro molecular modelling suite has been noted as a reliable and effective docking approach to consider flexibility in both ligands and the binding pocket residues in the binding pocket of target receptors (Khelil et al., 2020). During the IFD process, Glide/SP (Standard Precision) was performed for each ligand, the Prime refinement step specifically addressed the side chains of residues within a 5 Å radius of the ligand. Noteworthy is the retention of a maximum of 20 poses for each ligand.

➢ *Molecular Dynamics Simulation (MDS):*

The best compound in each plant exhibiting the highest IFD docking score was chosen for Molecular Dynamics Simulation (MDS). Recognizing the limitations of ligand docking in representing the biological system under aqueous conditions (Korb et al., 2012), a 100 ns simulation time for MDS was executed using the Desmond module within the Schrödinger suite (Bowers et al., 2006).

The MDS protocol comprised three essential steps: system builder, minimization, and MDS. In the system builder phase, the protein and ligand complex were selected and immersed in a biological environment. The transferable intermolecular potential with 3 points (TIP3P) solvent model, with the boundary condition maintained in an orthorhombic box form throughout the process with dimensions of 10 x 10 x 10 Å (Akbar et al., 2022). The OPLS-3e force field was consistently applied (Roos et al., 2019). The neutralization of model was conducted by addition of counter ions when needed and 0.15 M of NaCl salt was included to mimic the physiological state.

Subsequently, the NPT ensemble was utilized for energy minimization, maintaining pressure and temperature at 1.0132 bar and 300 K, respectively. Finally, MDS was conducted for the minimized protein-ligand complex (Halder et al., 2023).

➢ *Free Energy (MM-GBSA) Calculation:*

Upon completion of the dynamic simulation, an assessment of the free binding energy between the protein and the ligand was systematically undertaken utilizing the Prime MM-GBSA module within the Maestro molecular modelling suite (E. Wang et al., 2021). The calculation of ligand binding affinities was accomplished through the Molecular Mechanics/Generalized Born Surface Area ΔG (MM-GBSA ΔG) metric applied to the optimized Receptor-Ligand Complex. This computation, facilitated by the VSGB solvation model and the OPLS4 force field, stands as a methodologically rigorous approach for the comprehensive evaluation of molecular interactions and binding strengths (Gorla et al., 2021).

CHAPTER TWO: RESULTS AND DISCUSSION

1. Introduction:

This chapter delves into the findings regarding the extraction yield, characterization, and comprehensive exploration of the anticancer and antibacterial properties inherent in two essential oils derived from the aerial constituents of *Cotula cinerea* and *Origanum Majorana L*. The overarching aim of this investigation was to assess the potential anticancer and antibacterial efficacy exhibited by essential oils as significant agents in the domain of novel pharmaceutical development.

The elucidations provided herein furnish intricate insights into the chemical constitution of the essential oils, thereby enabling their utilization in the *in-silico* study. This involved the application of advanced computational techniques such as Induced Fit Docking (IFD) and Molecular Dynamics Simulation (MDS) for each compound, capabilities not feasible in traditional *in vitro* studies.

2. Extraction Yield:

Figure 2 illustrates the yields of essential oils obtained through hydrodistillation of the aerial parts of *Cotula cinerea* and *Origanum Majorana*. variety of Eloued.

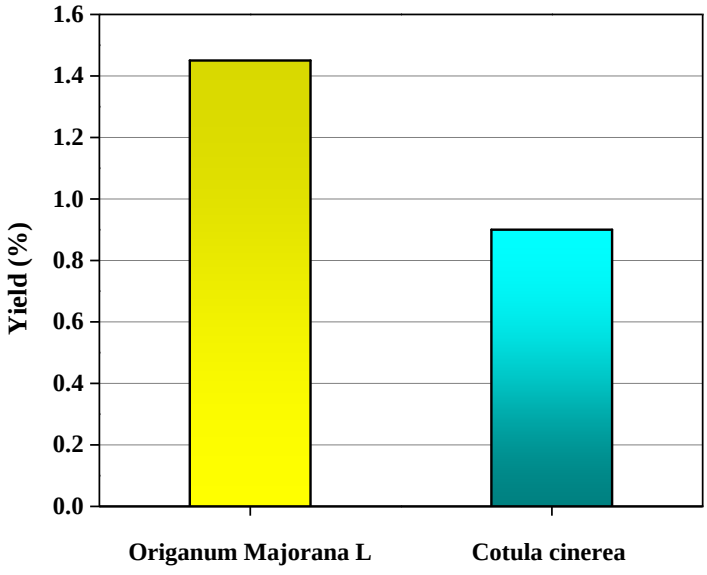


Figure 13. The yields of extracted essential oils

We observe that *Origanum Majorana L* exhibits a higher essential oil yield than *Cotula cinerea* (1.45% and 0.90% respectively). However, the difference between the two yields is non-significant.

Nevertheless, findings reported by (Alimi et al., 2022) indicate lower yields of essential oils extracted by hydrodistillation from *Origanum Majorana L* and *Cotula cinerea* at ambient temperature.

Similar results to ours were, however, obtained by (Naima et al., 2019) for the essential oil extracted from the same variety using the same method.

Furthermore, significantly higher yields were achieved by (Vera & Chane-Ming, 1999) during the hydrodistillation of *Origanum Majorana L*, Indian variety.

These variations in results can be explained by the fact that essential oil yields are influenced by several factors during extraction: either factors related to the plant (species, variety, chemical composition, etc.) or factors associated with experimental conditions (extraction process, extraction duration, etc).

3. Chemical composition of essential oils:

3.1. Organoleptic characteristics:

Through the conducted work, it has been revealed that the essential oil extracted from the studied plants exhibits the following (Table 2) organoleptic properties:

Table 8. The organoleptic characteristics of essential oils

Plant	Smell	Aspect	Colour
Cotula cinerea	A distinctive aroma	liquid at room temperature	Dark-yellow
Origanum Majorana L	A pleasant fragrance	liquid at room temperature	Light-yellow

3.2. Physicochemical Properties:

The physicochemical properties were meticulously determined according to the standards of the French Association for Standardization (AFNOR) using established methodologies to measure relative density, refractive index, acidity index, and ester index, as depicted in the following Table 3.

Table 9. The physicochemical properties of essential oils

Plant	Relative Density	Refractive Index	Acidity Index	Ester Index
Cotula cinerea	0.958	1.4720	5.01	43.72
Origanum Majorana L	0.908	1.4512	4.39	41.23

By comparing these results to those obtained by (Naima et al., 2019) (density 0.953, refractive index 1.474, acidity index 4.93 and ester index 45.96) and (Alimi et al., 2022) (density: 0.834 ± 0.02, 0.835 ± 0.02, refractive index, at 25°C 1.4596 ± 0.03, 1.4622 ± 0.04),

and considering that the refractive index was measured at a temperature of 23°C, we can conclude that these values are in accordance with the standards described by AFNOR (Afnor, 1982).

3.3. Gas Chromatography-Mass Spectrometry (GC/MS) analysis:

The analysis focused on the essential oil constituents extracted from two specific plant species using gas chromatography-mass spectrometry (GC/MS). Mass spectra corresponding to each chromatographic peak were juxtaposed with spectra from relevant scientific literature and the Weily electronic database for mass spectra (Horai et al., 2010). Retention indices were employed to ascertain compound identities. Moreover, utilizing the identical non-polar HP5 stationary phase in the gas chromatography column facilitated the preservation of consistent peak numbers and elution sequences for the compounds under investigation.

Figure 3 and Figure 4 depict the chromatograms illustrating the essential oil compositions of *Cotula cinerea* and *Origanum Majorana L*, respectively, as obtained through gas chromatography-mass spectrometry (GC/MS) analysis:

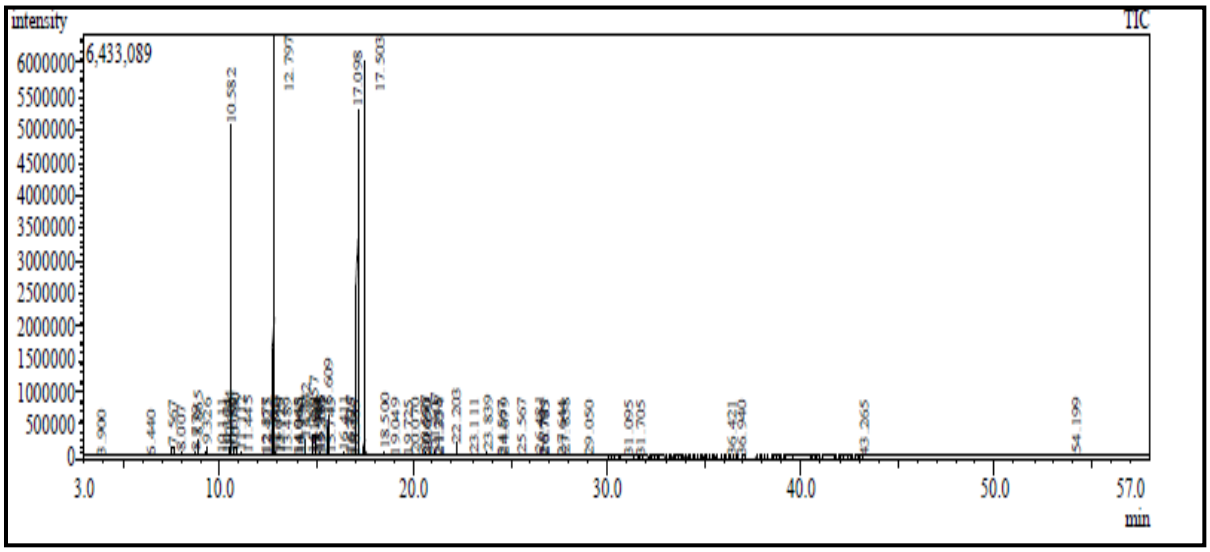


Figure 14. Chromatogram of the essential oil of *Cotula cinerea* plant obtained by GC/MS

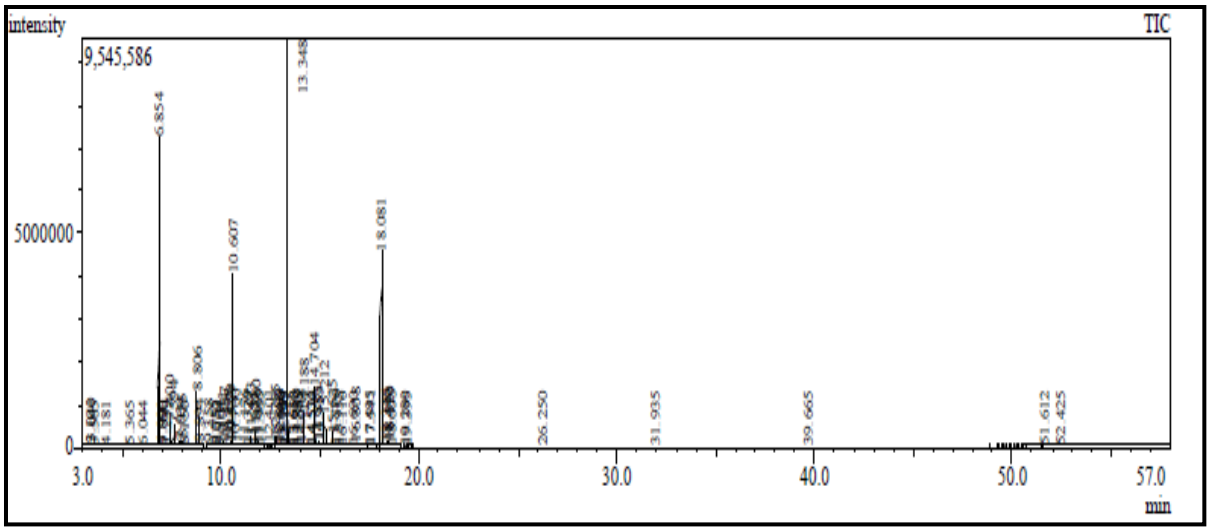


Figure 15. Chromatogram of the essential oil of *Origanum Majorana L* plant obtained by GC/MS

Upon scrutinizing the two chromatograms, notable resemblances are evident, with discernible bands interspersed with overlapping ones. Generally, distinct regions can be identified, delineating three segments:

1. The initial segment, delimited within the time frame of (4 min to 12.4 min), hosts several bands denoting the presence of hydrocarbon monoterpenes.
2. The succeeding segment, spanning from (12.4 min to 24 min), encompasses the predominant bands, indicative of oxygenated monoterpenes prevalent in the plant's essential oil.
3. The final segment, spanning (24 min to 32.5 min), exhibits minimal banding, suggesting a negligible presence of hydrocarbon sesquiterpenes in the plant's essential oil.

The pertinent compounds in each essential oil, extracted from *Cotula cinerea* and *Origanum majorana L*, have been identified and collated as follows

3.3.1. *Cotula cinerea:*

The hydrodistillation extraction of *Cotula cinerea* yielded a dark yellow oil with a yield of 0.91%. Gas chromatography-mass spectrometry (GC/MS) analysis identified 31 compounds, collectively constituting 95.64% of the oil's composition (as presented in Table 4). Predominantly, oxygenated monoterpenes accounted for 68.16%, followed by hydrocarbon monoterpenes at 23.27%, while the percentage of oxygenated sesquiterpenes was minimal at 0.15%. The remaining 4.06% comprised various other compounds.

Table 10. Essential oil constituents of *Cotula cinerea* identified by GC/MS

No	Compounds	IR_{Exp}	IR_{Ref}	(%)
01	**Santolina triene**	**908**	**914**	**10.6**
02	Alpha-thujene	931	935	0.88
03	**Alpha-pinene**	**939**	**943**	**2.02**
04	camphene	953	956	0.85
05	**Sabinene**	**976**	**976**	**6.17**
06	Beta- pinene	980	981	0.58
07	Dehydro-1.8-cineole	991	988	0.64
08	Myrcene	991	993	0.07
09	Meta mentha-1(7)-8dien	999	997	0.06
10	Alpha-terpinene	1018	1017	0.83
11	Ortho cymene	1022	1020	0.43
12	Para cymene	1026	1029	0.6
13	**1.8-cineole**	**1033**	**1033**	**5.34**
14	**Delta-terpinene**	**1062**	**1057**	**1.57**
15	Cis sabinene hydrate	1068	1069	0.46
16	Terpinolene	1088	1088	0.36
17	Cis thujone	1102	1100	0.52
18	**Trans thujone**	**1114**	**1117**	**51.86**
19	**Camphor**	**1143**	**1140**	**2.63**
20	**Beta-terpineol**	**1163**	**1160**	**1.39**
21	**Terpin-4-ol**	**1177**	**1178**	**1.73**
22	Alpha-terpineol	1189	1190	0.58
23	Myrtenol	1194	1195	0.13
24	Neo iso dihydro carveol	1226	1224	0.53
25	Carvotanacetone	1246	1247	0.9
26	**Cis verbenyl acetate**	**1262**	**1264**	**5.07**
27	Iso pulegol acetate	1273	1274	0.06
28	Para cymen-7-ol	1287	1285	0.08
29	Neryl acetate	1365	1367	--
30	Cis jasmone	1394	1390	0.15

31	Germacrene D	1480	1481	0.06
	Total		**95.64**	

Our analysis unveiled ten compounds with concentrations surpassing 1% (Table 4), with trans-thujone emerging as the predominant compound at 51.86%, followed by santolina triene at 10.69%, and sabinene at 6.17%. Additionally, 1,8-cineole constituted 5.34%, while four compounds α-pinene, terpin-4-ol, β-terpineol, and camphor appeared in lesser proportions at 2.02%, 1.73%, 1.39%, and 2.63%, respectively.

Comparison with other studies revealed some congruence, particularly with the percentage of santolina triene in Ekhlil et al.'s (Ekhlil et al., 2016) investigation (11.67%). However, disparities arose in the primary compound, with iso-thujanol predominating at 47.38% in their study. Conversely, El bouzidi et al.'s (Bouzidi et al., 2011) findings aligned closely with ours, particularly regarding trans-thujone (41.4%), 1,8-cineole (8.2%), and santolina triene (7.2%), while reporting camphor at 5.5%. Notably, Fournier et al.'s (Fournier et al., 1989) study diverged, identifying camphor as the principal compound at 50%.

For a visual representation of the top major compounds (more than 1%) and their structures, refer to Figure 5, which depicts the chemical structures and IUPAC names of the predominant constituents identified in the essential oil of *Cotula cinerea*.

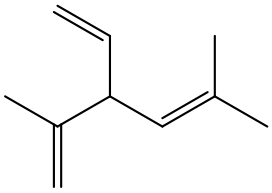
3.3.2. Origanum Majorana L:

The hydrodistillation extraction method was employed to obtain the essential oil from *Origanum Majorana L.*, yielding a noteworthy 1.5% output. Analysis revealed the identification of 98.84% of the constituents, comprising a total of 42 compounds (as presented in Table 5). Oxygenated monoterpenes dominated the composition, constituting over 57%, followed by hydrocarbon monoterpenes at 25.14%.

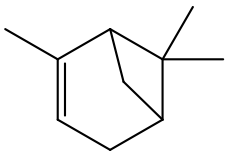
Of particular interest is the prominent presence of trans-Thujone, constituting 33.3% of the essential oil, a compound conspicuously absents in the majority of prior studies. For instance, J. Chane et al.'s (Vera & Chane-Ming, 1999) investigation reported Terpinen-4-ol as the primary compound at 38.4%, a constituent entirely absents in our study. Similarly, Santolina triene emerged as the second major compound in our analysis at 16.40%, a finding not corroborated in extant literature.

Despite these disparities, concordance exists with previous studies regarding the occurrence of Sabinene, which manifested at 3.12% in our study, compared to 15% in J. Chane et al.'s (Vera & Chane-Ming, 1999) study and 17% in K.H.C Baser et al.'s study (Baser

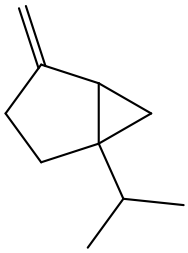
et al., 1993). Additionally, Sellami et al (Sellami et al., 2009) documented Sabinene at 2.14%. Supplementary compounds identified include α-Thujene (1.44%), α-Pinene (1.19%), and β-Pinene oxide (4.42%).



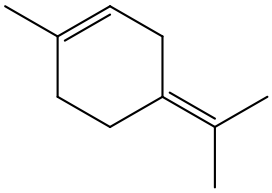
2,5-dimethyl-3-vinylhexa-1,4-diene
(Santolina triene)



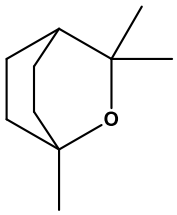
2,6,6-trimethylbicyclo[3.1.1]hept-2-ene
(Alpha-pinene)



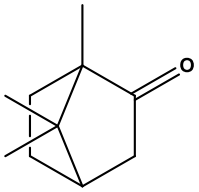
1-isopropyl-4-methylenebicyclo[3.1.0]hexane
(Sabinene)



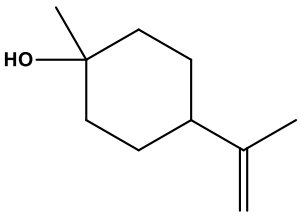
1-methyl-4-(propan-2-ylidene)cyclohex-1-ene
(Delta-terpinene)



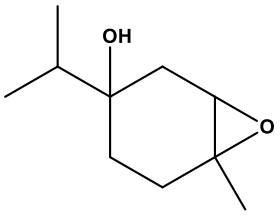
1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane
(1.8-cineole)



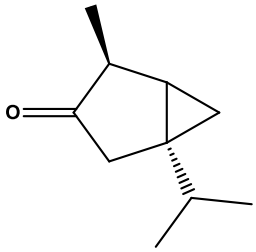
1,7,7-trimethylbicyclo[2.2.1]heptan-2-one
(Camphor)



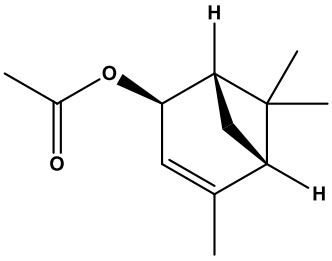
1-methyl-4-(prop-1-en-2-yl)cyclohexanol
(Beta-terpineol)



3-isopropyl-6-methyl-7-oxabicyclo[4.1.0]heptan-3-ol
(Terpin-4-ol)



(1*S*,4*S*)-1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-one
(Trans thujone)



(1*S*,2*R*,5*S*)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-yl acetate
(Cis verbenyl acetate)

Figure 16. Chemical structures and IUPAC names of the top major compounds identified in the essential oil of *Cotula cinerea*

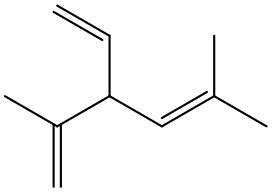
In summary, the essential oil derived from *Origanum Majorana L.* exhibits distinctive chemical compositions in Eloued region compared to oils from other geographical locales,

characterized notably by the prevalence of trans-Thujone and Santolina triene as major constituents. This variance likely stems from multifactorial influences such as soil quality, climatic conditions, and harvest timing. For an illustrative overview of the major compounds identified, Figure 6, displaying the structures and IUPAC nomenclature of these constituents.

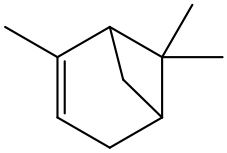
Table 11. Essential oil constituents of *Origanum Majorana L* identified by GC/MS

No	Compounds	IR_{Exp}	IR_{Ref}	(%)
01	Pentanol	759	762	0.12
02	Cis-2-Penten-1-ol	762	765	0.03
03	Hexanal	801	801	0.02
04	(Z)-Salvene	844	847	0.02
05	Isopentyl acetate	869	869	0.02
06	**Santolina triene**	**898**	**906**	**16.42**
07	Tricyclene	912	921	0.02
08	**Alpha-Thujene**	**918**	**924**	**1.44**
09	**Alpha-Pinene**	**925**	**932**	**1.19**
10	Camphene	941	946	0.43
11	**Sabinene**	**969**	**969**	**3.12**
12	Beta-Pinene	972	974	0.22
13	Beta-Myrcene	989	988	0.14
14	Alpha-Phellandrene	1003	1002	0.03
15	Propanoic acid, 2-methyl-, 3-methylbutyl ester	1012	1007	0.02
16	Alpha-Terpinene	1016	1014	0.36
17	Para cymene	1024	1020	0.20
18	Ortho Cymene	1026	1022	0.45
19	Sylvestrene	1028	1025	0.20
20	**1,8-Cineole**	**1031**	**1026**	**10.71**
21	Trans-decahydroNaphthalene	1054	1053	0.03
22	Terpinene	1059	1054	0.76
23	Cis Sabinene hydrate	1068	1065	0.89
24	Para-Mentha-2'4(8)-diene	1088	1085	0.11
25	Trans-Sabinene hydrate	1100	1198	0.86
26	Isopentyl-2-methylbutanoate	1104	1100	0.14
27	cis-Thujone	1107	1101	0.24
28	**trans-Thujone**	**1119**	**1112**	**33.30**
29	iso-3-Thujanol	1136	1134	0.04
30	**Camphor**	**1146**	**1141**	**2.85**
31	**Beta pinene oxide**	**1163**	**1154**	**4.42**
32	3-Thujanol	1168	1164	0.48
33	Alpha Terpineol	1192	1186	0.88
34	Dihydrocarveol	1197	1192	0.06
35	Safranal	1203	1197	0.01
36	Cis-3-Hexenyl 2-methyl butanoate	1233	1229	0.25
37	trans-Myrtanol	1257	1258	0.04
38	2-(1E)-propenyl Phenol	1258	1264	0.09
39	**cis Verbenyl acetate**	**1277**	**1280**	**15.05**

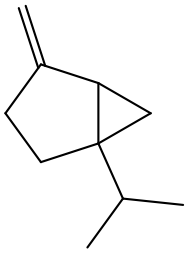
40	neoiso-3Thujanol acetate	1287	1281	0.09
41	Isobornyl acetate	1290	1283	0.21
42	Lavandulyl acetate	1293	1288	0.32
	Total		**95.64**	



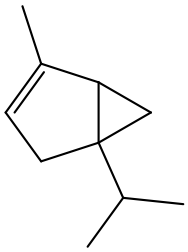
2,5-dimethyl-3-vinylhexa-1,4-diene
(Santolina triene)



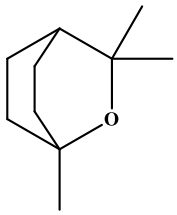
2,6,6-trimethylbicyclo[3.1.1]hept-2-ene
(Alpha-pinene)



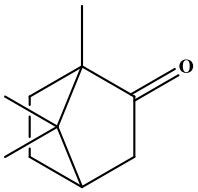
1-isopropyl-4-methylenebicyclo[3.1.0]hexane
(Sabinene)



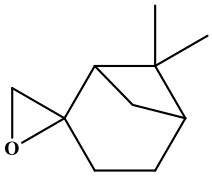
5-isopropyl-2-methylbicyclo[3.1.0]hex-2-ene
(Alpha-Thujene)



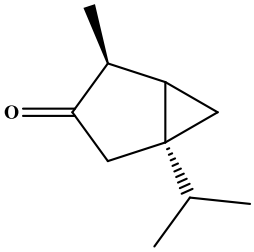
1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane
(1.8-cineole)



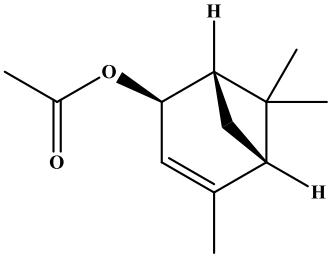
1,7,7-trimethylbicyclo[2.2.1]heptan-2-one
(Camphor)



6,6-dimethylspiro[bicyclo[3.1.1]heptane-2,2'-oxirane]
(Beta pinene oxide)



(1*S*,4*S*)-1-isopropyl-4-methylbicyclo[3.1.0]hexan-3-one
(Trans thujone)



(1*S*,2*R*,5*S*)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-yl acetate
(Cis verbenyl acetate)

Figure 17. Chemical structures and IUPAC names of the top major compounds identified in the essential oil of *Origanum Majorana L*

4. Assessment of Antibacterial Activity:

The *in vitro* assessment of the antimicrobial efficacy of *Cotula cinerea* and *Origanum Majorana* essential oils was conducted utilizing the disk diffusion technique against two prevalent bacterial strains associated with infections, Escherichia coli (E. coli), Bacillus subtilis (B. subtilis) and Staphylococcus aureus (S. aureus). Two distinct methodologies were employed to gauge the antibacterial activity: quantifying the zone of inhibition surrounding the EOs-impregnated disk and measuring the optical density of the bacterial suspension subsequent to treatment with varying concentrations of EOs. Comparative analysis was performed with amoxicillin, a widely utilized antibiotic. This study presents the findings derived from the diverse methodologies employed to evaluate the antibacterial efficacy of the studied essential oils and juxtaposes these outcomes with those obtained with amoxicillin.

5.1. Determination of the Diameter of the Inhibition Zone:

Following a 24-hour incubation period at 37°C, the zones of inhibition surrounding the disks impregnated with *Cotula cinerea* and *Origanum Majorana* essential oils and amoxicillin were measured. The diameters of the inhibition zones obtained with EOs and amoxicillin against E. coli, B. subtilis and S. aureus are summarized below (Table 10). The inhibition zones are identified by clear areas devoid of bacterial growth surrounding the disks.

Table 12. Diameters of the inhibition zones of the studied essential oils and amoxicillin against E. coli, B. subtilis and S. aureus

Antibiotics	Bacteria	Diameter of the inhibition zone (mm)
Cotula cinerea	E. coli	18.5
	B. subtilis	20.1
	S. aureus	10.9
Origanum Majorana L	E. coli	20.2
	B. subtilis	21.6
	S. aureus	13.8
Amoxicillin	E. coli	22.3
	B. subtilis	25.6
	S. aureus	24.6

The table provides a systematic examination of the antimicrobial efficacy of natural compounds, specifically *Cotula cinerea* and *Origanum Majorana L*essential oils, alongside the conventional antibiotic Amoxicillin, against three bacterial strains: Escherichia coli (E.

coli), Bacillus subtilis (B. subtilis), and Staphylococcus aureus (S. aureus). The diameter of the inhibition zones, measured in millimeters, serves as a quantitative indicator of antimicrobial activity (Cooper, 1963).

Analysis of the data reveals discernible trends in antimicrobial effectiveness across the tested antibiotics and bacterial strains. *Origanum Majorana L* and Amoxicillin consistently demonstrate larger inhibition zones compared to *Cotula cinerea*, indicating superior antimicrobial potency. Furthermore, B. subtilis exhibits the largest inhibition zones among the bacterial strains tested, suggesting varying susceptibility to the antibiotics among different bacterial species.

These findings underscore the importance of considering both the antimicrobial spectrum and potency of antibiotics when selecting appropriate treatment options for bacterial infections (Giurazza et al., 2021). Moreover, the observed efficacy of natural compounds like *Origanum Majorana L* highlights their potential as viable alternatives or adjuncts to conventional antibiotics.

5.2. Electronic Spectroscopy Interaction Study:

5.2.1. Bacterial inhibitory activities (IC_{50}):

The antibacterial activity of *Cotula cinerea* and *Origanum Majorana L* was assessed using the absorbance measurement method to determine the IC_{50}. This methodology entails measuring bacterial growth in wells containing varying concentrations of EOs. The resulting data facilitated the determination of the minimum concentration of EOs required to inhibit bacterial growth by 50%.

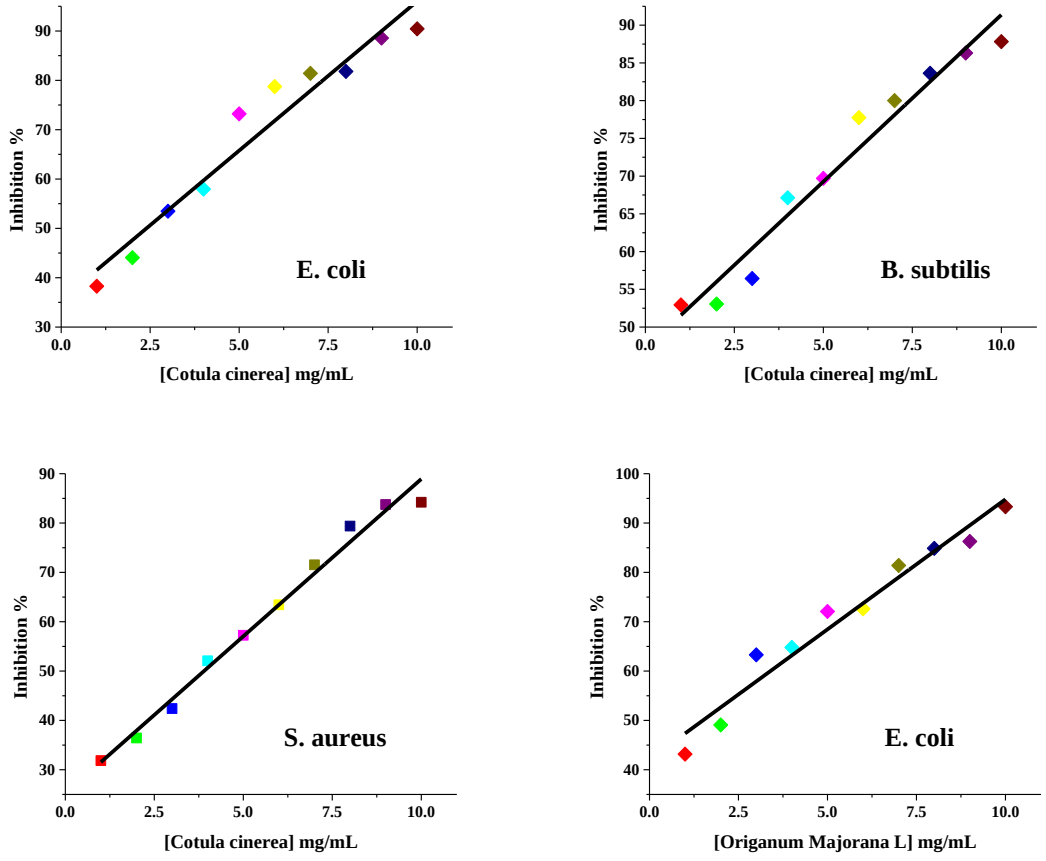
To this end, cultures of E. coli, B. subtilis and S. aureus were incubated with different concentrations of EOs and amoxicillin, ranging from 1 to 10 mg/ml, for 24 hours at 37°C. Subsequently, the optical density of each sample was measured at 620 nm using a spectrophotometer. The findings obtained are summarized in the following Table 7.

Table 13. Absorbance values sorted from the antibacterial assays

C (mg/ml)	*Cotula cinerea*			*Origanum Majorana L*			Amoxicillin		
	E. coli	*B. subtilis*	*S. aureus*	*E. coli*	*B. subtilis*	*S. aureus*	*E. coli*	*B. subtilis*	*S. aureus*
0	2.571	2.571	2.571	2.571	2.571	2.571	2.117	2.209	1.954
1	1.587	1.21	1.752	1.461	1.089	1.576	1.718	2.191	1.816
2	1.438	1.207	1.634	1.309	0.965	1.42	1.658	2.183	1.626
3	1.196	1.12	1.481	0.944	0.822	1.2	1.103	2.176	1.389

4	1.081	0.845	1.232	0.905	0.788	0.97	0.828	2.169	1.344
5	0.689	0.779	1.099	0.718	0.673	0.843	0.677	2.153	0.868
6	0.547	0.572	0.94	0.704	0.55	0.652	0.638	2.144	0.71
7	0.478	0.514	0.732	0.478	0.5	0.526	0.395	2.133	0.697
8	0.468	0.421	0.53	0.389	0.398	0.419	0.198	2.103	0.587
9	0.294	0.352	0.418	0.353	0.317	0.317	0.127	2.098	0.477
10	0.246	0.313	0.406	0.172	0.222	0.268	2.117	2.209	1.954

This table presents the outcomes of the investigation into the antibacterial efficacy of *Cotula cinerea* and *Origanum Majorana L* essential oils and amoxicillin against E. coli, B. subtilis and S. aureus, employing the absorbance measurement technique. The results depict a gradual decline in optical densities alongside escalating concentrations of EOs or amoxicillin, indicative of diminished bacterial proliferation. Subsequently, IC_{50} values, delineating the concentration of EOs or amoxicillin necessary to impede 50% of bacterial growth, were derived from these observations by plotting the inhibition percentage against the compound concentrations (refer to Figure 7). The resultant of IC_{50} values is meticulously documented in Table 8.



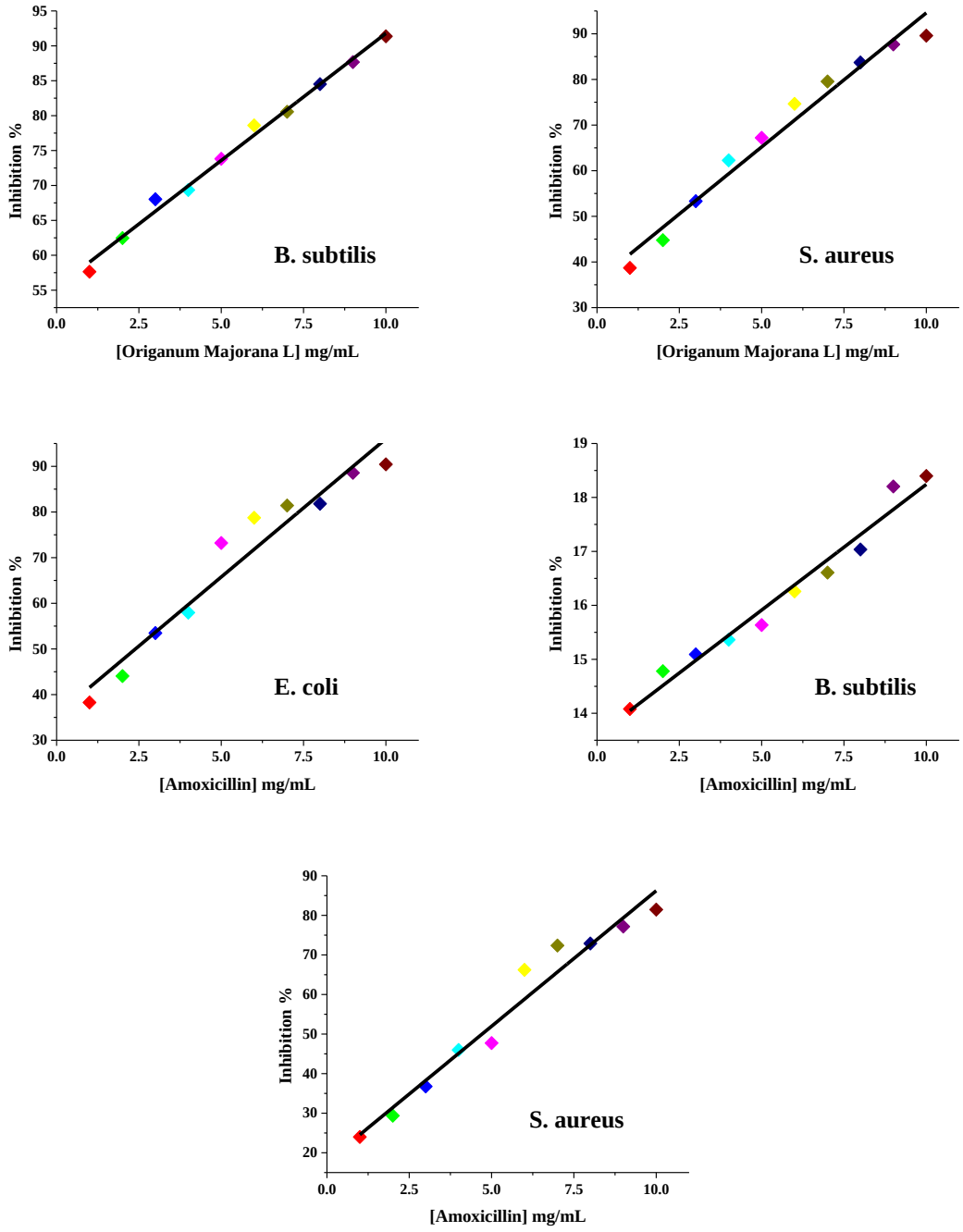


Figure 18. The linear regression curves depicting the percentage inhibition of bacteria versus the concentration of *Cotula cinerea* and *Origanum Majorana L* essential oils and Amoxicillin

Table 14. The antibacterial activity of *Cotula cinerea* and *Origanum Majorana L* essential oils and amoxicillin through the inhibition test against E. coli, B. subtilis and S. aureus

Cotula cinerea			***Origanum Majorana***			**Amoxicillin**		
E. coli	*B. subtilis*	*S. aureus*	*E. coli*	*B. subtilis*	*S. aureus*	*E. coli*	*B. subtilis*	*S. aureus*
38.273	52.937	31.855	43.174	57.643	38.701	17.658	14.080	23.998
44.068	53.053	36.445	49.086	62.466	44.769	33.178	14.780	29.366

53.481	56.437	42.396	63.283	68.028	53.326	35.511	15.091	36.756
57.954	67.133	52.081	64.800	69.350	62.271	57.098	15.364	45.974
73.201	69.701	57.254	72.073	73.823	67.211	67.795	15.636	47.725
78.724	77.752	63.438	72.618	78.608	74.640	73.668	16.258	66.239
81.408	80.008	71.529	81.408	80.552	79.541	75.185	16.608	72.384
81.797	83.625	79.385	84.870	84.520	83.703	84.636	17.036	72.890
88.565	86.309	83.742	86.270	87.670	87.670	92.299	18.203	77.168
90.432	87.826	84.208	93.310	91.365	89.576	95.060	18.398	81.447
IC_{50} = 2.935 mg/ml	IC_{50} = 0.641 mg/ml	IC_{50} = 3.895 mg/ml	IC_{50} = 1.500 mg/ml	IC_{50} = 1.477 mg/ml	IC_{50} = 2.412 mg/ml	IC_{50} = 3.961 mg/ml	IC_{50} = 8.154 mg/ml	IC_{50} = 4.712 mg/ml

The findings reveal the growth inhibitions of E. coli, B. subtilis, and S. aureus bacteria in the presence of *Cotula cinerea* and *Origanum Majorana L* essential oils, alongside amoxicillin. The results demonstrate a notably potent efficacy of *Cotula cinerea* and *Origanum Majorana L* compared to amoxicillin, with IC_{50} values of 3.961 mg/ml, 8.154 mg/ml, and 4.712 mg/ml, respectively.

Concerning *Cotula cinerea*, the outcomes indicate bacterial growth inhibition of up to 90% for E. coli, 87% for B. subtilis, and 84% for S. aureus at the highest tested concentration (10 mg/ml). This suggests that *Cotula cinerea* may possess antimicrobial activity, albeit at relatively elevated concentrations.

In contrast, *Origanum Majorana L* results in bacterial growth inhibition of up to 93% for E. coli, 81% for B. subtilis, and 89% for S. aureus at the highest tested concentration (10 mg/ml). This underscores the superior efficacy of this essential oil as an antibiotic against bacterial infections.

In conclusion, the findings of this study highlight the notable antibacterial effects exhibited by the two essential oils under investigation, *Cotula cinerea* and *Origanum Majorana L*, surpassing those of amoxicillin. This suggests their potential as promising alternatives or adjuncts in combating bacterial infections.

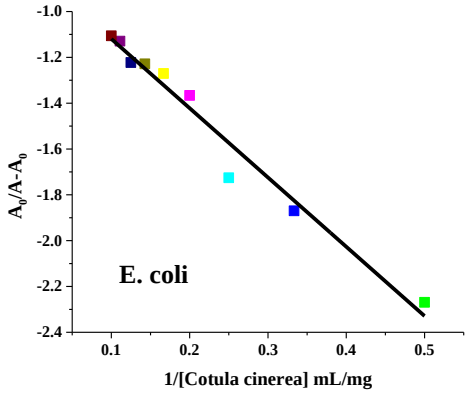
5.2.2. Binding constants:

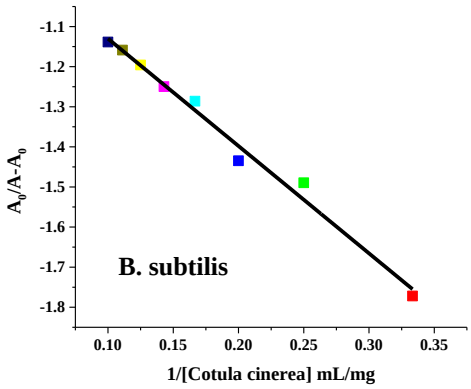
The spectrophotometric absorption method was employed to assess the binding constant (K_b) of *Cotula cinerea* and *Origanum Majorana L* essential oils with the three types of bacteria under investigation. This constant was expressed in M^{-1}. Decreasing concentrations of EOs and amoxicillin resulted in a gradual decrease in the absorption of the solution containing the bacteria. By plotting $A_0/(A-A_0)$ against 1/C (Figure 8) (Tyagi et al., 2015), the slope was calculated as $\varepsilon/(\varepsilon_0-\varepsilon).Kb$, where ε represents the absorbance of the solution containing the compound, and ε_0 denotes the absorbance of the control solution. The "y"

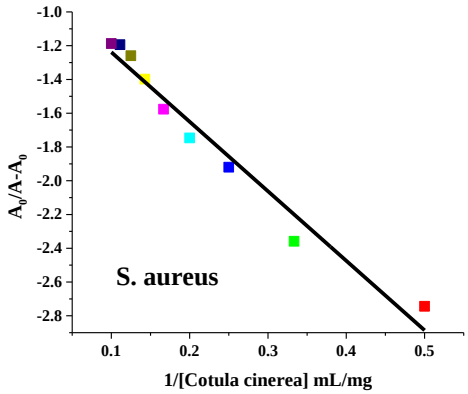
intercept was also calculated as $\varepsilon/(\varepsilon_0-\varepsilon)$. Subsequently, the binding constant was determined by dividing the slope by the intercept (K_b = slope/intercept).

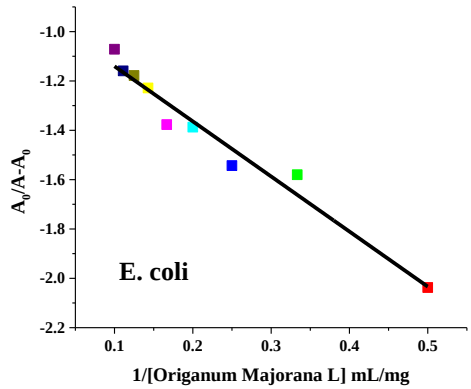
5.2.3. Binding free energy:

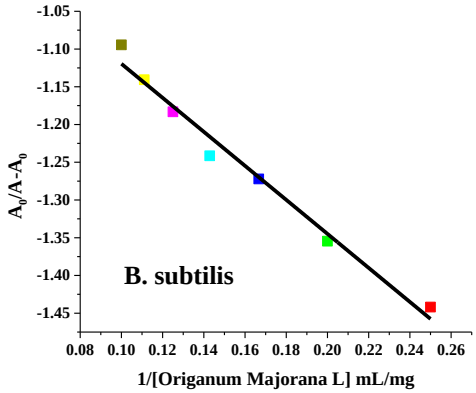
Utilizing Equation 10, the variation in binding free energy was calculated. Negative values of ΔG signify the spontaneity of the interaction between the specified bacteria and the studied compounds, with their magnitude indicative of a robust binding affinity between the protein and the ligands. The binding constants alongside their corresponding binding free energies are succinctly presented in Table 13.

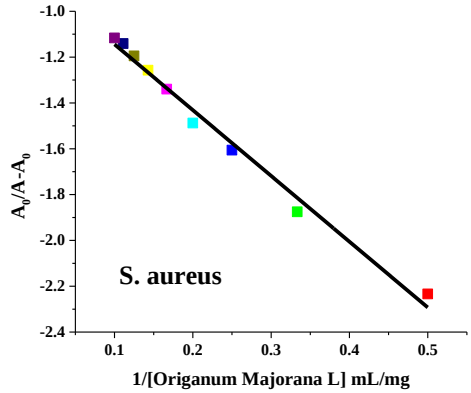












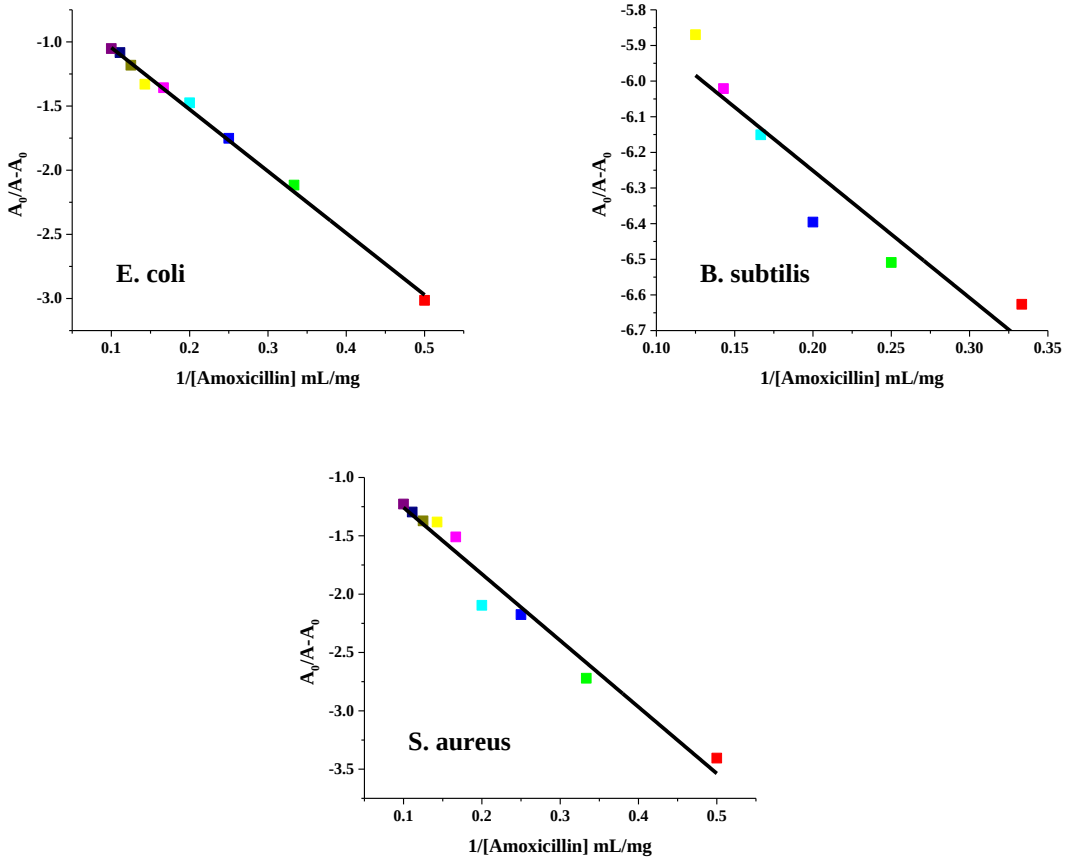


Figure 19. Plots of $A_0/(A - A_0)$ against 1/[*Cotula cinerea*], 1/[*Origanum Majorana L*] and 1/[Amoxicillin] were constructed to calculate the binding constants

Table 15. Binding constants and binding free energies of the studied compounds with bacterial strains

Compounds	Bacteria's	Equations	R^2	K (M^{-1})	-ΔG ($KJ.mol^{-1}$)
Cotula cinerea	E. coli	y = -3.024x – 0.817	0.968	2.70×10^5	31.01
	B. subtilis	y = -2.677x – 0.862	0.985	3.22×10^5	31.45
	S. aureus	y = -4.118x – 0.827	0.962	2.00×10^5	30.27
Origanum Majorana L	E. coli	y = -2.232x – 0.917	0.960	4.11×10^5	32.05
	B. subtilis	y = -2.254x – 0.894	0.977	3.97×10^5	31.96
	S. aureus	y = -2.871x –	0.986	2.98×10^5	31.26

		0.857		
Amoxicillin	E. coli	y = -4.817x – 0.563	0.995	1.17×10^5	28.93
	B. subtilis	y = -3.566x – 5.538	0.853	1.55×10^5	35.35
	S. aureus	y = -5.697x – 0.688	0.964	1.21×10^5	29.01

The table provides a comprehensive overview of the binding interactions between various compounds, including *Cotula cinerea*, *Origanum Majorana L*, and amoxicillin, with different bacterial strains, namely E. coli, B. subtilis, and S. aureus. Each entry includes the equation of the linear regression line obtained from the plots of $A/(A_0 - A)$ against 1/[EOs], the coefficient of determination (R^2) indicating the goodness of fit of the regression model, the binding constant (K) expressed in M^{-1}, and the corresponding negative change in Gibbs free energy ($-\Delta G$) in kJ/mol (Ozer, 1985).

The high values of R^2 across all entries indicate a strong correlation between the experimental data and the fitted regression lines, suggesting the reliability of the models in describing the binding behavior (Plonsky & Ghanbar, 2018). Additionally, the binding constants (K) provide quantitative measures of the strength of interaction between the compounds and the bacterial strains, with higher values indicating stronger binding affinities (Lanez et al., 2018).

The negative values of ΔG underscore the spontaneity of the binding interactions, with larger magnitudes indicating more favorable and energetically stable associations between the compounds and the bacteria (Kedadra et al., 2022). Notably, Amoxicillin exhibits relatively lower binding constants and ΔG values compared to the essential oils, suggesting potentially weaker interactions with the bacterial strains studied.

In the context of this study, higher values of K indicate stronger binding affinities between the compounds and the bacterial strains (Adaika et al., 2019). *Cotula cinerea* and *Origanum Majorana L* essential oils exhibit notably higher K values compared to amoxicillin across all bacterial strains tested. This suggests that the essential oils have a greater propensity to bind to the bacterial receptors, potentially leading to enhanced antimicrobial efficacy. Conversely, amoxicillin demonstrates relatively lower K values, indicating comparatively weaker binding interactions with the bacterial strains. This observation aligns with the known mechanism of action of amoxicillin, which primarily targets specific bacterial cell wall components rather than interacting directly with bacterial receptors.

5.3. Molecular Docking Interaction Study:

In order to validate the experimentally measured antibacterial activity against E. coli, B. subtilis, and S. aureus, an in-silico molecular docking study was conducted on the proteins of the respective bacteria. The objective of this study was to predict the molecular interactions between the compound of interest and the studied bacteria, and to assess their antibacterial potential using Maestro version 11.7 user interface of the Schrödinger suite (Small-Molecule Drug Discovery Suite 2021-4, Schrödinger, LLC, New York, NY, 2021) (Schrödinger, 2015). Additionally, a comparison of the results obtained with amoxicillin, a commonly used antibiotic, was performed. The results obtained after the docking assays are presented in the Table 14.

Table 16. Rigid and induced fit docking scores of the studied compounds against E. coli, B. subtilis, and S. aureus

Compound	E. coli		B. subtilis		S. aureus	
	Rigid Docking Score (Kcal/mol)	IFD scores (Kcal/mol)	Rigid Docking Score (Kcal/mol)	IFD scores (Kcal/mol)	Rigid Docking Score (Kcal/mol)	IFD scores (Kcal/mol)
trans thujone	-7.728	-8.228	-6.894	-7.394	-5.828	-7.128
alpha-Terpineol	-7.694	-8.194	-9.551	-10.051	-5.794	-7.094
beta-Terpineol	-6.894	-7.394	-8.969	-9.469	-4.994	-6.294
cis Verbenyl acetate	-9.551	-10.051	-8.569	-9.069	-7.651	-8.951
Camphor	-8.969	-9.469	-8.062	-8.562	-7.069	-8.369
Sabinene	-8.569	-9.069	-7.536	-8.036	-6.669	-7.969
gamma terpinene	-8.062	-8.562	-8.062	-8.562	-6.162	-7.462
alpha-Pinene	-7.536	-8.036	-5.636	-6.936	-5.636	-6.936
1,8-Cineole	-6.994	-7.494	-5.094	-6.394	-5.094	-6.394
Santolina triene	-6.716	-7.216	-4.816	-6.116	-4.816	-6.116
alpha-Thujene	-6.616	-7.116	-4.516	-6.036	-4.516	-6.011
Beta Pinene oxide	-6.601	-7.101	-4.511	-6.022	-4.510	-6.009

The table presents the results of rigid docking scores and induced fit docking (IFD) scores for various compounds against three bacterial strains: Escherichia coli, Bacillus subtilis, and

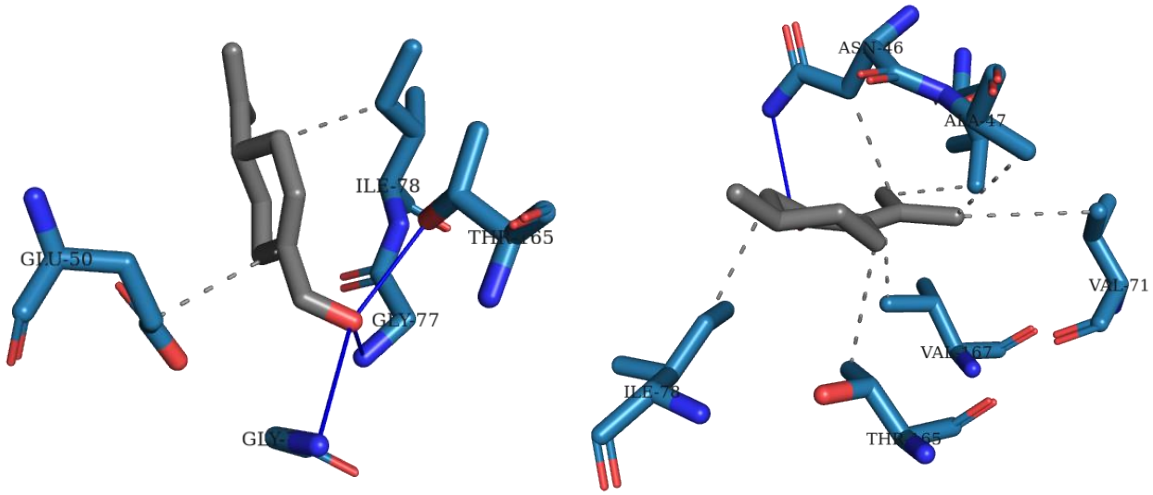
Staphylococcus aureus. These scores provide insights into the potential antibacterial activity of each compound by indicating the strength of interaction with the target proteins or receptors within the bacteria.

Observing the rigid docking scores, it is evident that compounds such as Pulegone and D-Limonene exhibit the most favorable scores across all three bacterial strains, suggesting strong binding affinities towards their respective targets. Conversely, Linalool acetate and 1,8-Cineole demonstrate comparatively weaker rigid docking scores, indicating potentially weaker interactions with the bacterial proteins.

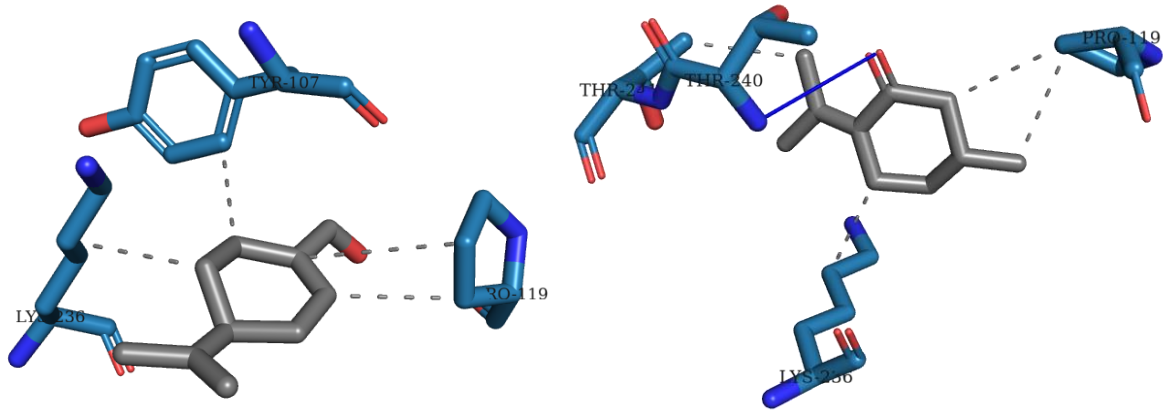
The induced fit docking (IFD) scores further validate the binding affinities of the compounds by considering the flexibility of both the ligands and the target proteins during the docking process. Notably, compounds like Pulegone and D-Limonene maintain their favorable IFD scores, affirming their potential as promising antibacterial agents.

Comparing the results obtained for each compound across the three bacterial strains, variations in docking scores are observed, suggesting potential differences in the binding interactions with the specific target proteins or receptors present in each strain. This highlights the importance of considering the target specificity of antibacterial agents in rational drug design.

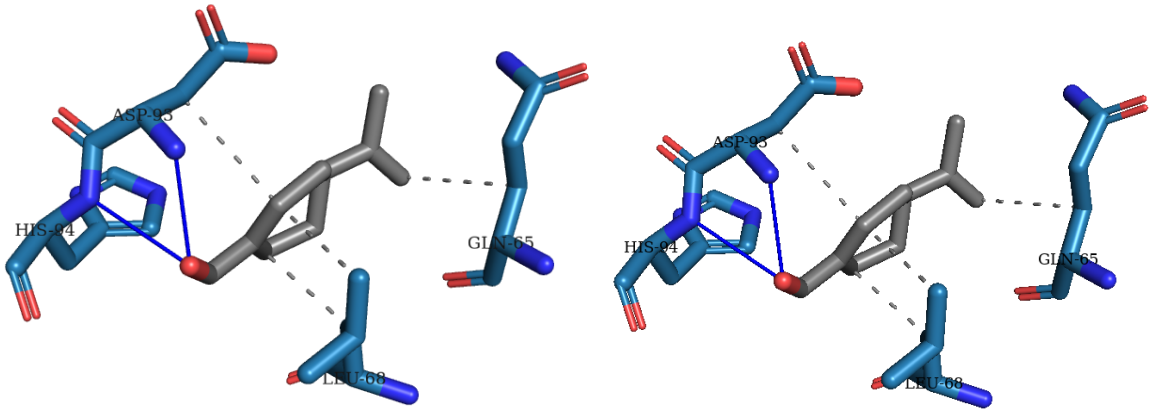
The photographs depicting the interaction of the best compound in each essential oil with the studied bacteria are presented in Figures below:



Docking complex and interaction plot for compound trans thujone and Pulegone with E. coli



Docking complex and interaction plot for compound trans thujone and Pulegone with B. subtilis



Docking complex and interaction plot for compound trans thujone and Pulegone with S. aureus

6. ADMET and drug-likeness prediction:

Modern drug discovery involves assessment of competence of the dynamic molecules and their strength to reach target site in bioactive form, which involves cellular, animal and human clinical trials which are highly priced and encumbered with risks (Ranjith & Ravikumar, 2019; Ranjith D & Viswanath S, 2019). Presently computer aided drug development encouraged the estimate of absorption, distribution, metabolism and excretion of drugs (ADME), they postulate anticipatory and dependable data very quickly and compliment for experimental approaches (Ranjith & Ravikumar, 2019; Sliwoski et al., 2014). It has been determined that the initial appraisal of ADME properties in the discovery period diminishes remarkably the fraction of pharmacokinetics related failures in the clinical phase (Hay et al., 2014; Ranjith & Ravikumar, 2019).

In the present study we evaluated the ADME properties of the major compounds (more than 10%) present in both essential oils using SwissADME web tool. A total of 4 potent phytoconstituents were analysed to study general characteristics (Table 11), Physicochemical properties (Table 12), lipophilicity and water solubility characteristics (Table 13 & 14), pharmacokinetic parameters (Table 15), drug likeness rule and bioavailability score (Table 16) and medicinal chemistry properties (Table 17), respectively. General characteristics of the studied compounds revealed all the compounds having molecular weight less than 500 Da, which is a good prime property to be called as drug likeness of the small molecules.

Table 17. General characteristics of the phytoconstituents of essential oils

SI. No	Compounds	Molecular formula	Canonical SMILES	Molecular weight (g/mol)
1	Santolina triene	$C_{10}H_{16}$	CC(=CC(C=C)C(=C)C)C	136.23
2	trans-Thujone	$C_{10}H_{16}O$	CC1C2CC2(CC1=O)C(C)C	152.53
3	1,8-Cineole	$C_{10}H_{18}O$	CC1(C2CCC(O1)(CC2)C)C	154.25
4	cis-Verbenyl acetate	$C_{12}H_{18}O_2$	CC1=CC(C2CC1C2(C)C)OC(=O)C	194.27

Table 18. Physicochemical properties of the phytoconstituents of essential oils

Properties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate
Num. heavy atoms	10	11	11	14
Num. arom. heavy atoms	0	0	0	0
Fraction Csp3	0.40	0.90	1.00	0.75
Num. rotatable bonds	3	1	0	2
Num. H-bond acceptors	0	1	1	2
Num. H-bond donors	0	0	0	0
Molar refractivity	48.76	45.90	47.12	56.12
TPSA (Å²)	0.00	17.07	9.23	26.30

Table 19. Lipophilicity characteristics of the phytoconstituents of essential oils

Properties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate
iLOGP	2.90	2.27	2.58	2.53
XLOGP3	4.22	2.27	2.74	3.73
WLOGP	3.33	2.26	2.74	2.54
MLOGP	3.56	2.30	2.45	2.65
SILICOS-IT	2.88	2.63	2.86	2.26
Consensus Log Po/w	3.38	2.35	2.67	2.74

Table 20. Water Solubility characteristics of the phytoconstituents of essential oils

Small molecules	ESOL				Ali				SILICOS-IT			
	Log S (ESOL)	Solubility		Class	Log S (Ali)	Solubility		Class	Log S (SILICOS-IT)	Solubility		Class
		mg/ml	mol/L			mg/ml	mol/L			mg/ml	mol/L	
Santolina triene	-3.15	9.75e-2	7.16e-4	S	-3.93	1.60e-2	1.17e-4	S	-2.04	1.24e-0	9.10e-3	S
trans-Thujone	-2.15	1.08e-0	7.11e-3	S	-2.27	8.27e-1	5.43e-3	S	-2.15	1.08e-0	7.10e-3	S
1,8-Cineole	-2.52	4.63e-1	3.00e-3	S	-2.59	3.98e-1	2.58e-3	S	-2.45	5.45e-1	3.53e-3	S
cis-Verbenyl acetate	-3.26	1.06e-1	5.47e-4	S	-3.97	2.06e-2	1.06e-4	S	-2.11	1.51e-0	7.78e-3	S

Table 21. Pharmacokinetics parameters of the phytoconstituents of essential oils

Proprieties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate
GI absorption	Low	High	High	Low
BBB permeant	Yes	Yes	Yes	Yes
P-gp substrate	No	No	No	No
CYP1A2 inhibitor	No	No	No	No
CYP2C19 inhibitor	No	No	No	No
CYP2C9 inhibitor	No	No	No	Yes
CYP2D6 inhibitor	No	No	No	No
CYP3A4 inhibitor	No	No	No	No

Log Kp (Skin Permeation) (cm/s)	-4.13	-5.62	-5.30	-4.84

Table 22. Druglikeness rule and bioavailability score of the phytoconstituents of essential oils

Proprieties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate
Lipinski	Yes; 0 violations	Yes; 0 violations	Yes; 0 violations	Yes; 0 violations
Ghose	No; 1 violation: MW<160	No; 1 violation: MW<160	No; 1 violation: MW<160	Yes
Veber	Yes	Yes	Yes	Yes
Egan	Yes	Yes	Yes	Yes
Muegge	No; 2 violations: MW<200, XLOGP3>3.5	No; 2 violations: MW<200, Heteroatoms<2	No; 2 violations: MW<200, Heteroatoms<2	No; 1 violation: MW<200,
Bioavailability score	0.55	0.55	0.55	0.55

Table 23. Medicinal Chemistry properties of the Phytoconstituents of essential oils

Proprieties	Santolina triene	trans-Thujone	1,8-Cineole	cis-Verbenyl acetate
PAINS	0 alert	0 alert	0 alert	0 alert
Brenk	1 alert: isolated_alkene	0 alert	0 alert	1 alert: isolated_alkene
Leadlikeness	No; 2 violations: MW<250, XLOGP3>3.5	No; 1 violation: MW<250	No; 1 violation: MW<250	No; 2 violations: MW<250, XLOGP3>3.5
Synthetic accessibility	3.22	2.79	3.65	4.50

Lipophilicity property of the compounds portrays an important role for molecular discovery activities in multifarious domains. The quantitative descriptor of the lipophilicity is the partition coefficient P of a given molecule between *n*-octanol and water system (Daina et al., 2014). Because of its amphiphilic nature, *n*-octanol is considered a good mimic of phospholipid membrane characteristics (Liu et al., 2011). Multifarious algorithms are accessible to compute log Po/w, which rely on factual methodologies. The classic log P predictors branched in to two division, first ones split molecular structures into molecular fragments includes fragmental approach e.g. KLOGP (Klopman et al., 1993), KOWWNIN (Meylan & Howard, 2000) or atomic approach e.g. ALOGP (Ghose et al., 1998; R. Wang et al., 1997), XLOGP (Cheng et al., 2007; Moriguchi et al., 1994). The second division gathers the topological methods in which, the molecules description is related to its topology being as count or flags for specific atoms, groups or structural properties e.g. MLOGP (Brenk et al., 2008; Moriguchi et al., 1992), the prediction attained by manifold linear regression trained on large molecular data sets. The SILICOS-IT is a hybrid technique which combines both molecular fragments and topological parameters, which confide on 27 fragments and 7 topological descriptors, it was disciplined on 23,455 molecules with experimental *n*-octanol/water partition values (Daina et al., 2014). The version three of the XLOGP atomic model is established on a system of 87 fragments and two corrective factors. If the input structures are similar to a reference compound, the fragments differentiating them are treated and the corresponding log P contributions added to the reference structure log P value (Cheng et al., 2007). Lipophilicity estimated as consensus Log P, which is the average value of all Log P evaluated with various lipophilicity criteria, determined trans-Thujone as most lipophilic whereas santolina triene as least lipophilic and water solubility of the small molecules ranged from highly water soluble to water soluble.

The SwissADME model returns “Yes” or “No” if the compound under examination has greater probability to be a substrate or non-substrate of P-gp or inhibitor or non-inhibitor of Cytochrome P450 isoenzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4).

The pharmacokinetics and drug likeness performed using SwissADME showed a low level of GI absorption and BBB permeant with santolina triene and cis-Verbenyl acetate while a high absorption detected with trans-Thujone and 1,8-Cineole. All the compounds present in the essential oils are not the substrates for P-gp except contrarily to the trans-Thujone (Table 12), so they are not susceptible to the efflux mechanism performed by this transporter which is used by many tumours cell lines as a drug-resistance mechanism (Ranjith & Ravikumar, 2019)

All of the small molecules returned as non-inhibitors for inactivation for CYP isoenzymes. The skin permeability coefficient (Log Kp), a multiple linear regression, the more negative the log Kp (with Kp in cm/s), the less skin permeant is the molecule. Among the phytoconstituents, trans-Thujone (-5.62) is the least permeant and santolina triene (-4.13) is highly permeant respectively. This SwissADME section gives access to five different rule-based filters, with diverse ranges of properties inside of which the molecule is defined as drug-like. The Lipinski (Pfizer) filter is the pioneer rule-of-five implemented and with the Ghose (Amgen), Veber (GSK), Egan (Pharmacia) and Muegge (Bayer) methods. Multiple estimations allow consensus views or selection of methods best fitting the end-user's specific needs in terms of chemical space or project-related demands. Any violation of any rule described here appears explicitly in the output panel. All the four compounds followed the filtered rule invoked in the SwissADME; the violation shown by the molecules are minimal.

SwissADME interpretation posts 0 PAINS alert of the 4 studied compounds. Brenk considered compounds that are smaller and less hydrophobic and not those defined by "Lipinski's rule of 5" to widen opportunities for lead optimization. This was after exclusion of compounds with potentially mutagenic, reactive and unfavourable groups such as nitro groups, sulphates, phosphates, 2-halopyridines and thiols (Brenk et al., 2008). All the compounds examined flouted Brenk's rule with only one alert, all the compounds failed Lead-likeness criteria due to their molar weight.

In silico toxicity study aims to help in optimizing compounds regarding their toxicity proprieties. The study could offer an important improvement to the awareness of the full perspective of virtual screening for the identification of target compounds with negligible or no toxicity, which may open a path for the selection of novel nontoxic phytoconstituents present in *Cotula cinerea* and *Origanum Majorana L* essential oils with high antidiabetic activity. In silico toxicity study of the chosen compounds was performed using the ProTox-II web server (Drwal et al., 2014). It aims to predict hepatotoxicity (Dili), carcinogenicity (Carcino), immunotoxicity (Immuno), mutagenicity (Mutagen), cytotoxicity (Cyto), median lethal dose (LD50), and toxicity class (TC).

According to in silico toxicity profiles presented in Table 19, the toxicity class of all the phytoconstituents was detected to be equal to 5 except the trans-Thujone which predicted to be 4. Santolina triene, trans-Thujone**,** 1,8-Cineole and cis-Verbenyl acetate were predicted to be nontoxic except in immunotoxicity for the last wrote compound.

Table 24. In silico toxicity profiles of the studied compounds

Molecule	Dili	Carcino	Immuno	Mutagen	Cyto	LD_{50} ($mg.Kg^{-1}$)	TC
Santolina triene	Inactive	Active	Inactive	Inactive	Inactive	2610	5
trans-Thujone	Inactive	Inactive	Inactive	Inactive	Inactive	500	4
1,8-Cineole	Inactive	Inactive	Inactive	Inactive	Inactive	2480	5
cis-Verbenyl acetate	Inactive	Inactive	Active	Inactive	Inactive	2600	5

7. Molecular Dynamics Simulation:

Molecular Dynamics Simulation (MDS) investigations were conducted for the premier compound show the best IFD score, trans thujone, in complex with E. coli. The principal objective of these MDS endeavours was to subject the ligand-receptor complex to physiological conditions, a feat unattainable through the confines of molecular docking (Dumitrică & James, 2007). Throughout the MDS protocol, a trajectory frame was generated at intervals of 100 picoseconds (Tu et al., 2008), accumulating a total of 1000 frames over the course of a 100-nanosecond simulation. Analysis encompassed metrics such as 'Root Mean Square Deviation (RMSD), and 'Root Mean Square Fluctuation (RMSF). Specifically, RMSD values were computed by aligning the frames of the protein and ligand-protein complex with the reference frame, respectively. These analytical approaches provide a detailed understanding of the structural stability and dynamic fluctuations exhibited by the trans thujone _E. coli complex throughout the simulation period (Schreiner et al., 2012).

The Root Mean Square Deviation (RMSD) values for the complex consistently fall within the acceptable range of 0 – 2 Å. Examination of the analysed complex revealed no significant conformational alterations; however, an initial minor deviation was discerned in the vicinity of 8 – 20 nanoseconds, and a subtle drift was observed approximately between 45 – 55 nanoseconds. Beyond these periods, the complex demonstrated marked stability throughout the entirety of the simulation process, with no notable occurrence of major conformational changes. (Figure 9).

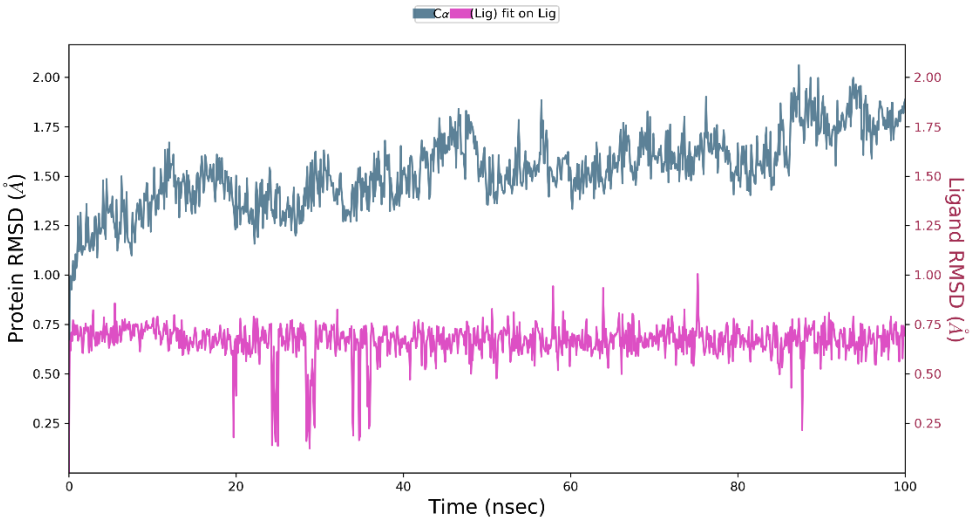


Figure 20. RMSD plot of trans thujone and E. coli complex

The Root Mean Square Fluctuation (RMSF) analysis provides insights into local variations along the protein chain, facilitating the identification of residues contributing to structural fluctuations within the complex (Dey et al., 2021). In the examined complex, the observed fluctuation variation remained below 3 Å. Major fluctuations (2.20-2.63 and 1.81-2.96Å) were observed in the C-terminal and loop regions (223-225 and 363-366), respectively, which are positioned away from the binding pocket of alpha amylase. Except for the loop regions, the RMSF values of most residues are less than 1.5 Å, indicating that the residue conformation is relatively stable during the simulation. A graphical representation of the RMSF plot depicting the specific ligand interactions with amino acid residues in the protein is presented in Figure 10.

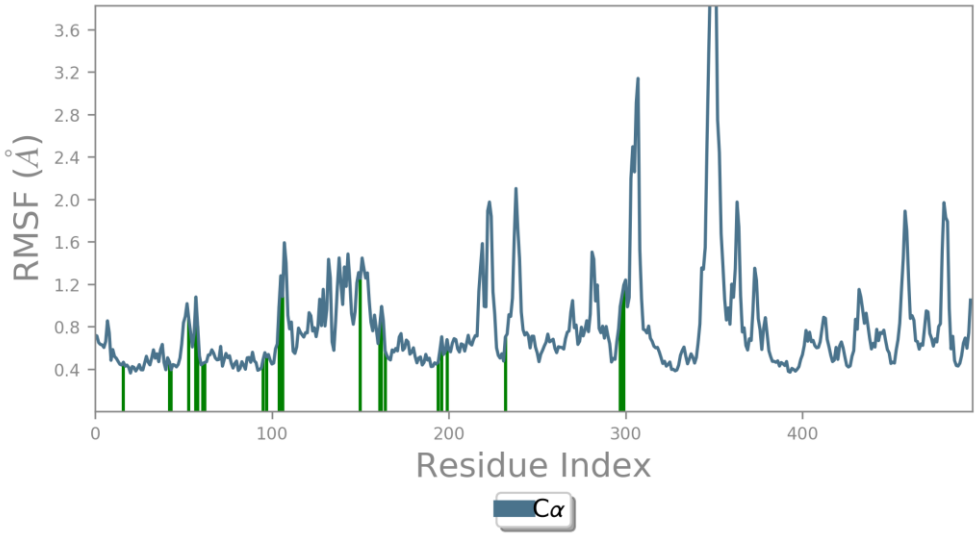


Figure 21. RMSF plot of trans thujone and E. coli complex

Within the investigated complex, notable hydrogen bond interactions were observed involving HIS299, ASP197, and TRP58. Additionally, water-mediated hydrogen bond interactions were formed, encompassing the same residues, as visually depicted in Figure 11.

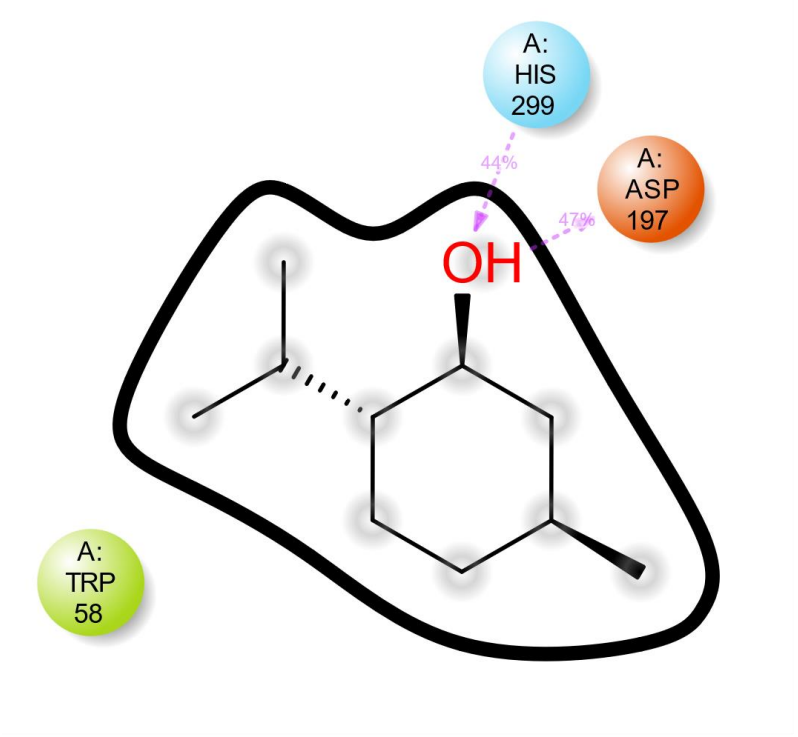


Figure 22. Interaction diagram of protein-ligand after MDS

The analysis of Protein-ligand contact serves to elucidate the temporal continuity of interactions between the ligand and protein amino acids throughout the simulation. A numerical representation is employed, where a value of 0.5 denotes that a specific interaction was sustained for 50% of the simulation duration. Conversely, a value exceeding 1 suggests that protein amino acids may establish multiple contacts of the same subtype with the ligand (Hatmal & Taha, 2017).

In the context of the investigated complex, the interaction values for the residues GLU240, HIS201, ASP197, GLN63, GLU233, and THR163 are recorded as 1.25, 1, 1.75, 1, 0.60, and 1.5, respectively. These values signify varying degrees of sustained interactions between the ligand and corresponding amino acid residues. The graphical representation of the protein-ligand contact histograms for the complex is depicted in Figure 12 for visual elucidation.

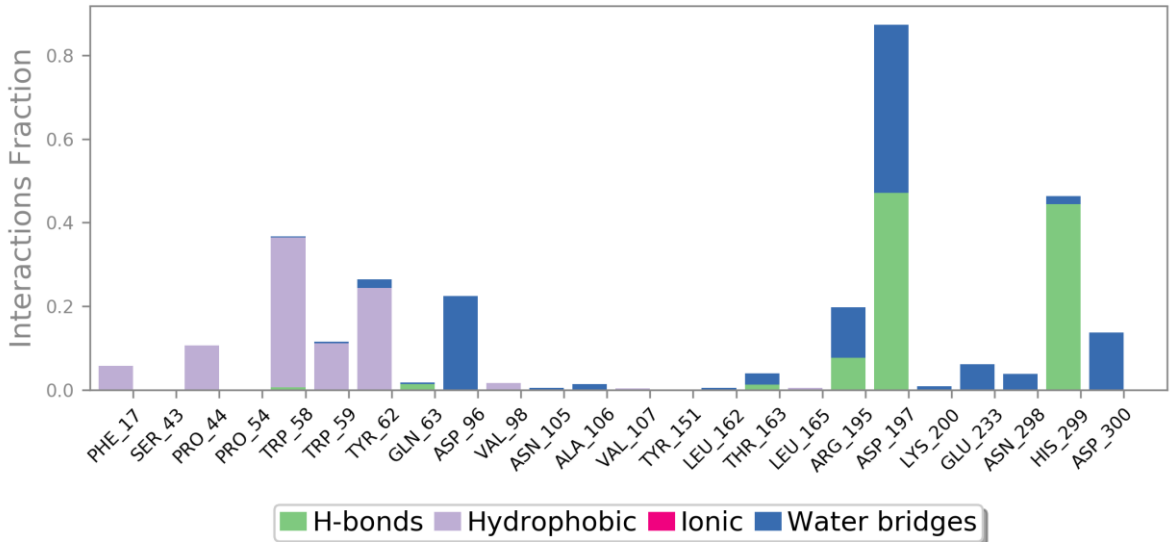


Figure 23. Histogram of protein-ligand complex

To comprehensively investigate the interactions between the ligand and alpha-amylase receptor, two supplementary panels were introduced by Schrödinger post Molecular Dynamics Simulation (MDS), as depicted in Figure 13. The upper panel provides insights into the total number of specific contacts established by the protein with the ligand throughout the trajectory. Conversely, the lower panel delineates the residues engaged in interactions with the ligand in each trajectory frame.

Notably, certain residues exhibit multiple specific contacts with the ligand, visualized through varying shades of orange on the plot, in accordance with the scale positioned to the right of the diagram. This nuanced representation captures the dynamic nature of the interactions, offering a detailed portrayal of the residues that consistently contribute to the ligand-receptor interface. The incorporation of these supplementary panels enhances the granularity of our understanding of the molecular dynamics and specific contacts governing the ligand-receptor interaction over the course of the simulation.

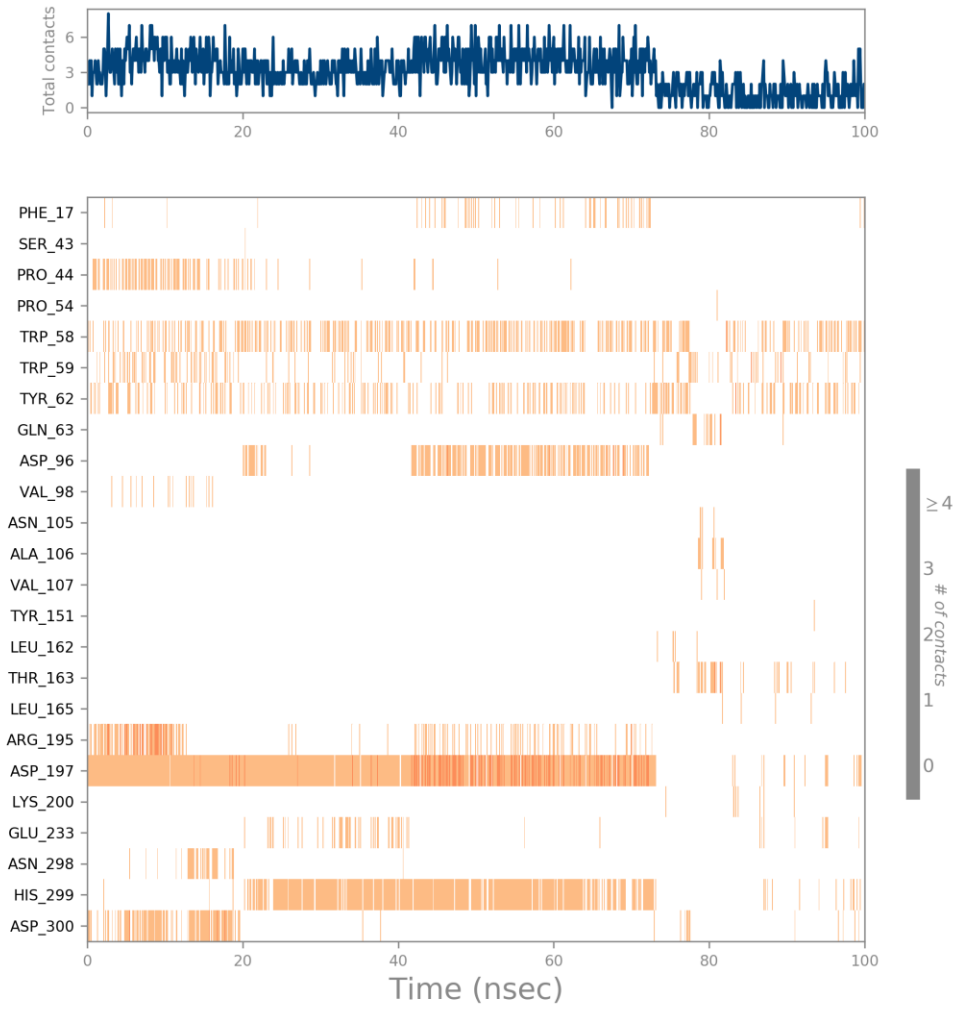


Figure 24. Details of the protein ligand contact

7.1. Free Energy (MM-GBSA) Calculation:

To evaluate the binding efficacy of the preeminent compound, trans thujone, with the E. coli protein, the Prime-MM-GBSA method was applied to compute the binding free energies. The establishment of stable complexes is evidenced by favourable binding interactions between the compound and the protein. The determination of binding free energy entails the consideration of various energy components, encompassing van der Waals (vdW), lipophilic (lipo), Generalized Born electrostatic solvation (Solv GB), Coulomb, and hydrogen-bonding (hbond) energies. The discrete contributions of these energy components to the overall binding free energy of the Pulegone and E. coli complex are delineated in Table 19.

Table 25. Energy components of the studied complex

Component	Energy (kcal/mol)
ΔG Bind	-61.14
ΔG Bind Coulomb	-30.03
ΔG Bind Solv GB	48.10
ΔG Bind vdW	-54.91
ΔG Bind lipo	-20.51
ΔG Bind hbond	-3.79

Notably, ΔG Bind is calculated at -61.14 kcal/mol, indicating a robust favourable binding interaction. Individual positive contributions include Coulombic energy at -30.03 kcal/mol, van der Waals interactions at -54.91 kcal/mol, lipophilic energy at -20.51 kcal/mol, and hydrogen-bonding energy at -3.79 kcal/mol. However, solvation energy through Generalized Born model contributed negatively at 48.10 kcal/mol. This collective interplay of energy components underscores the strong binding affinity observed in the complex.

CONCLUSION

Conclusion:

in the exploration into the antibacterial attributes of medicinal plants through *in vitro* examinations, ADMET prediction, molecular docking analysis, and molecular dynamics simulation, several key conclusions emerge.

Firstly, the study underscores the rich potential of medicinal plants as a source of antibacterial agents. Through rigorous *in vitro* examinations, it was observed that certain plant extracts possess significant antibacterial activity against a range of pathogens. This highlights the importance of traditional medicinal knowledge and the need for further scientific investigation into plant-based remedies.

Secondly, the integration of computational methods such as ADMET prediction, molecular docking analysis, and molecular dynamics simulation provides valuable insights into the underlying mechanisms of antibacterial action. By elucidating the interactions between plant compounds and bacterial targets at the molecular level, these approaches contribute to our understanding of structure-activity relationships and aid in the rational design of novel antibacterial agents.

Furthermore, the study emphasizes the importance of considering pharmacokinetic and toxicity profiles in the development of medicinal plant-derived therapeutics. ADMET prediction offers a valuable tool for assessing the absorption, distribution, metabolism, excretion, and toxicity of bioactive compounds, guiding the selection of candidates with favourable drug-like properties.

Lastly, molecular dynamics simulation provides dynamic insights into the behaviour of plant compounds within the bacterial environment, offering a more comprehensive understanding of their efficacy and potential resistance mechanisms. This dynamic perspective is crucial for predicting long-term therapeutic outcomes and informing strategies to combat antibiotic resistance.

In conclusion, the exploration into the antibacterial attributes of medicinal plants represents a multidisciplinary approach that integrates experimental and computational methodologies to unravel the therapeutic potential of natural sources. By combining traditional knowledge with modern scientific techniques, this research contributes to the ongoing quest for effective antibacterial agents and underscores the importance of biodiversity conservation and sustainable use of medicinal plants in combating infectious diseases.

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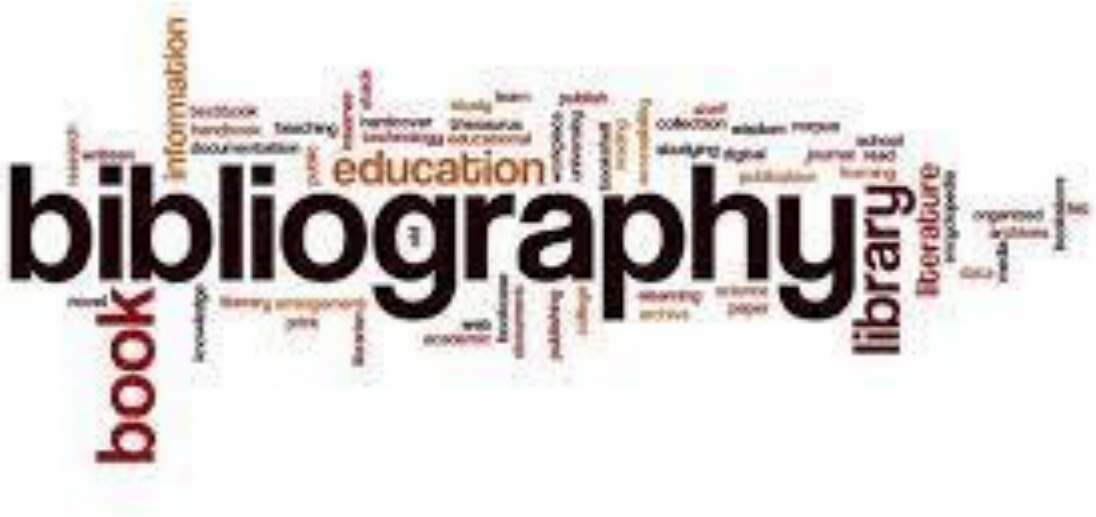
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