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Effect of Essential Oil of *Achillea Ligustica (Asterales, Asteraceae)* to Inhibit Growth and Infestation of Certain Fungi, Bacteria and Insect

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Dedecation

I dedicate this modest work:

To my dear Parents,

To my dear siblings ***Mima, Hanan, Halim, Aziz, Soufiane, Basma, Amira, Ahlem,***
Without forgetting ***Nariman, Amina***

To my dear nephews ***Djihad eddin, Yahia, Saif eddin, Ahmed adem,*** *my cutie* ***Eline,***
Anes, *the coming Baby* ***Rima junior,***

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Abbreviation List

AD: Anno domini (latin): in the year of our lord.
BC: Before christ.
CLSI: Clinical and laboratory standards institute.
CNR-ISA: Institute of food science-national research council.
E. coli: Escherichia coli.
E.D: Distilled water witness.
EO: Essential oil.
GN: nutrient agar.
HIV: human immunodeficiency virus.
I: Intermediate.
LD50: Lethal dose.
LPS: Lipopolysaccharides.
MAP: Aromatic and medicinal plants.
MH: Mueller hinton agar.
MM: Millimeter
UL: Micro liter newline **ML:** Milliliter
C°: Degree Celsius
G: Gram **OECD:** Organisation for economic cooperation and development.
OM: Outer membrane.
PDA: Potato dextrose agar.
R: Resistant.
S: Distilled water + 1 percent of acetone (witness).
Se: Sensitive.

Introduction

Achillea ligustica is an herbaceous *Asteraceae* widespread in the Mediterranean area. Yarrow (*Achillea*) species had been utilized in hnopharmacology for thousands of years all around the world. The genus *Achillea* is rich in essential oil (EO); Where Phytochemical studies of this EOs have shown high variability in its constituents **(Mohammadhosseini et al, 2017)**.

It has been used in traditional Sardinian medicine since ancient times, mainly as an anthelmintic, against gastric pains and neuralgias, and as an anti-inflammatory on skin diseases **(Atzei et al, 1994)**. Essential oils from different species of yarrow have been widely investigated, and herbal, food, and cosmetic uses are reported in many scientific papers **(Tzakou et al, 1995)**.

Essential oils are becoming increasingly popular to be used for a wide variety of purposes, including aromatherapeutic et alternative natural medicines. During the past years, some studies have been carried out concerning the application of essential oils as antimicrobial agents **(Barrtta et al., 1998)**.

The valorization of essential oils in the therapeutic field goes through a preliminary stage of determining their chemical composition (to characterize them, and to highlight their possible specification), as well as by studying their biological properties; to use them as an antifungal, antibacterial or anti-insect agent, which would prevent the phenomenon of the growth of a fungus, a bacterium or the infestation of an insect responsible for several problems**(Allali et al, 2021)**.

Essential oils can even be used as a flavoring agent thanks to their fragrant property, as well as an antifungal, antibacterial, and insecticidal agent thanks to their power, which allows them to replace fertilizers and antibiotic treatments, especially since they are synthetic chemical origin, and their consequences on plants and public health are harmful **(Abdalla et al, 2020)**.

This work aims to study the effect of *Achillea ligustica* essential oils and know if this oil can inhibit the growth of bacteria, and fungi, and stop insect infestation.

The study includes three parts. The first concerns the bibliographical study, which comprises two chapters: general information on medicinal plants, the essential oils of medicinal plants. A second part, relating to the experimental study, includes the selection of bacteria, fungi and insects, and presents the methodology and techniques used throughout this work, namely: the extraction of essential oils, the preparation of bacteria, fungus and insects, and finally the evaluation of the antibacterial, antifungal and insecticidal activity.

Part I

Bibliography Part

potential to prevent or treat disease in humans or animals **(Moreau, 2003)**. These are plants used in traditional medicine that have medicinal effects in some form. Their action is based on chemical molecules (primary or secondary metabolites) or the interaction of the various substances contained **(Sanago, 2006)**.

A medicinal plant, according to **Danton** and **Baffray (1995)**, is one where one of the organs, such as the leaf or the bark, has healing properties when utilized at a certain dosage and in a careful way. The medicinal plant comes from two different places. Plants that grow naturally, sometimes known as "wild" plants, and cultivated plants **(Bezanger et al., 1986)**.

There are other different definitions **(Farnsworth et al, 1986; Anton, 1999; Gayet, 2013)**:

- Medicinal plant: The definition of a medicinal plant is very simple. In fact it is of a plant that is used to prevent, cure or relieve various ailments. Plants Medicinal drugs are herbal drugs, at least a part of which has properties medicated.
- Phytotherapy: this word comes from the Greek phyton which means "plant" and therapeia which means "treatment". It is therefore a technique of care that uses plants to come understand the causes and symptoms of various diseases. It is one of the oldest therapies.
- Plant drugs: are essentially whole, fragmented plants or thallophytes or powder, used for therapeutic purposes, either most often in the dried state, or in the fresh state.
- Active ingredient: a herbal drug in the state or in the form of a preparation is considered as an active principle in its entirety, only its components having a therapeutic effect are known to no.
- Herbal medicines: these are medicines whose active ingredients are exclusively herbal drugs and/or herbal drug preparations.

1.3 Components and Active Ingredient of Medicinal Plants

1.3.1 Definition of Active Ingredient

It is a molecule of curative or preventive therapeutic interest for humans or animals. The active ingredient is contained in a herbal drug or herbal drug preparation **(Pelt, 1980)**.

1.3.2 Types of Active Ingredient of Medicinal Plants

1.3.2.1 Essential Oils

These are molecules with an aromatic nucleus and a volatile character giving the plant a characteristic odor and these molecules are found in the secretory organs **(Iserin et al., 2001)**. These oils play a role in protecting plants against excess light and attracting pollinating insects **(Dunstan et al., 2013)**. They are used to treat inflammatory diseases such as allergies, eczema, and to relieve intestinal problems **(Iserin et al., 2001)**. Their use is also present in the cosmetics and food industry **(Kunkele and Lobmeyer, 2007)**.

1.3.2.2 Flavonoids

Are a frequent group among natural substances. They are responsible for the coloring of leaves, flowers, fruits, and other plant parts. Flavonoles, flavonones, and flavones are the three main groups that exist **(Kunkele and Lobmeyer, 2007)**. Flavonoids are antibacterial **(Wichtl and Anton, 2009)**. They can be exploited in several ways in the cosmetic and food industry, and in the pharmaceutical industry, as some flavonoids also have anti-inflammatory and antiviral properties **(Iserin et al., 2001)**.

1.3.2.3 Alkaloids

Are natural nitrogenous substances with frequent basic reactions derived from amino acids. In general, they bear the name of the plant that contains them **(Kunkele and Lobmeyer, 2007)**. All alkaloids have an intense physiological, medicinal, or toxic action. Very active, alkaloids have given rise to numerous drugs **(Delille, 2013)**.

1.3.2.4 Bitter substances

Which form a very diversified group of components whose common point is the bitterness of their taste. This bitterness stimulates secretions from the salivary glands and digestive organs, these secretions increase appetite and improve digestion. With better digestion, and the absorption of suitable nutrients, the body is better nourished **(Iserin et al., 2001)**.

1.3.2.5 Tannins

This is a term that comes from an ancient practice that used plant extracts to tan animal hides *(Hopkins, 2003)*. It is an amorphous substance contained in many plants. It is used in the manufacture of leather because it makes the skin rot-proof. It also has antiseptic properties but also antibiotic, astringent, anti-inflammatory, anti-diarrheal, hemostatic, and vasoconstrictor (reduction in the caliber of blood vessels) **(Delille, 2013)**. Examples of plants containing tannins are oak and walnut **(Kunkele and Lobmeyer, 2007)**.

1.3.2.6 Glucosides

Are very widespread organic compounds, contained in a large number of pharmaceutical preparations. In addition to sugars (simple and compound) **(Kunkele and Lobmeyer, 2007)**.

1.3.2.7 Resins

Materials are born from a fluid whose function is to limit water loss from the plant from which they come. The best-known resin is amber, a fossil resin from conifers **(Delille, 2013)**.

1.3.2.8 Phenols

Are small molecules made up of a benzene nucleus and at least one hydroxyl group, these phenols are soluble in polar solvents, their biosynthesis derives from benzoic acid and acid cinnamic **(Wichtl and Anton, 2009)**. Phenols possess anti-inflammatory, antiseptic, and analgesic activities **(Iserin et al., 2001)**.

1.3.2.9 Glucosinolates

Cause an irritating effect on the skin, causing inflammation and blisters. Applied as a poultice on painful joints, they increase blood flow in the irritated area, thus promoting the evacuation of toxins **(Iserin et al., 2001)**.

1.3.2.10 Starch

Is the most common active element in the plant kingdom and covers a large proportion of the body's carbohydrate needs. The pharmaceutical industry widely uses starch in the manufacture of tablets or as a base for powders and ointments **(Kunkele and Lobmeyer, 2007)**.

1.3.2.11 Mucilages

Form solutions with a viscous and colloidal appearance that calms the irritations of coughing and bronchitis. They have a mild laxative action, reduce heartburn, and have a lubricating effect. The plants that contain it are used in the treatment of infectious diseases of the digestive tract, such as ulcers for example **(Kunkele and Lobmeyer, 2007)**.

1.3.3 Methods of Preparation and Use of Medicinal plants

Medicinal plants can be used in different ways. Here is the list of the most common preparations **(Iserin et al., 2001)**:

1.3.3.1 Infusions

Infusion is the easiest way to accommodate leaves and flowers to obtain fortifying or calming remedies or drinks. It is prepared exactly like tea, from a single plant or a mixture of several, and drunk hot or cold **(Iserin et al., 2001)**.

1.3.3.2 Decoctions

To extract the active ingredients from the roots, bark, stems, and berries, they usually need to be treated more vigorously than leaves or flowers. A decoction is to boil in water dried or fresh plants previously cut into small pieces. It can be consumed hot or cold **(Iserin et al., 2001)**.

1.3.3.3 Essential oils

Before using essential oils, they should be diluted in neutral oil **(Iserin et al., 2001)**.

1.3.3.4 Tinctures

Are fresh, dried, grated, or pounded vegetable parts **(Kunkele and Lobmeyer, 2007)**. These are traditional medicinal preparations, and to obtain a tincture, it is enough to let a plant macerate in alcohol: the active substances thus dissolve easily, and tinctures are more effective than infusions or decoctions. For simple use, they are kept for two years.

1.3.3.5 Medicinal Powders

Plants (leaves, flowers, seeds bark) prepared in the form of powder obtained by spraying, in a mortar, or a mill, can be used for internal or external care. Powders are sometimes compressed into tablets and sometimes used as is **(Dellile, 2013)**. Powders can also be sprinkled on food or diluted. They are applied to the skin, such as talc, or, mixed with tinctures, as a poultice.

1.3.3.6 Syrups

Honey and unrefined sugar are effective preservatives that can be mixed with infusions and decoctions to give syrups and cordials. They additionally have to soften properties that make them excellent remedies for relieving sore throat. The sweet flavor of syrups masks the bad taste of some plants so that children absorb them more willingly **(Iserin et al., 2001)**.

1.3.3.7 Medicinal oils

The infusion of a plant in oil makes it possible to extract the active ingredients soluble in the oil. Medicinal oils made hot are brought to a low boil, while those made cold are heated naturally by the sun. Medicinal oils should not be confused with essential oils, natural constituents of plants that have their own medicinal properties and a distinct aroma. The latter can be added to medicinal oils to enhance their therapeutic effectiveness **(Iserin et al., 2001)**.

1.3.3.8 Ointments

Are creamy preparations made from oil or any other fatty substance, in which the active ingredients of plants are dissolved. They include active medicinal constituents, such as essential oils. They are applied to wounds to prevent inflammation **(Iserin et al., 2001)**.

1.3.3.9 Poultices

Preparations of pasty consistency that are applied to the skin. They are particularly useful in the case of wounds that are difficult to heal, or in the case of deep bruises **(Delille, 2013)**.

1.3.3.10 Creams

A cream is prepared by combining oil or another fatty substance with water, through an emulsion process **(Iserin et al., 2001)**.

1.3.3.11 Inhalations

Steam from herbal infusions that contain ethereal oils **(Kunkele and Lobmeyer, 2007)**. Inhalations are effective against bronchitis, sinusitis, hay fever, and asthma The combined action of water vapor and antiseptic substances clears the sinuses and respiratory tract.

1.3.3.12 Gargles and Mouthwashes

In general, gargles and mouthwashes are prepared from astringent plants that tighten the mucous membranes of the mouth and throat.

1.3.3.13 Baths

Plant baths are prepared from diluted essential oils or infusions. Eye baths are recommended for irritation or inflammation of the eye **(Iserin et al., 2001)**. It can be aromatic, stimulating, fortifying, relaxing, or even sedative. Effective in the case of rheumatism, baths stimulate and refresh the body **(Delille, 2013)**.

1.3.3.14 Macerations

Since heat destroys the active principles of certain plants, cold maceration is sometimes more indicated than a decoction. This method is particularly suitable for plants rich in essential oils et allows you to take full advantage of the vitamins and minerals they contain **(Delille, 2013)**.

1.4 The Parts Used and Method of Harvesting

Scientific studies have made it possible to define the optimal moment of harvest. Thus, are preferably harvested: **(Anton, 1999)**

- The roots at the time of vegetative rest (autumn, winter);
- The aerial parts, most often at the time of flowering;
- The leaves, just before flowering;
- Flowers in full bloom, see bud (hawthorn);
- The seeds, when they have lost most of their natural humidity.

1.5 Traditional Extraction Method of the Active Parts of Medicinal Plant

Extraction means the separation of the active parts of the plant or animal tissues from the inactive or inert components using selective solvents, traditionally water, vegetable oils, or animal fats. The products thus obtained are relatively impure in the form of liquids, semi-solids, or powders exclusively intended for oral or external use. These are preparations known as herbal teas and medicinal oils **(Handa, 2008)**.

1.5.1 Infusion

This is the simplest form of preparation, it is prepared by pouring boiling water over the parts of fresh or dried plants and soaking them well to extract their medicinal principles. It is suitable for the extraction of delicate or finely chopped parts of plants: leaves, flowers, seeds, bark, and roots, having volatile or heat-labile constituents such as essential oils **(Baba-Aïssa, 2000; Kraft and Hobbs, 2004)**.

1.5.2 Decoction

It is suitable for the extraction of hard or very hard plant matter: wood, bark, roots, or plants with poorly soluble constituents (e.g. silicic acid). It consists of boiling fresh or dried plants in water for 10 to 30 minutes, to fully extract the medicinal principles **(Baba-Aïssa, 2000; Kraft and Hobbs, 2004)**.

1.5.3 Maceration

It consists of putting a plant or part of a plant in cold water (aqueous maceration) or vegetable oil (oily maceration) for several hours, or even several days, to allow the active constituents to diffuse well. It is suitable for the extraction of plants containing mucilage, such as linseed or sand plantain seeds, their high concentration of starch or pectin can cause gelatinization if they are prepared in boiling water. Also used to prevent the extraction of unwanted constituents that dissolve in hot water **(Kraft and Hobbs, 2004)**. It also concerns plants whose active substances risk disappearing or degrading under the effect of heat by boiling **(Baba-Aïssa, 2000)**.

1.5.4 Poultice

The plants are coarsely chopped, then put to heat in a saucepan covered with a little water. Let simmer for two to three minutes. Squeeze the herbs, then place them in the area to be treated. Cover with a strip or piece of gauze **(Nogaret, 2003)**.

1.6 *Achillea Ligustica*

Ligurian Yarrow *(Achille Ligustica All)* is a perennial, pubescent herbaceous plant belonging to the family of Asteraceae. It naturally grows throughout the western Mediterranean region, and especially in the Tyrrhenian region of Italy, from Liguria to Sicily. With pinnatipartite leaves, a slightly aromatic perfume, and a general bitter taste, the plant doesn't grow higher than one meter. Flowers are arranged in flat-topped clusters, and both disk and ray flowers are small and white, like those of the common yarrow To all *(Achillea Millefolium L.)* **(Atzei, 1994; Pignatti,1982)**.

There are about five species of *Achillea* which are widely distributed in Algeria; *A. Ligustica All., A. Leptophylla M.B., A. Odorata L., A. Santolinoïdes Lag.* and *A.Santolina L.* **(Quezel et al. 1962)**.

Achille Ligustica prefers sunny or slightly shaded grassy fields, roadsides, and other edges. It can be found growing in a variety of habitats, often from sea level to 1000 m. It starts to bloom at the beginning of summer. It has been used in traditional Sardinian medicine since ancient times, mainly as an anthelmintic, against gastric pains and neuralgias, and as an anti-inflammatory for skin diseases. Moreover, "magical uses", especially against bad luck**(Atzei, 1994, Atzei, 2003)**. The majority of aspects of *Achillea* are medicinal plants with therapeutic applications **(Sheidai et al., 2009)**.

1.6.1 Botanical Description and Distribution

Perennial plant of 30-100 cm, with a short and not very branchy stump. Angled rods. Rosette and basilar leaves have 5-7 segments on each side. Inflorescences in relatively loose corymbs, corolla flowers not or hardly covering the ovary or achene, it grows in clear forests and at the edges of streams **(Figure 1)** **(Quezel and Santa, 1963)**.

Figure 1: *Achillea Ligustica* Distribution **(GBIF, 2021)**.

1.6.2 Systematics

The systematic position of the species *Achillea ligustica* is as follows **(GBIF, 2021)**:

Kingdom: Plantae

Clade: Tracheophytes

Clade: Angiosperms

Clade: Eudicots

Clade: Asterids

Order: Asterales

Family: Asteraceae

Genus: *Achillea*

Species: *A. ligustica*

1.6.3 Phytochemical Constituents

Phytochemical studies on *Achillea* species have revealed that many components of this genus are highly bioactive (**Falk et al., 1975**). The literature research shows that flavonoids, terpenoids, lignans, amino acid derivatives, fatty acids, et alkamides such as p-hydroxyphenylamide IV have been identified in the *Achillea* species. The main constituents of most species have already been examined (**Si et al., 2006**). Therefore, other minor or rare compounds and in particular their medicinal or industrial uses that have been less described are reviewed. Among them, alkamides, lipophilic and nitrogen compounds, are responsible for the insecticide, anti-inflammatory and certain immunological activities of *Achillea* plants (**Greger, 1984**). The genus *Achillea* includes flavored species that produce intense essential oils.

Volatile *Achillea* oils contain monoterpenes as the most representative metabolites. However, there are reports of high levels of sesquiterpenes compared to monoterpenes (**Nemeth, 2005; Bakkali, 2008**). Several pharmacological actions have been mainly attributed to the presence of sesquiterpene lactones azulenogenic in Yarrow essential oil.

1.6.4 Use

Since the genus *Achillea* is widespread throughout the world, its species have been used by local people as traditional or herbal medicines. They are reported as tonic, anti-inflammatory, antispasmodic, diaphoretic, diuretic, and emmenagogic agents, and have been used for the treatment of hemorrhage, pneumonia, rheumatic pain, and wound healing in traditional Persian literature (**Zargari, 1996; Saeidnia et al., 2005**). Herbal teas prepared from certain species of *Achillea* are traditionally used for abdominal pain and flatulence in Turkey (**Honda et al., 1996**).
In terms of Chinese medicine, *Achillea* can have three main actions: clear external wind (diaphoretic), toning deficit (tonic), and clear heart phlegm (anti-hypertension) (**Ross, 2003**). Many of these therapeutic uses have been confirmed by new experimental and clinical studies. The consumption of herbal teas of different species of *Achillea*, especially for the treatment of the gastrointestinal tract, is common in folk medicine (**Skocibusic et al., 2004**).

CHAPTER 2

Essential Oils

2.1 History

The history of aromatic and medicinal plants (MAP) is linked to the evolution of civilizations. The history of people shows that these plants are still important in medicine, in the composition of perfumes, and culinary preparations. The first evidence of the manufacture and use of essential oils (EO) dates from the year 3000 BC **(Baser and Buchbauer, 2015)**.

According to **Ntezurubanza (2000)**, the history of aromatherapy, which is that of essential oils, can be summarized in the following four eras:

- The period during which aromatic plants were used as such or in the form of infusions or decoctions.
- The one in which aromatic plants were burned or left to infuse or macerate in vegetable oil. At this time, the notion of activity linked to the odorous substance intervenes.
- The third corresponds to the search for the extraction of this fragrant substance. It appears the concept of "Essential Oil" leads to the creation and development of distillation.
- Finally, the last is the modern period in which the knowledge of the components of essential oils intervenes and explains the physical, chemical, biochemical, and physiological effects.

In 2001, the book "Aromatherapy Exactly by **Franchomme and Pénoël (2001)**, evokes the term "aromatic medicine" which contains chemotypes and therapeutic indications based on scientific bases.
Thus, the medicinal plant industry has become, in a short time, the sector of the pharmaceutical industry experiencing the strongest annual growth, ie 15 to 20 percent.

2.2 Definition

The term "Essential Oil" was coined empirically. The word "oil" emphasizes the viscous and hydrophobic nature of these substances; however, the word "essential" is understood to be the main character of the plant **(Bernard, 1988)**.
According to French Standardization Association **(AFNOR, 1981)**, these are generally fragrant products, obtained either by steam distillation of plants or parts of plants or by expression of the fresh pericarp of certain citrus fruits. This definition excludes species obtained by other extraction processes.
An essential oil (EO) is an aromatic and highly volatile plant extract, marked by a strong odor. It is a product with a complex composition, obtained from a vegetable raw material. Essential oils contain a considerable number of biochemical families including alcohols, phenols, esters, oxides, coumarins, monoterpenes, sesquiterpenes, ketones, and aldehydes. **(Funk and Wagnalls, 2004)**.

According to **Theis and Lerdau (2003)**, the term "essential oil" is strictly reserved for the aromatic product resulting from distillation, taking into account the fact, that other extraction techniques do not make it possible to obtain the essential oil as such. Before its extraction, when it is in the plant, the essential oil is called essence. Essences are complex mixtures of secondary metabolites characterized in this way because they do not participate directly in plant growth, they are involved in plant defense or attraction mechanisms (defense against herbivores or phytopathogens (fungi, viruses, and bacteria) or the attraction of pollinators).

They are stimuli for beneficial microorganisms and also participate in the protection of the plant against UV rays.
The amount of essential oil contained in plants is always in very low concentrations. It is important to make a difference between essential oils and vegetable oils. Essential oils are obtained by expression (reserved for citrus fruits) or by steam distillation. Vegetable oil is obtained by pressing and consists mainly of fatty substances **(Bastien, 2008)**.

2.3 Physical Properties of Essential Oils

Essential oils have in common several Physical properties:

- They are soluble in alcohol, and most organic solvents, but are poorly soluble in water to which, however, they communicate their smell.
- Their boiling point varies from 160° to 240°C.
- Their density is usually lower than that of water.
- These are perfumes and are of limited conservation.
- These are substances of oily consistency, more or less fluid.
- At ambient temperature, they are usually liquid, colorless, or pale yellow, there are, however, some exceptions, for example, azulene essential oil blue coloration **(Bekbecbi and Abdelouabid, 2014)**.

2.4 Location of EOs Constituents in Tissues

The constituents of EOs can accumulate in isolated cells which are distinguished from ordinary cells by their more yellow tint and their thick, slightly suberized walls. They can form fine droplets dotting the protoplasm of epidermal cells (upper epidermis of rose petals). But generally, the epidermis of the petals of fragrant flowers does not contain large reserves of essences. The essences are vaporized continuously during their formation **(Alilou, 2012)**.

2.5 Methods of Extracting Essential Oils

Essential Oils are obtained by steam distillation water or hydrodistilation, the oldest technique, but also by; simple hydrodistillation, saturated steam distillation, and hydrodiffusion **(Belyakoobi, 2006; Benayad, 2008)**.

2.5.1 Hydrodistillation

To isolate essential oils by hydrodistillation, the aromatic plant material is packed in a still and a sufficient quantity of water is added and brought to a boil; alternatively, live steam is injected into the plant charge. Due to the influence of hot water and steam, the essential oil is freed from the oil glands in the plant tissue. The vapor mixture of water and oil is condensed by indirect cooling with water. From the condenser, distillate flows into a separator, where the oil separates automatically from the distillate water **(Figure 2.1) (Benayad, 2008)**.

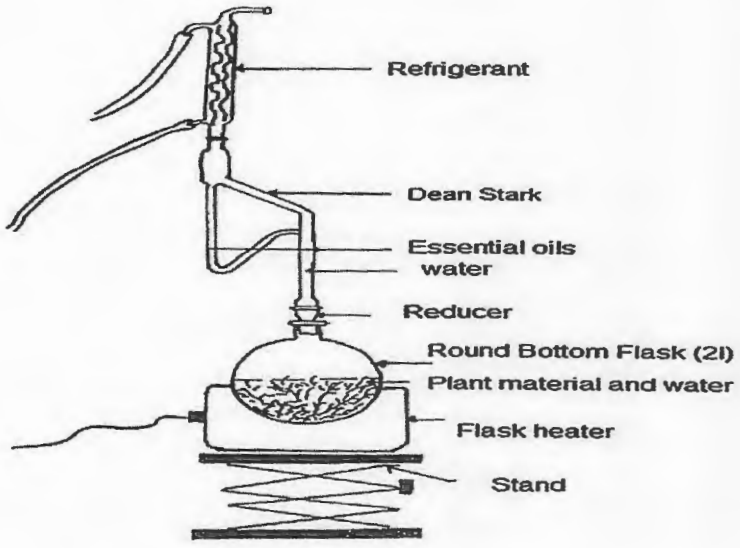

Figure 2: Hydrodistillation of Essential Oils: Clevenger-type Apparatus Materials (**Farhat, 2010**).

2.5.2 Hydrodiffusion

Hydrodiffusion consists of passing a stream of water vapor at very low pressure through the plant mass. The composition of the products obtained is significantly different qualitatively from that of the products obtained by the previous methods (**Figure 3**) (**Alilou, 2012**).

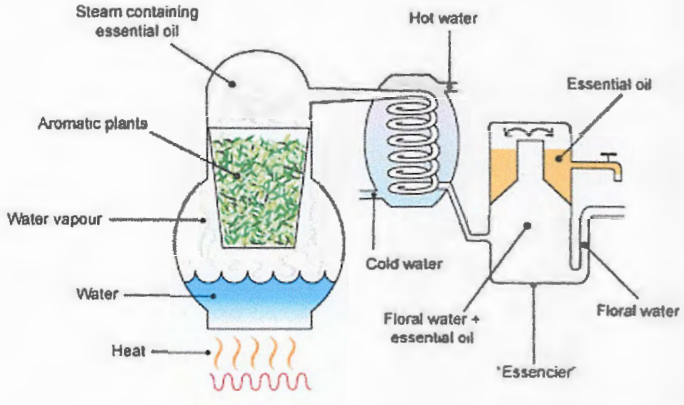

Figure 3: Hydrodiffusion of Essential Oils (**Farhat, 2010**).

2.5.3 Extraction by volatile solvents

It's done using volatile organic solvents in devices called Soxhlet extractors. This procedure consists in exhausting the vegetable matter of its fragrant constituents using a solvent, then separating it from the extract by a separator, this is linked to the property of essential oils to be soluble in most organic solvents, particularly aliphatic hydrocarbons (hexane, petroleum ether, etc.) which are the most widely used (**Figure 4**) (**Mohamed, 1997**).

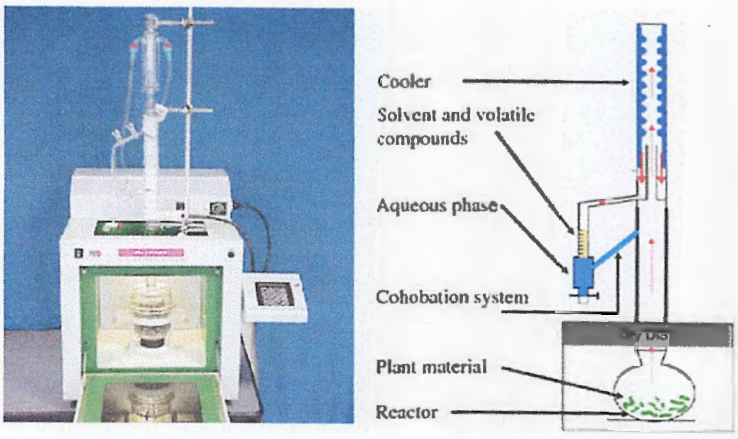

Figure 4: Extraction of Essential Oils by Volatile Solvents(**Farhat, 2010**).

2.6 Biological Activities of Essential Oils

2.6.1 Antibacterial Activity

Resistance against antimicrobial agents has increasingly become a major and urgent problem worldwide (**Tim et al., 2005**), which has directed research by health agencies and authorities towards plant genetic resources to find a solution to this problem (**Sudano et al., 2004**). According to **Ghasemi et al (2010)**, the use of EOs as antibacterial agents appears to be an interesting alternative solution for controlling the presence of pathogenic bacteria in food, many of which have remarkable antibacterial activities against a broad spectrum.
EOs that have antibacterial activities destabilize the phospholipid bilayer of the bacterial membrane, although they are involved in the enzymatic systems and genetic material of bacteria (**Kim et al., 1995**). In addition, several essential oil constituents exhibit significant antibacterial properties when tested separately by intervening by different mechanisms (**Ultee et al., 1998**). It is obvious that the antibacterial properties of essential oils are more strongly explained by the additive effect of their main antimicrobial compounds because of their minor constituents that appear, to play a significant role in synergy (**Lattaoui et al., 1994**).
Some of the constituents such as carvacrol, thymol, and eugenol (phenolic compound) have been proven antibacterial. The Aldehyde compounds in essential oils are somewhat antibacterial; aldehyde constituents: neral, geranial, citronellal, and cuminal are the most widely used. The antibacterial action of ethers is certain but irregular; terpenes are interesting, but are mostly diffused into the air (**Kim et al., 1995**).

2.6.2 Antiviral Activity

Essential oils of different botanical families exhibit antiviral actions, but the degree of effectiveness varies depending on the strain and viral structure. It is because of particular molecular structures found in each viral type, that essential oils enter entities to varying degrees (**Davidson et al., 2005**).
Research has found that many essential oils have antiviral activity against certain viral strains of influenza, adenoviruses, glandular fever strains, viral enteritis, viral enterocolitis, and HIV-1 (**Schnitzler et al., 2001**). Researchers have shown that certain compounds specific to essential oils, tested separately, possess remarkable antiviral activity. These are anethole acetate, carvone, beta-caryophyllene, citral, eugenol, limonene, linalool, and linalyl (**Belaiche, 1979**).

Viruses are highly sensitive to aromatic molecules and some serious viral infections can show great improvement through herbal medicine **(Hayashi et al., 1994)**. The synergy between oil compounds such as cineole-monoterpenol has been used to treat viral infections of the respiratory tract; Ketones and cryptone compounds in essential oils have shown an ability to fight naked viruses **(Bhaskara et al., 1998)**.
Several antiviral methods of action have been proposed for essential oils although for their compounds. Some oils interfere with the surface glycoprotein in the viral envelope, preventing the virus from attaching to the host cell. Other oils are thought to attack the virus in the host cell, possibly at the cell membrane **(Belaiche, 1979)**.

2.6.3 Anti-fungal Activity

Fungal infections have increased in recent years due to the increasing number of high-risk patients, particularly immunocompromised hosts (people with deficient immune systems) **(Rapp, 2004)**.
The essential oils of many plants are known to possess antifungal activity **(Kalemba and kunicka, 2003)**, however, there is limited information on activity towards human pathogenic fungi **(Okoh, 2010)**.
According to the Institute of Food Science-National Research Council **(CNR-ISA)**; EOs can represent one of the most promising natural products for fungal inhibition. Many kinds of EOs obtained from different plants or herbs exhibited intense antifungal properties.
Essential Oils, like the other phytochemicals, could attenuate microbial growth and biofilm development through specific mechanisms. The antifungal activity of essential oil might be caused by the properties of terpenes/terpenoids, that due to their highly lipophilic nature and low molecular weight are capable of disrupting the cell membrane, causing cell death or inhibiting the sporulation and germination of food spoilage fungi. Therefore, several in vitro tests indicate that terpenes/terpenoids show ineffective antimicrobial activity when used as singular compounds compared to the whole EO.

2.6.4 Insecticidal Activity

The preservation of stored food is usually provided by synthetic insecticides, which can be the most effective and least expensive way to control insects. However, the misuse of chemical insecticides has harmful effects **(Guarrera, 1999)**, which directs current work toward the search for substances extracted from plants that exhibit insect-insect-repellent, insect-repellent, or insect-repellent activity **(Barkire, 1996)**.
Indeed, plants are a source of natural substances that has great potential for application against insects and other plant and plant pests.

2.6.5 Antioxidant Activity

2.6.5.1 The Origin of Oxidative Stress

Oxidative stress is the imbalance between the generation of reactive oxygen species and the body's ability to neutralize them and repair oxidative damage **(Boydand et al., 2003)**. It corresponds to a disturbance of intracellular oxidative status **(Morel and Barouki, 1999)**.
Lipid peroxidation is a complex process of oxidative stress, which occurs in cells, aerobically and reflects the interaction between molecular oxygen and polyunsaturated fatty acids **(Janero, 1990)**. Asthma, inflammation, arthritis, neurodegeneration, Parkinson's disease, Mongolism, and dementia are the results of the free radical-forming effect.

2.6.5.2 Free Radicals

A free radical is a molecule or atom with one or more unpaired electrons, making it extremely reactive **(Vansant, 2004)**. The set of free radicals and their precursors is often referred to as reactive oxygen species **(Favier, 2003)**. The name is not restrictive, it includes the free radicals of oxygen proper, but also certain reactive non-radical oxygen derivatives whose toxicity is important such as hydrogen peroxide (H2O2), peroxynitrite (ONOO) **(Novelli, 1997)**.
radicals are produced by various physiological mechanisms (inflammation, redox cycle,... etc.), to destroy bacteria within phagocytic cells (macrophage, polynuclear) or to regulate lethal cellular functions such as programmed cell death or apoptosis **(Favier, 2003)**.
The ingestion of alcohol is followed by the formation of free radicals through various mechanisms, also antibiotics, anticancer drugs **(Hadi, 2004)** HIV infection has the effect of increasing the production of free radicals in the body **(Hosein and Lytle, 2001)**.

2.6.5.3 Antioxidants

An antioxidant is defined as any substance that can delay or prevent the oxidation of biological substrates **(Boyd et al., 2003)**. These are compounds that react with free radicals and thus render them harmless **(Vansant, 2004)**.
Antioxidants are preventative agents. They block initiation, by complexing catalysts, reacting with oxygen, or terminating agents capable of deflecting or trapping free radicals. They act by forming non-radical finished products. Others by interrupting the peroxidation chain reaction or reacting quickly with a fatty acid radical before it can react with a new fatty acid. While other antioxidants absorb excess energy from the singlet oxygen to transform it into heat **(Berset and Cervelier, 1996)**.
In recent years, there has been a growing interest in the search for natural antioxidants. The antioxidant properties of many herbs and spices have been reported to be effective in this regard, including species belonging to the Lamiaceae family that possess appreciable antioxidant activity **(Gutteridge and Halliwell, 1994)**.

2.7 Toxicity of Essential Oils

The toxicity of essential oils is a term generally used when abusing this natural product. Some indeed confuse a little quickly plants with essential oils and essential oils: the safety of the former is almost always, a fact; the toxicity of the seconds is quite often demonstrated. Self-medication is encouraged by the fact that many of these products are distributed outside the pharmaceutical sector.
Essential oils have low or very low acute oral toxicity, the majority of which have an LD50 rating between 2 and 5g/kg.
The chronic toxicity of essential oils is not well known, at least concerning their use in practice such as aromatherapy, regardless of the route of administration; possible adverse reactions are not only rarely reported **(Bruneton, 2009)**.

2.8 Field of Use of Essential Oils

Essential oils are used in the composition of perfumes, cosmetics (shampoos, shower gels, creams, milk, body deodorants), cleaning products (soaps, detergents, detergents, fabric softeners), and any other product, such as insecticides, air fresheners, diffusers, and candles. They are also used as flavorings to add odors and/or flavors to foods. Finally, they have certain therapeutic properties and applications in aromatherapy **(Schauenberg, 2006)**.

2.9 Essential Oils of *Achillea Ligustica*

Essential oils are attracting increasing attention due to their importance in several applications in sectors such as aromatherapy, pharmaceuticals, cosmetics, and foods. They have a range of pharmacological properties, such as antibacterial, antifungal, insecticidal, anti-inflammatory, antioxidant, and cytotoxic activities. They can be extracted from different parts of the plant materials using classical and advanced techniques (**Mohammadhosseini, 2017**). Hydrodistillation is among the most important traditional approaches to extracting the EOs.
The genus *Achillea* is rich in essential oil (EO) with high chemical diversity. Different species of *Achillea* are known for their pharmaceutical, cosmetic and aromatic properties.
This plant has been used in several traditional systems, such as Italian folk medicine, for skin disorders and rheumatism (**Maggi, 2009**). It is also used in Sicily against intestinal worms (**Tuttolomondo et al. 2014**). In Sardinia, an infusion of *A. ligustica* is traditionally used for gastralgia and neuralgia (**Bruni, 1997**). In addition, it has traditionally been used to relieve sprains and insect bites, as well as to stop bleeding (**Filippi, 2006**).
A.ligustica contains several secondary metabolites, such as essential oils, flavonoids, sesquiterpene lactones, piperidine amides, and guaianolides, with the essential oils used as traditional herbal remedies as well as in food and cosmetics (**Conforti, 2005**).
It has been reported that several biological activities are due to essential oils or methanolic extracts of *A. ligustica*. Essential oils have been found to possess antifungal properties, as well as antibacterial activity towards both Gram-positive and negative bacteria (**Tuberoso, 2005**). Moreover, anti-proliferative activities have been reported for essential oils, which could make them candidates for anti-carcinogenic formulations (**Maggi, 2009**).
Phytochemical studies of the essential oil (EO) of *A. ligustica* have shown high variability in their constituents. This high variability in the chemical composition of the EOs obtained has an impact on their biological activity, demonstrated by different ranges of inhibition of microbial growth, which largely depend on geographical and seasonal factors (**Maggi, 2009**).

2.9.1 Main Components of Essential Oils, Volatile Constituents and Extracts from *Achillea Ligustica*

Phytochemical studies of the essential oil (EO) of *A. ligustica* have shown high variability in their constituents. This high variability in the chemical composition of the EOs obtained has an impact on their biological activity, demonstrated by different ranges of inhibition of microbial growth, which largely depend on geographical and seasonal factors (**Table 2.1**) (**Maggi, 2009**).

Table 1: Main Components of Essential Oils, Volatile Constituents and Extracts from *Achillea Ligustica*

Main components	Area/country	Ref
Santolina alcohol, borneol, sabinol, transsabinyl acandate, alpha-thujone and viridiflorol	Eight localities of Sardinia, Italy	**(Tuberoso et al., 2005)**
Camphane derivatives: camphor, borneol, bornyl acandate, artemisia kandone, and santolina alcohol	U Rugnicone, near Ajaccio, Corsica	**(Filippi et al., 2006)**
Leaves: 4-Terpineol, 4-terpineol, carvone, carvone, alpha-terpinene, phellandrene, phellandrene, Flower: linalool, and cedrol	Fantina, Messina, Sicily, Italy	**(Bader et al., 2007)**
Viridiflorol, terpinen-4-ol, pinene, 1,8-cineole	Central Italy	**(Maggi et al., 2009)**
Leafy stems: Artemisia kandone, camphor, santolina alcohol, camphene, viridiflorol, trans-sabinyl acandate	Corsican, France	**(Muselli et al., 2009)**
Aerial Parts: Camphor, santolina alcohol, artemisia kandone, viridiflorol, trans-sabinyl acandate, camphene Flowers: camphor, trans-sabinyl acandate, viridiflorol, artemisia kandone, santolina alcohol, camphene	Sardinian, Italy	**(Muselli et al., 2009)**
(Z)-Chrysanthenyl acandate, viridiflorol, bornyl acandate; 1,8-cineole	Lipari, Aeolian Islands, Italy	**(Ben Jemia et al., Stems: 2013)**

2.10 Majority Compounds of EO of *Achillea ligustica*

All the essential oils showed a very varied chemotype distribution, in agreement with previously published studies **(Tuberoso,2005)**. Moreover, this marked variability of the EO composition among specimens belonging to the same species has also been reported for other *Achillea* species, such as *A. millefolium* **(Saeidi et al, 2018)**, *A. cartilaginea* **(Gudaityt,2007)**, A. biebersteinii **(Gudaityt,2012)**, *A. crithmifolia* **(Mirahmadi,2012)**, *A. ageratum* **(Grandi,1976)**, and *A. wilhelmsii* **(Saeidi et al, 2018)**. This chemical polymorphism, thus, appears to be a distinctive trait of this genus, whose EO profile seems to be strongly influenced by the growing environment. the studies carried out at the university of Jijel by **Laater and Bousmaha (2012)**, on the same species of *Achillea* shows the following components: Camphene, Eucalyptol, Chrysanthenon, R-carvone, and Caryophyllene
in the **(Table 2)** there are the majority compounds of EO of *Achillea ligustica* that have a very important biological activity:

CHAPTER 3

Material and Methods

3.1 Material

3.1.1 Plant Material

In this study, *Achillea ligustica* plant was used for the extraction of essential oils. it's a perennial plant of 30-100 cm, grows in clear forests and at the edges of streams **(Quezel and Santa, 1963)**.
During April in 2022, the adult leaves and flowers of *A chillea l igustica* used in this study were collected in the Jijel region (Chekfa), then transported in bags to the microbiology laboratory of the University of Jijel for experimentation. The parts of the plants used are cleaned and dried in the shade, away from humidity at room temperature for a few days, to remove the water they contain**(Figure 5)**. Then, ground into a powder using an electric grinder, to be ready to extract the essential oils.

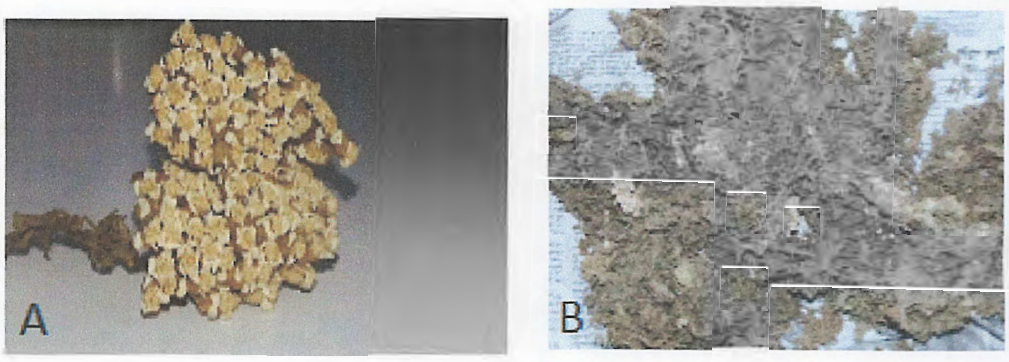

Figure 5: Dried *Achillea ligustica* Plant (A: Flower, B: Leaves).

3.1.2 Microorganisms Tested

It should be noted that it was supposed to isolate microorganisms from the bean plant that was available at that time, but all methods of isolating bacteria and fungi failed and this is due to the contamination that occurred in all microbiological laboratories, after the continuous failure and due to the short time remaining to do the laboratory work, Clinical bacteria were brought from Majdoub Said Hospital and fungi isolated from oak trees were used.

3.1.2.1 Bacteria Material

To test the antibacterial activity of *Achillea ligustica* essential oil. The two bacterial strains selected in this study are responsible for several infections. These strains were supplied from Medjdoub Said Hospital, (Taher, Jijel). They include clinical bacterial strains: *Escherichia coli* **(27.05.22.1)** and *Staphylococcus sp* **(27.05.22.7)** **(Figure 6)**.The choice of strains to be tested was based on pathogenicity, studies already carried out, and their availability in the laboratory. More information about those two strains can be found in **Appendix N°2**.

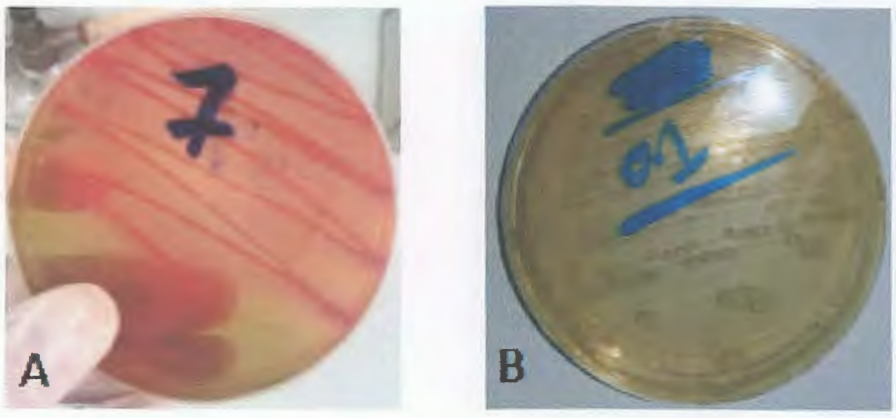

Figure 6: Strains of Bacteria (A:*Staphylococcus sp*, B: *E. coli*).

A. Culture Media

The culture of bacteria required the use of the following media: Mueller Hinton agar (MH), and nutrient broths. The chemical composition of these different growing media can be found in the **Appendix N°1**.

B. Activation of the studied strains

From a storage medium, a 1ml sample is taken and fed into 9ml of nutrient broth, incubated at 37°C for 2 hours. 0.1 ml of the latter solution is surface-seeded in a solid medium (MH) petri dish, which is incubated at 37°C for 24 hours **(Moroh et al, 2008)(Figure 6)**.

C. Transplanting Bacterial Strains

The different bacterial strains were transplanted by the striation method and incubated in the oven at 37°C for 18 to 24 hours to obtain a young culture and isolated colonies. Isolated colonies were used to prepare the inoculum **(Moroh et al, 2008)(Figure 7)**.

Figure 7: Troublement in Nutrient broth Tubes.

D. Preparation of the Inoculum

After 24 hours of incubation at 37°C, three to four well-isolated colonies are removed and emulsified in 5 ml of nutrient broth. Dilutions of bacterial suspension are performed to standardize the inoculum.

3.1.2.2 Fungi Material

To test the antifungal activity of *Achillea ligustica* essential oil. The two fungi strains selected in this study are responsible for several infections. These strains were supplied from the *Cork Oak* forest located in the Jijel region. They include *Acremonium sp* and *Diplodia sp.* The choice of strains to be tested was based on the importance of host plants for the forest and the significant damage caused by these fungi to it.

A. Fungi isolation

Samples of the *Cork Oak* plants were brought to the laboratory and stored at 4°C in a refrigerator until analysis. The insulation method used is that described by **(Vajna, 1986; Valencia, 2004)**. Branches, stems, and leaves show symptoms of blackening of the xylem and stains with varying shapes.

Longitudinal and transverse sections of the symptomatic branches were made (5-2mm) and fragments of 1 cm in diameter were made from the leaves, were sterilized with 0.1 percent sodium hypo-chlorite for one minute, followed by immersion in sterile distilled water for ten minutes (10min). The fragments were then dried using sterile filter paper. All fragments, ranging from five to six (4-5) per Petri dish, with 2 replicates per sample, were grown on PDA culture media. The seeded boxes were incubated in an oven at 25°C for 6 days.

B. Purification of Fungal Colonies

The isolates obtained are purified by successive subculturing, which consists of aseptically transferring the microorganism to a sterile Petrie dish to isolate it or maintain it in pure culture, it is better to take a few spores or a mycelial fragment with a sterile platinum loop. and transfer it to a new Petrie dish **(Botton et al., 1990)**.

C. Identification of Fungal Isolates

The identification was based on two types of criteria (macroscopic and microscopic).

- A macroscopic criteria:
  These criteria are based on the observation of colonies with the eyes and by using a binocular magnifying glass.
  The observation of characters' concerns:
  - The appearance of the colony (color of the surface and the back of the box, the texture of the surface of the colonies, topography,...).
  - Presence or absence of droplets on the mycelium.
  - Pigment production.
  - Growth rate (colony diameter at 7 days: fast more than 3 cm; moderate: between 1 and 3 cm and slow less than 1 cm) **(Guillaume, 2006)**.
- A microscopic criteria: The identification of fungi requires observation under an optical microscope based on the microscopic identification criteria carried out by **Botton et al (1990)** and when it is possible to identify. For this, and using a sterile platinum handle, a fragment of the culture is superficially removed and placed on a slide. then we put a drop of lactophenol or lactic acid. Then the slide is covered with a Lamella, then observed under an optical microscope at a magnification G X 40.
  The microscopic criteria are:
  - Hyphae: septate or not.
  - Conidiophores: absent, simple, branched.

- Conidiogenous cells: annelid, phialide...
- Conidia: uni- or multicellular, solitary, in clusters or chains, shape (round, oval, club).

3.1.3 Animal Material

The insects used are *Corn aphids (Rhopalosiphum Maidis).* These insects are harvested from corn, planted in the Jijel region (Ouled Fadel). This experiment is carried out in the microbiology laboratory at Mohamed Seddik Ben Yahia University, Jijel. The collection of insects must be carried out continuously during the tests to guarantee the vitality of the sample and ensure the reliability of the results **(Appendix N°3)**.
The selection of insects was based on:

- The economic importance of host plants.
- The significant damage caused by these insects on host plants.
- These insects are very common and easy to find.

3.2 Methods

3.2.1 Extraction of Essential Oils

The extraction of essential oils of Achillea ligustica was carried out at the educational laboratory at the University of Jijel by Hydrodistillation method (Clevenger type)**(Figure 3.4)**.

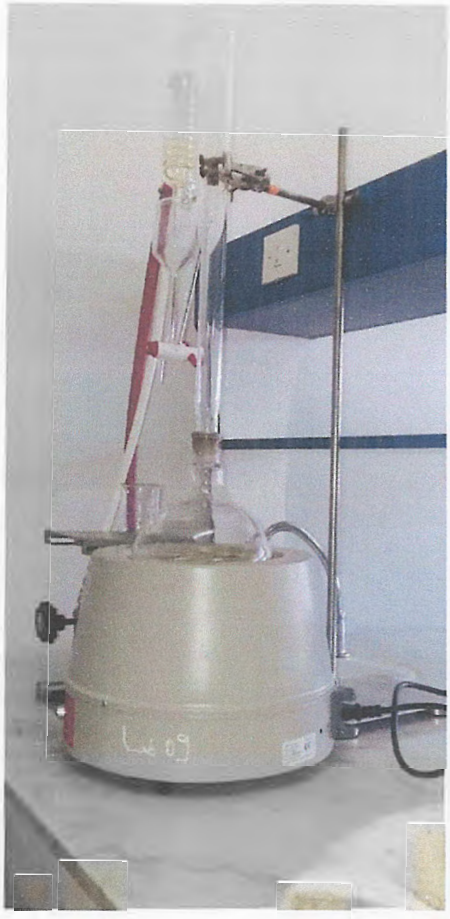

Figure 8: Hydrodistillation of Essential Oils: Clevenger-type Apparatus Materials.

3.2.1.1 Experimental Protocol

Hydrodistillation remains the most widely used technique for extracting oils essential and provides better returns. Hydrodistillation is carried out by a Clevenger type **(Figure 3.2)**. The principle consists of immersing the raw material in distilled water contained in a flask. The whole is then brought to a boil and the operation is generally carried out at atmospheric pressure. The vapors are condensed in a cooler and the essential oils separate from the water by density difference **(Benayad, 2008)**. Then, batches of 50 g of plant material, prepared beforehand, are subjected to Hydrodistilation, using a Clevenger-type device. The plant material was introduced into a 1000 ml flask containing 300 ml of distilled water. The whole is then boiled for 1 h 30 min. Decanting the distillate gives two phases:

- an organic phase: consisting of the essential oil.
- An aqueous phase: consisting of aromatic water.

The essential oil obtained is dried over anhydrous sodium sulfate and is stored in tubes covered with aluminum foil, at a temperature of +4°C until its subsequent use. The essential oil yield was determined concerning the quantity of dry matter, evaluated from the 100 g samples.

3.2.1.2 Yield Calculation

The yield of essential oils is defined as the ratio between the mass of essential oil obtained and the dry mass of the plant material to be treated **(Kaid, 2004)**. The return, expressed as a percentage, is calculated by the following formula:

R=P2/P1x100

- R: yield of essential oils expressed (percentage).
- P2: mass of essential oils in grams.
- P1: mass of sample in grams.

3.2.1.3 Preparation of Essential Oils Dilutions and Witnesses

To choose the dosages to be used, many preliminary tests were done. Accordingly, specific doses of (10, 30, 60, and 100) ul of EO were diluted in acetone and distilled water following a methodology inspired by **Tchoumbougnang's work (2008)**.

To do this, each preparation was made by introducing (10, 30, 60, 100) ul of EO into test tubes, and the volume is completed up to 20ml with distilled water, which corresponds into all cases to an acetone concentration of 1 percent concerning the mixture.
Two types of negative witnesses were prepared; the first is constituted by distilled water (E.D) and the second by a solution of distilled water+1 percent of acetone (S) **(Figure 9)**.

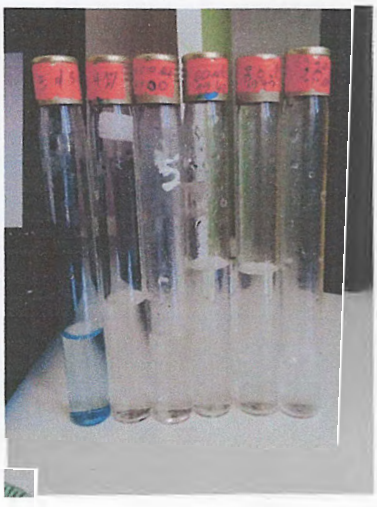

Figure 9: Essential Oils Dilution and Witnesses

3.2.2 Biological Tests

The experiments were carried out in Petri dishes that had been already prepared in the microbiology laboratory. They help decide the essential oils' toxicity to bacteria, fungi, and insects both by contact and/or inhalation.
The methodology used is based on the OECD (Organisation for Economic Co-operation and Development) guideline for the testing of chemicals **(OECD, 1998)**.

3.2.2.1 Antibacterial Activity Assay

The agar disk diffusion method (Kirby-Bauer method) was used to assess the bio-activity of essential oils by the formation of zones of inhibition **(CLSI, 2006)**. Filter paper discs 6 mm in diameter are impregnated with 10, 30, 60, and 100 µl of essential oil and deposited on the pre-seeded agar surface by swabbing with standardized microbial suspension.

A bacterial suspension is prepared from an 18-24 hour bacterial culture of *E.coli* and *Staphylococcus sp.* and is inoculated on a culture medium Muller Hinton or nutrient agar. In Petri dishes, the bacterial suspension is seeded by layer culture with a volume of 2 ml of

the inoculum, to cover the entire surface of the agar dish. The bacterial suspension is left in contact with the agar for 5 min at a very low temperature.

Subsequently, sterile discs impregnated with EO with different concentrations are deposited on the surface of the agar culture medium. The cans are incubated at 37°C for 24 hours **(Razakarivony et al, 2009)(Ponce et al, 2003)**. For reading these discs are prepared as follows**(Table 4)**:

Table 4: Experimental Design.

		Concentrations (ul)				Witnesses		
		10 ul	30 ul	60 ul	100 ul	E.D	S	Pure
Repetitions	R1	GN+EO	GN+EO	GN+EO	GN+EO	GN+ED	GN+S	GN
	R2	GN+EO	GN+EO	GN+EO	GN+EO	GN+ED	GN+S	GN

(GN= Nutrient Agar; E.D= Distilled Water; S= Distilled Water+ 1 Percent of Acetone).

The results obtained are expressed as sensitive (Se), intermediate (I), and resistant (R) to recommended standards. Antibiotic susceptibility is studied by the method of diffusion on a solid culture medium (method of discs), which allows the determination of the sensitivity of fast-growing bacteria to a range of antibiotics **(Cavallo et al., 2007)**. The strains tested are of clinical origin, suggesting their likely resistance to conventional antimicrobial agents.

Table 5: Resistance and Sensitivity of Bacteria to Essential Oils **(European Pharmacopoeia, 2002)**.

Strain sensitivity	Diameter of the inhibition zone
Sensitive (+++)	Greater than or equal to 15mm
Intermediate (+)	Less than 15 mm
Resistant (–)	non-existent

3.2.2.2 Antifungal Activity Assay

The method chosen to assess the antifungal activity of essential oils is the direct contact method **(Fandohan et al., 2004)**.

This method is based on the migratory power of fungi on a solid medium inside a Petri dish rich in essential oil. This method allows us to highlight the antifungal effect of the essential oil on the fungi, as well as the determination of the resistance or the sensitivity of these fungi to essential oil.

To prove the efficacy of the bioactive compounds of EOs against *Diplodia sp.* and *Acremonium sp.*, the final concentrations used in potato dextrose agar (PDA) were (10, 30, 60, and 100) ul. The controls were prepared with ED, S, and Witnesses. 0.5 ml of the different concentrations of essential oils are added to the PDA medium. The surface is completely covered to obtain a homogeneous distribution **(Remmal et al., 1993; Satrani et al., 2001)**. These concentrations are prepared as follows**(Table 6)**:

Table 6: Experimental Protocol .

		Doses (ul)				Witnesses		
		10 ul	30 ul	60 ul	100 ul	E.D	S	W
Repetitions	R1	PDA+EO	PDA+EO	PDA+EO	PDA+EO	PDA+ED	PDA+S	PDA
	R2	PDA+EO	PDA+EO	PDA+EO	PDA+EO	PDA+ED	PDA+S	PDA

(PDA = Potato Dextrose Agar; E.D= Distilled Water; S= Distilled Water+ 1 Percent of Acetone).

The influence of the antifungal activity of essential oils on the radial growth of Diplodia sp. and Acremonium sp. was performed by the agar medium assay according to **Tatsadjieu et al. 2009**. Potato Dextrose Agar (PDA) media with different concentrations of essential oils (10, 30, 60, and 100) ul were prepared by adding an appropriate quantity of essential oils to the melted media. About 16 ml of the medium were poured into Petri dishes. Then inoculated at the center with a mycelial disc (6mm of diameter) taken from the margins of 3–6 days old Diplodia sp. or Acremonium sp. cultures. two replicates were conserved for each treatment. Additionally, controls (without essential oils) were inoculated following the same procedure. Petri dishes were incubated at 25°C and the colony diameter was daily measured until control Petri dishes were fully covered with mycelia.

The antifungal activity was determined by measuring the fungal colony, using the following formulas:

- Evaluation of the mycelial growth inhibition rate:

  The antifungal activity was determined by measuring the inhibition of the growth of the fungal colony, using the formula described by **Leroux and Credet (2003)**.

  $$\mathbf{T=L\text{-}I/\ L\ X\ 100}$$

  - T: Rate of inhibition of the growth of the mycelium in percentage.
  - L: Diameter of the Witnesses' mycelial colony (cm).
  - I: Diameter of the mycelial colony in the experiment.

- Determination of mycelial growth rate (VC):

  $$\mathbf{CV = [D1/T1]+[(D2\text{-}D1)/T2]+\ [(D3\text{-}D2)/T3]+\ldots\ +\ [(Dn\text{-}Dn\text{-}1)/Tn].}$$

  - D = Diameter of the growth zone of each day (mm).
  - T = Incubation time (day).

3.2.2.3 Insecticidal Activity

Petri dishes were prepared to house the test insects. Indeed, for each box, a window has been dug. The window undergoes a double cover by a mosquito net, of a mesh of 1mm in diameter, to allow at the same time the ventilation of the box and to guarantee the captivity of the insects. A fresh uninfected host leaf is placed in each box (**Figure 10**).

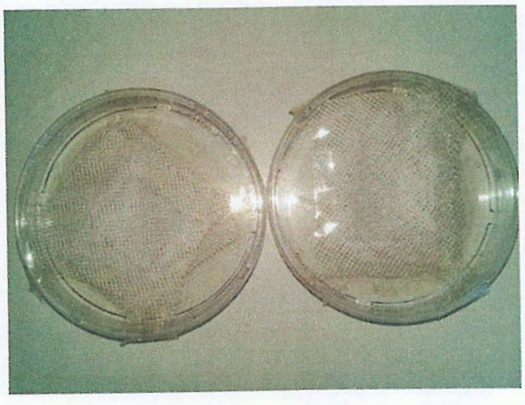

Figure 10: Insects Petri Dishes.

A batch of 10 homogeneous, unsexed insects, freshly sampled with the part of the leaf they colonize, was introduced into each Petri dish. The treatments were made by spraying EO preparations on the insects, where each box receives 1 ml of the corresponding preparation and will be immediately closed.
Two types of controls were also constituted, as described, under conditions identical to the test boxes. Two replicates were performed for each dose and dead insects were counted 3, 6, and 24 hours after the treatments **(Figure 11)**.

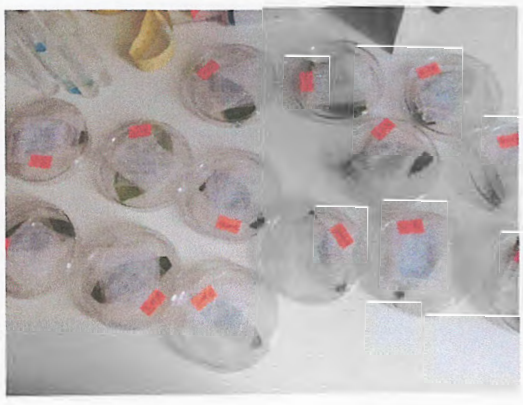

Figure 11: Experimental Test of Insect Treatments With EOs.

The insecticidal activity was determined by measuring the number of dead insects, using the following formulas:

- Mortality correction:

  The mortalities in the treated boxes (Mo) were expressed as corrected mortalities (Mc), taking into account the natural mortalities observed in the control boxes (Mt) according to Abbott's formula **(Habout et al., 2011)**.

$$\mathbf{Mc = (Mo\text{-}Mt/100 - Mt)\ 100}$$

- DL50 Determination:

  The letters DL denote "lethal Dose", LD50 is the amount of a material, given all at once, which causes the death of 50 percent (one half) of a group of test animals. The LD50 is one way to measure the short-term poisoning potential (acute toxicity) of a material.**(Habout et al., 2011)**.

CHAPTER 4

Results and Discussion

4.1 Yield in EO

The average yield of the extraction by Hydrodistillation is calculated concerning 100 g of the dry matter for the plant. The yield of *Achillea ligustica* essential oils is 0.75 percent. it is represented by the following diagram **(Figure 12)**:

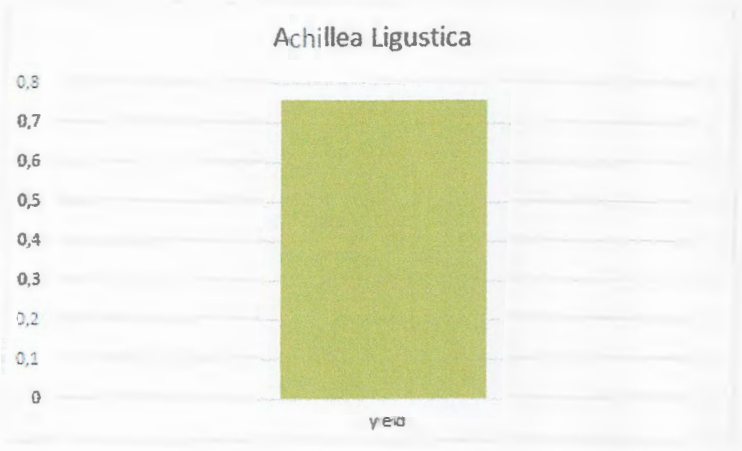

Figure 12: Average Yield of EO Extraction by Hydrodistillation.

The yield of essential oils can vary from one plant family to another, from one species to another, and even between plants of the same species. Furthermore, this difference i n EO content may be related to several factors such as the geographic area of collection, climate, development stage, and harvest season **(Belyagoubi, 2006; Khenaka, 2011)**.
The number of essential oils obtained from *Achillea ligustica* (0.75 percent) is higher than that in a study conducted in Italy by **Bader et al. (2007)**, (0.38 and 0.48 percent).
Achillea ligustica essential oils has been characterized by its color, smell and yield; these elements are presented in the **table 7**.

Table 7: Organoleptic Characteristics of *Achillea ligustica* EOs.

Essential oil of	Organoleptic characteristics			
Achillea ligustica	Color	Odor	Palatable	Yield
	Dark blue	Earthy smell, root and fresh carrot juice.	Bitter	0,75 percent

4.2 Fungi Isolation and Identification

4.2.1 Isolated Fungi

After the isolation and purification of the fungi, we tried to identify them from their macroscopic characters of colonies (appearance, colour, shape, contour,...) and on microscopic characters of the mycelium and conidia or spores (partition of mycelium, a form of spores, form of organs fruiting, etc.). Four fungi was purified, *Acremonium sp.*, *Diplodia sp.*, *Fusarium solani*, and *Verticilium sp.*. Only two strains were selected to do the biological test, namely *Acremonium sp.* and *Diplodia sp.*. More information about those two strains can be found in appendix N°4.

The two isolated species, *Acremonium sp.* and *Diplodia sp.* are found in the galleries dug into the wood. After their introduction into the host trees in the form of spores, these - finding favorable conditions for germination develop causing the phenomenon of decline of the *Cork oak*

trees, and considered mushrooms of nutritional interest **(Harrington, 2005); (Belhoucine, 2013)**.

4.2.2 Description of Identified Genera

4.2.2.1 *Diplodia sp.*

In culture on PDA, *Diplodia sp.* initially appears sponge white, turning to dark grey after about five days. The bottom becomes first olive green, then black **(Dreaden et al., 2011)**, the fungus fills the petri dish, and then stacks the mycelia in an aerial manner **(figure 13)**.

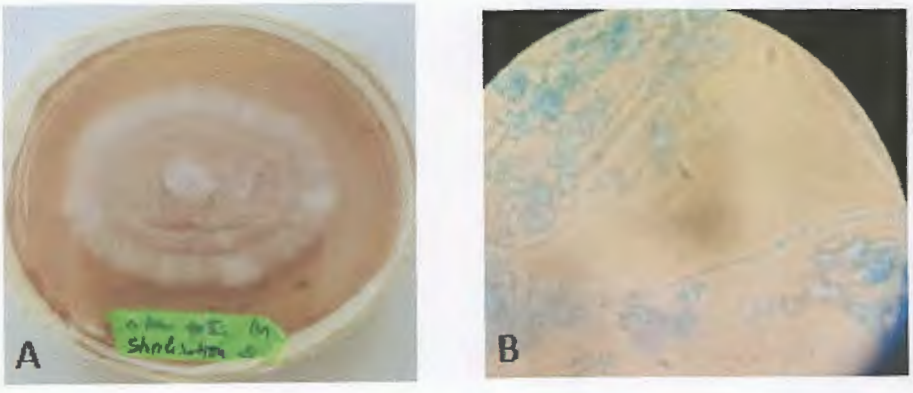

Figure 13: Characteristics of *Diplodia sp.* Fungal Isolate Obtained (A: Macroscopic aspect, B: Microscopic aspect).

4.2.2.2 *Acremonium sp.*

It is a fungus of the genus *Acremonium*, of the order Hypocreales, this species is a potential pathogen identified f or s everal t ree s pecies i ncluding t he *C ork o ak*. T he i solated f ungus is characterized by a hyaline and partitioned mycelium, with conidia which are usually unicellular, sometimes bicellular, hyaline, or pigmented. Colonies appear compact and sometimes moist, flat, or pleated; they are covered by loose hyphae, which give them a powdery appearance. Colonies may be white, pale grey, or pink depending on the species **(figure 14)** **(Kiffer and Morelet, 1997)**.

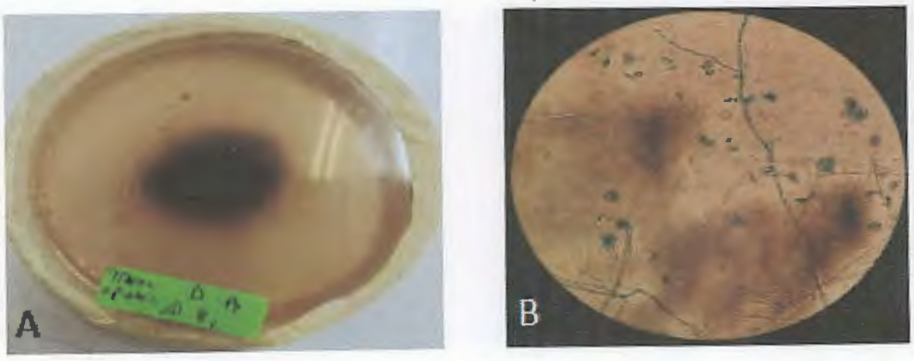

Figure 14: Characteristics of *Acremonium sp.* Fungal Isolate Obtained (A: Macroscopic aspect, B: Microscopic aspect).

4.3 Biological tests

4.3.1 Antibacterial Activity

The diameters of the zones of inhibition (mm) differ from one strain to another and from concentration to another. In a solid medium, the evaluation of the antibacterial activity of the oil of *Achillea ligustica* is determined by the measurements of the diameters of inhibition of the growth in millimeters around the discs (**table 10**).

Table 8: The Average Diameter of the Inhibition Zones.

Average diameters of inhibition zones (mm)				
Concentrations	*Staphylococcus sp.*		*E. coli*	
	48H	72H	48H	72H
10ul	7,75	8,125	7,875	8,25
30ul	7,875	8,375	8,75	8,875
60ul	8,25	8,625	9,125	9,625
100ul	8,75	9	10,125	10,375
S	7,5	7,625	7,875	8,25
E.D	6,875	6,875	7,625	7,875

The diameters of inhibition zones of the bacterial growth increase with the increase of the doses of the oils.
According to the results in **(Table 10)**, the essential oils of *Achillea ligustica* act differently on the strains tested. The largest inhibition zone was observed in *E. coli* with an inhibition zone of (10,375mm) in diameter. The weakest zone was observed in *Staphylococcus sp.* with a diameter of (9mm) **(Figure 16)**. The results of the antibiogram test show a variation in the inhibition efficiency of *Achillea ligustica* essential oil vis-a-vis the bacterial strains tested: *Escherichia coli* and *Staphyloccocus sp.*.
The work reported by **Carlo et al,(2005)** confirms the result found for the essential oil of *Achillea ligustica* against the bacterial strain *Staphylococcus sp.* and *Escherichia coli* where they found a weak activity.

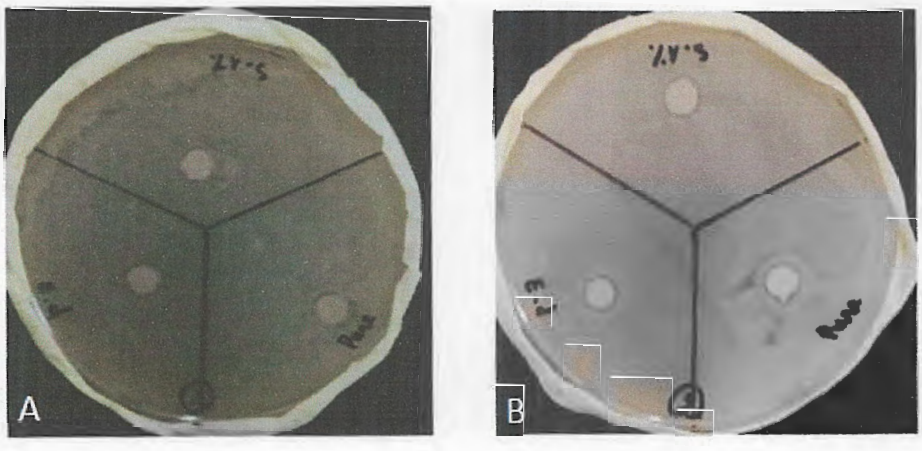

Figure 15: Witnesses Boxes of Two Bacteria Strains (A: *Staphylococcus sp*, B: *E. coli*).

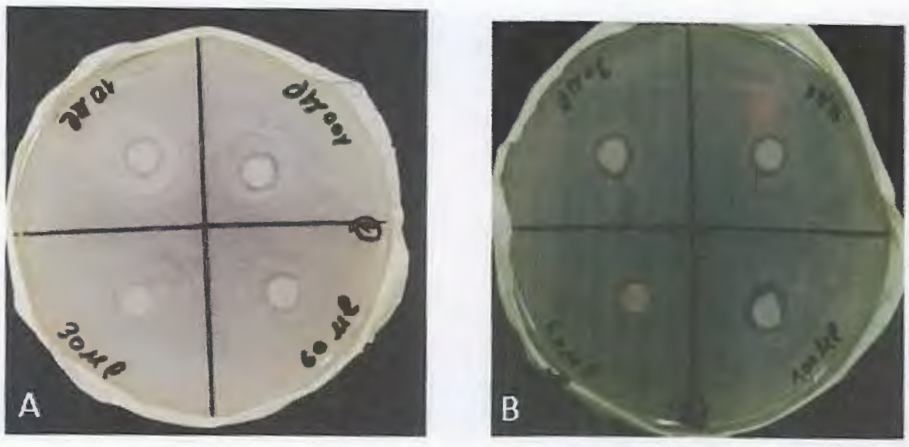

Figure 16: Test Boxes of Two Bacteria Strains (A: *Staphylococcus sp*, B: *E. coli*).

The inhibition diameters are represented in the histograms **(figure 17)**. It should be noted that the initial diameter of the disk deposited in each Petri dish is 0.6 cm, so if the diameter increases beyond this value it is considered that there is inhibition, otherwise, the diameter of 0.6 indicates a complete lack of inhibition.

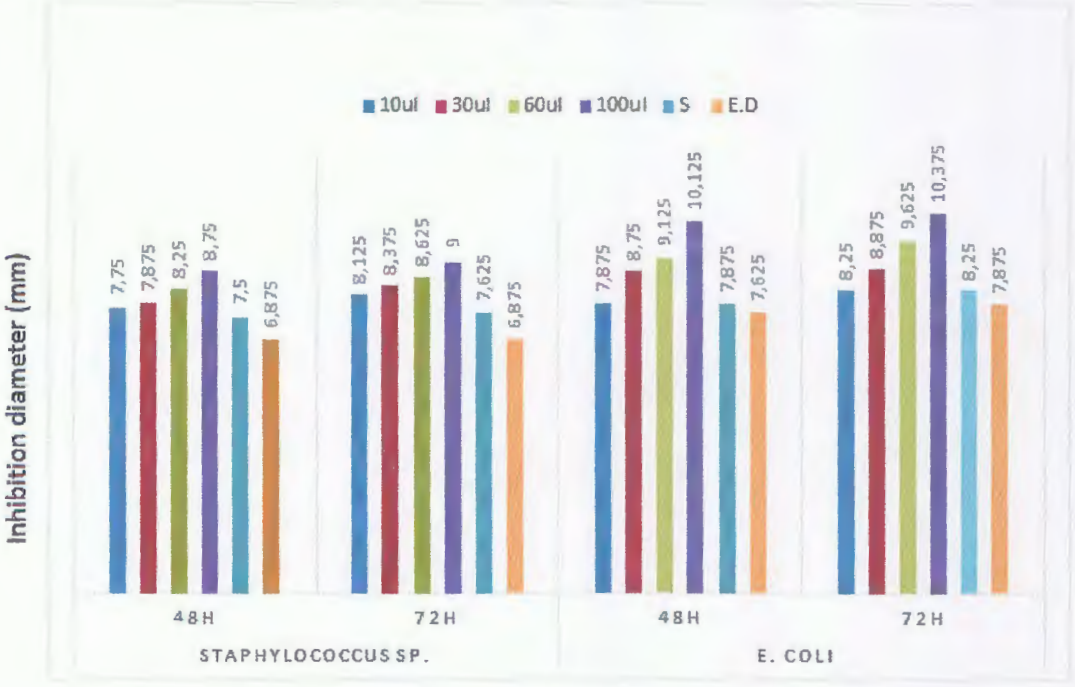

Figure 17: The Effect of the Four Concentrations of the Oils and the Three Witnesses on the Two Bacteria After 48 and 72 Hours (Inhibition diameter mm).

From the **(figure 15, 16, 17)**, and **(Table 10)** it can be seen that the higher the concentration of oils, the bigger the inhibition diameter compared to witnesses. It can also be noted that the concentrations affect more on *E. Coli* compared to *Staphylococcus sp.*.
In general, the action of *Achillea ligustica* on the growth of *E. coli* tested showed inhibition zones of (7,87, 8,75, 9,12, and 10,12) mm respectively for concentrations (10, 30, 60, and 100) ul during the first 48h of treatment. On the other hand, after 72h, an increase in the inhibitions zones diameters was observed which reached (9,62 and 10,37) mm by the concentrations (60 and 100) ul respectively and compared with that obtained with witnesses (7,87)mm.

In the current study, Gram-positive bacteria (*Staphylococcus sp.*) were found to be more resistant to the components of the EOs tested than Gram-negative bacteria(*E. coli*). This behavior was similar to what other authors had observed **(Borges et al, 2014; Borges et al, 2013; Zaika, 1988; Prabuseenivasan et al, 2006) (Appendix N°2)**.
In this sense, many studies suggest that the structure and composition of the cellular membrane (cytoplasmic membrane and/or outer membrane and cell wall) may explain the difference in resistance to antimicrobials between Gram-positive and Gram-negative bacteria **(Shrivastava et al, 2007; Tamboli and Lee, 2013)**. The cell wall is more complicated in Gram-negative bacteria. It is composed of an outer membrane (OM) made of phospholipids and lipopolysaccharides (LPS) and a thin peptidoglycan layer next to the cytoplasmic membrane **(Nazzaro et al, 2013)**.
The existence of hydrophilic channels known as porins, which typically prevent the entry of hydrophobic molecules, regulates the flow through the OM. In this study, the presence of an OM in the cytoplasmic membrane in Gram-negative bacteria did not cause an increase in resistance to the components of EOs. In fact, some compounds have now proven that they can disrupt OM through the release of LPS **(Nohynek et al, 2006)**.
Additionally, several phenolic components of EOs have shown a capacity to interact with OM and thus exhibit bactericidal activity **(Helander et al, 1998; Puupponen et al, 2005)**. While Gram-positive bacteria lack the OM, their cell walls are made of a thicker peptidoglycan layer that gives the cells more rigidity and makes it more difficult for antimicrobials to penetrate.

Another characteristic that plays a role in the resistance to antimicrobials is the shape of the cell. The activity of EOs and/or their components differs depending on the shape of the bacteria studied. Normally, rod-shaped (*E. coli*) bacterial cells are more sensitive to EOs than coccoid cells(*Staphylococcus sp.*) **(Hajlaoui et al, 2009)**. These characteristics can help to explain why the elements of EOs have less effect on the Gram-positive bacterium *Staphylococcus sp.*.

According to the results obtained from the **(table 8)** and histogram**(Figure 17)**, the resistance and sensitivity of bacteria to essential oils of *Achillea ligustica* can be represented by the **(Table 9) (European Pharmacopoeia, 2002)**.

Table 9: the resistance and sensitivity of *E. coli* and *Staphylococcus sp.*.

	Strain sensitivity	
Concentrations	*Staphylococcus sp.*	*E. coli*
10 ul	+	+
30 ul	+	+
60 ul	+	+
100 ul	+	+

From (Table 9) we can conclude that the resistance of the two strains (*E. coli* and *Staphylococcus sp.*) to essential oils of *Achillea ligustica* is intermediate.
It is inferred that the essential oil of *Achillea ligustica* showed an important activity antimicrobial. All these studies are in agreement with our studies for the essential oil of Achillea ligustica that have revealed different areas of inhibition against Gram (-) strain and resistance to Gram (+) bacteria such as *Staphylococcus sp.*.

4.3.2 Antifungal Activity

The antifungal activity of essential oils is mainly due to their composition in various bioactive molecules, belonging to different classes chemical, which can be used to reduce the contaminating fungal flora **(Ouraini et al., 2005)**.

The results obtained from the antifungal activity of *Achillea ligustica* essential oils to certain fungal strains have shown an important sense of the strains *Acremonium sp.* and *Diplodia sp.* at a concentration greater than 30ul. Therefore, concentrations required to perform an activity antimicrobial are therefore higher molecules isolated from plants compared to bacteria and fungi. Indeed, a phytochemical molecule is considered "antimicrobial" when it inhibits the growth of microorganisms at minimum inhibitory concentrations (CMI) between 100 and 1000 ug/mL.
The two fungal strains after incubation in the absence (Witnesses) and in the presence of different concentrations of essential oils of *Achillea ligustica* shown in the **Figure (18, 19, 20, 21)** bellow.

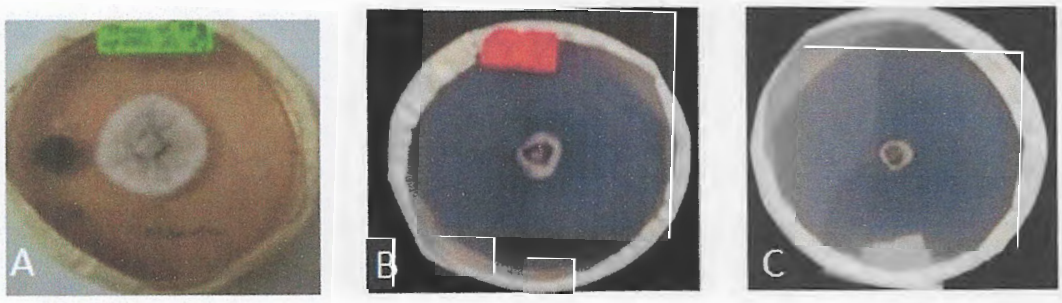

Figure 18: *Acremonium sp.* Witnesses (A: T(no concentration), B: S(solvent witness), C: E.D(distilled water witness)).

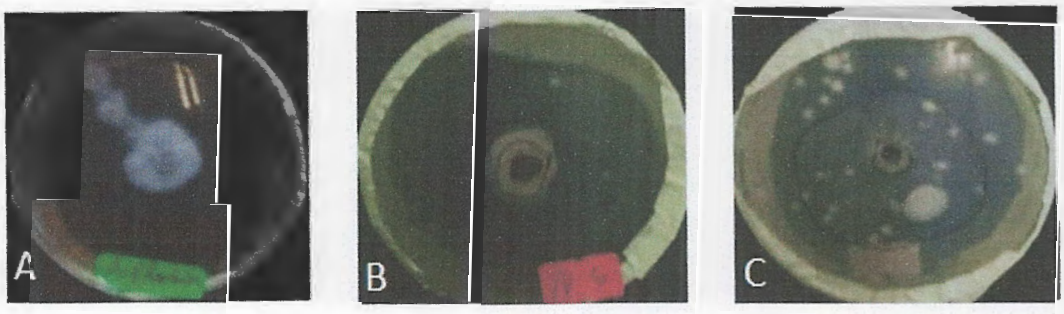

Figure 19: *Diplodia sp.* Witnesses (A: T(no concentration), B: S(solvent witness), C: E.D(distilled water witness)).

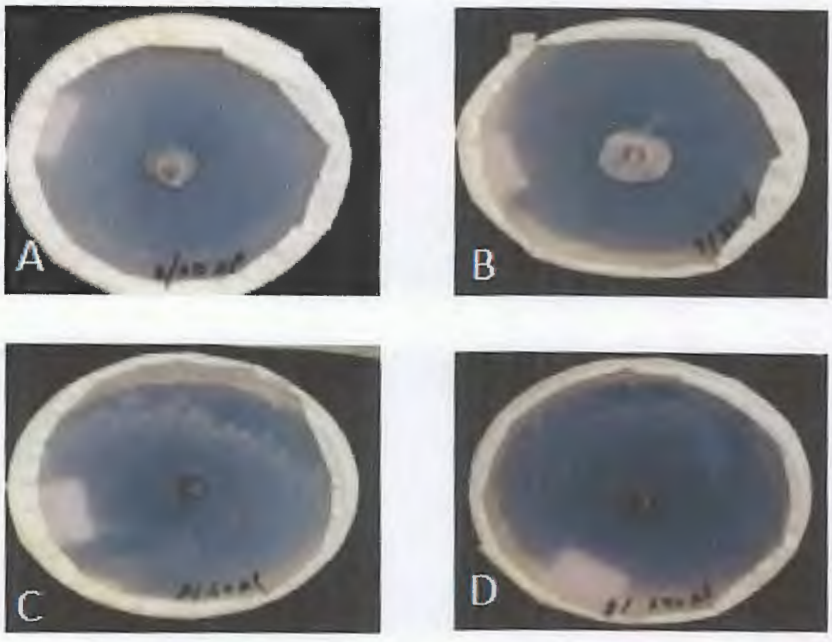

Figure 20: *Acremonium sp.* Tested (A: 10 ul, B: 30 ul, C: 60 ul, D: 100 ul).

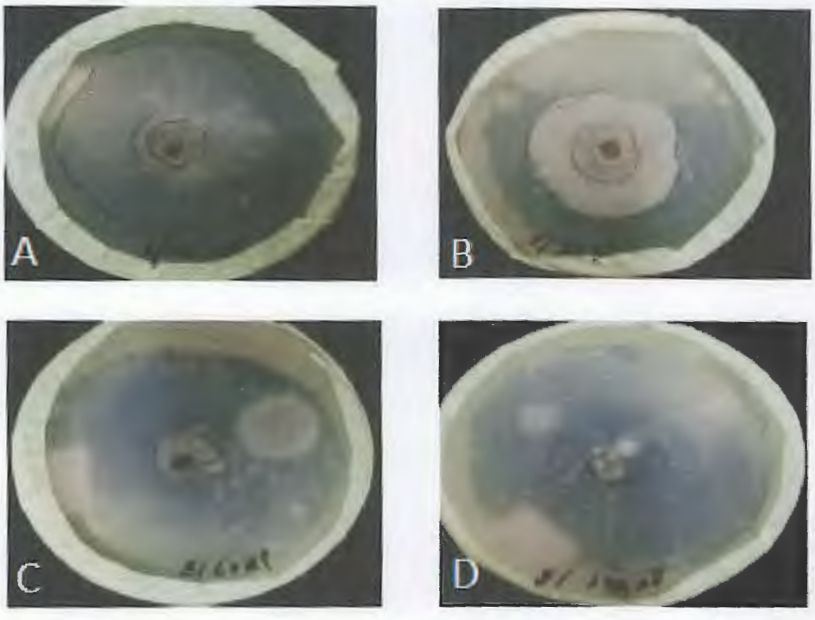

Figure 21: *Diplodia sp.* Tested (A: 10 ul, B: 30 ul, C: 60 ul, D: 100 ul).

4.3.2.1 The Action of Essential Oils on Mycelial Growth of the Isolates Tested

The extracts' in vitro fungicide activity was evaluated by determining the capacity of inhibition of mycelial growth of each of the species under study. In the treatment of *Achillea ligustica* essential oil against *Acremonium sp.*, the biological activity was proportional to the increase in concentration. Observed a mycelia growth of (8,5 and 8)mm at concentrations of (60 and 100) ul on the first day respectively, and with mycelial growth significantly reduced with the same

concentration (19 and 12) mm during the second day of incubation, the essential oil showing greater inhibition compared to the growth of the corresponding control sample. However, the mycelial growth in the control samples was (16,5 and 56,5) mm on the first and the second day respectively.
The same thing for *Diplodia sp.*. During the first d ay o f i ncubation, a t t he c oncentrations (60 and 100) ul the mycelial observed growth of (7,75 and 7) mm respectively, and the growth diameter of *Diplodia sp.* mycelial on the second day was (11,75 and 10) mm at the same concentrations. However, the mycelial growth of the wetness was (11,75 and 17,5) mm as shown in **(Table 10) (figure 22)**.

Table 10: The Average Growth Diameters (mm) of *Diplodia sp.* and *Acremonium sp.*.

Average growth diameter (mm)				
Concentrations	*Diplodia sp.*		*Acremonium sp.*	
	DAY1	DAY2	DAY1	DAY2
10ul	9,25	13,25	9,5	21,75
30ul	8,25	12,5	9,25	20
60ul	7,75	11,75	8,5	19
100ul	7	10	8	12
S	10,5	14,5	11,75	24
E.D	11,25	15,5	14,25	41,5
T	11,75	17,5	16,5	56,5

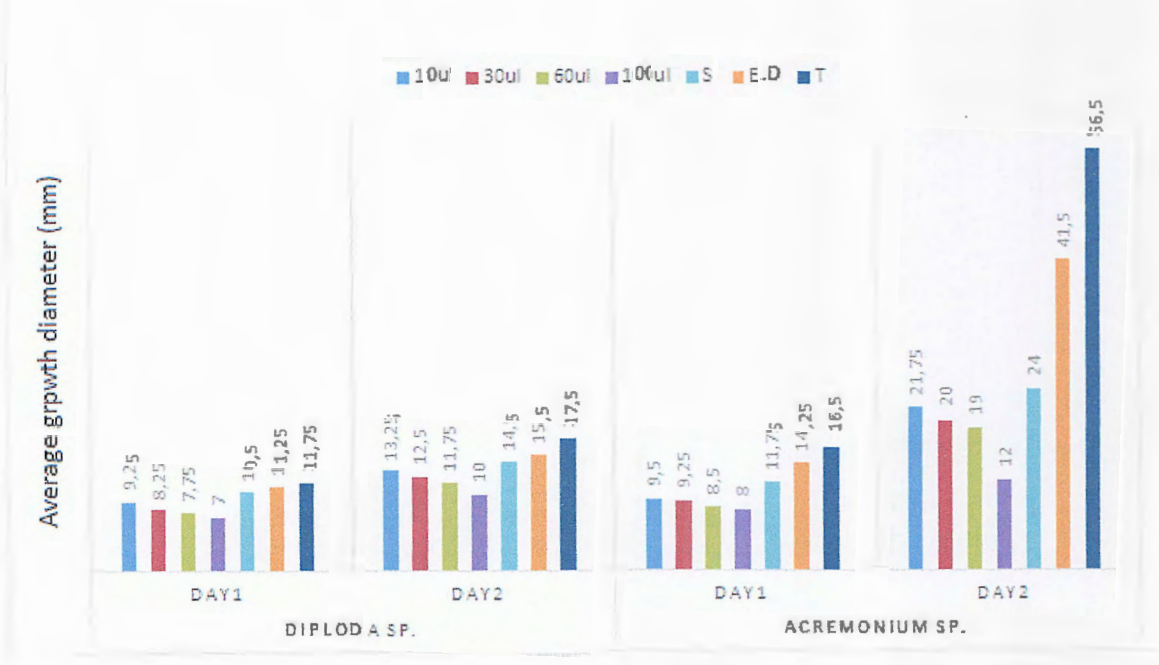

Figure 22: Average Growth Diameter (mm) of Four Concentrations and Three Witnesses on *Acremonium sp.* and *Diplodia sp.*.

4.3.2.2 Inhibition Rate of Essential Oils on Growth of Tested Isolates (T Percent)

The result of **(Figure 23) (Table 11)**, shows that after two days of treatment, all concentrations tested show effects n ot s imilar t o u ntreated witnesses.

Table 11: Inhibition Rate Percent.

Inhibition rate (T percent)				
Concentrations	*Diplodia sp.*		*Acremonium sp.*	
	Day1	Day 2	Day 1	Day 2
10ul	21,3	24,3	42,4	61,5
30ul	29,8	28,6	44	64,6
60ul	34	32,9	48,5	66,4
100ul	40,4	42,8	51,5	78,8
S	10,6	17	28,8	57,5
E.D	4,2	11,4	13,6	26,5

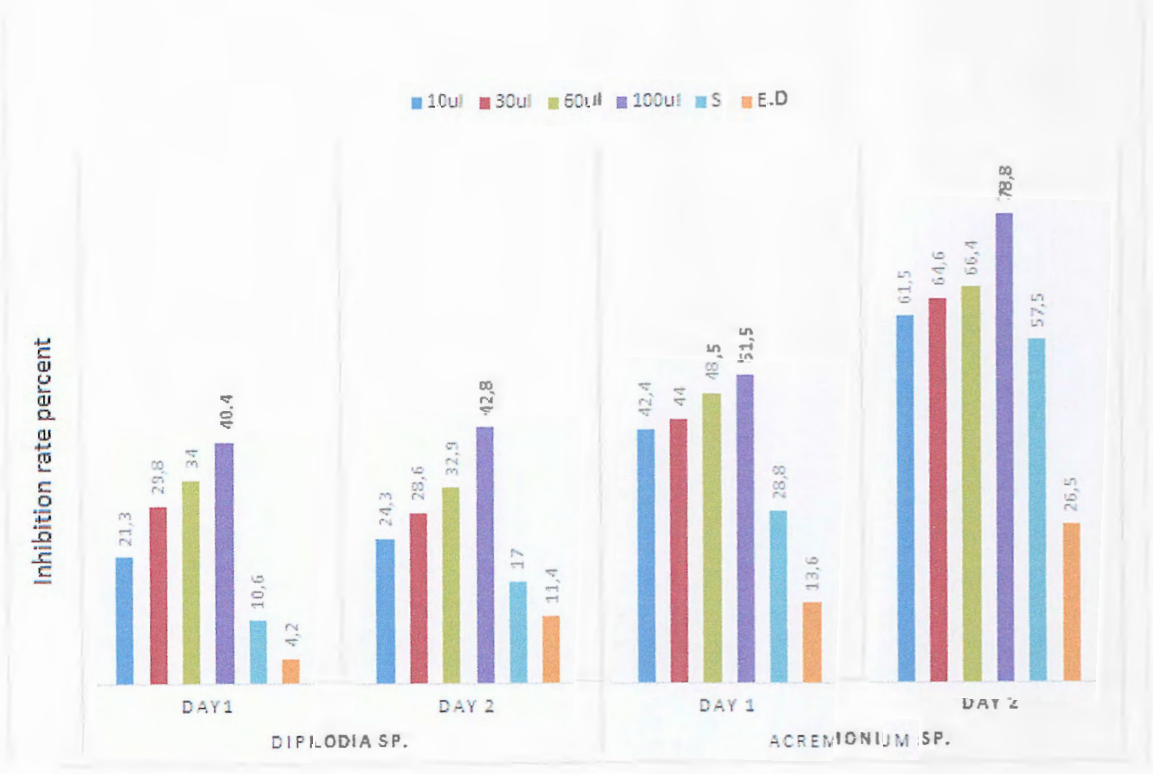

Figure 23: The Effect of the 4 Concentrations of Essential Oils on the Growth of *Diplodia sp.* and *Acremonium sp.* After 1 and 2 Days of Incubation (inhibition rate percent).

The results of **(Figure 23)** and **(Table 11)** show that the essential oils have a different inhibitory activity, but the total inhibition (100 percent) was absent by the application of the concentrations (10, 30, 60 and 100 ul).

The results obtained after testing different concentrations of essential oils of *Achillea ligustica* against *Acremonium sp.* have remarkable percentages (42,4- 44- 48,5 and 51,5) percent for (10- 30- 60 and 100) ul respectively on the first day of treatment. However, on the second day, it is noted the graduated effect of essential oils on mycelial growth. Despite this, there was considerable variability in the effect of essential oils on mycelial growth of *Diplodia sp.* on the first day, compared with that recorded with *Acremonium sp.* as a gradual increase in inhibition rate was observed on the second day of treatment (61,5- 64,6- 66,4 and 78,8) percent for (10- 30- 60 and 100) ul respectively and comparatively with witnesses (57,5 and 26,5). This shows the effectiveness of the oils in inhibition of fungus growth tested. Based on the inhibition rates generated by the studied essential oils, these present the best activity on the two strains tested.

4.3.2.3 Determination of mycelial growth rate (VC)

According to the results shown in **(Figure 24)** and **table 12**, for *Diplodia sp.* a maximum speed of mycelial growth (11,25 mm/d) for the concentration (10 ul) is observed, thereafter the speed is reduced with the increase in the concentration of aqueous extract, we recorded a lower value (8,5 mm/d) for the concentration (100 ul).
As for *Acremonium sp.*; a maximum rate of mycelial growth (15,6 mm/d) is observed for the dose (10 ul). Then a increase of the speed (14,6mm/d, 13,75mm/d, 10mm/d) for the doses (30 ul, 60 ul, 100 ul) respectively.
The speed of mycelial growth of *Diplodia sp.* and *Acremonium sp.* is very low comparing to witnesses(14,6 and 36,5 mm/d). This proves the effectiveness of essential oils on the two fungal strains.

Table 12: Mycelial Growth Rate (VC).

Mycelial growth rate (VC)		
Concentrations	Diplodia sp.	Acremonium sp.
10ul	11,25	15,6
30ul	10,4	14,6
60ul	9,75	13,75
100ul	8,5	10
S	12,5	17,9
E.D	13,4	27,9
T	14,6	36,5

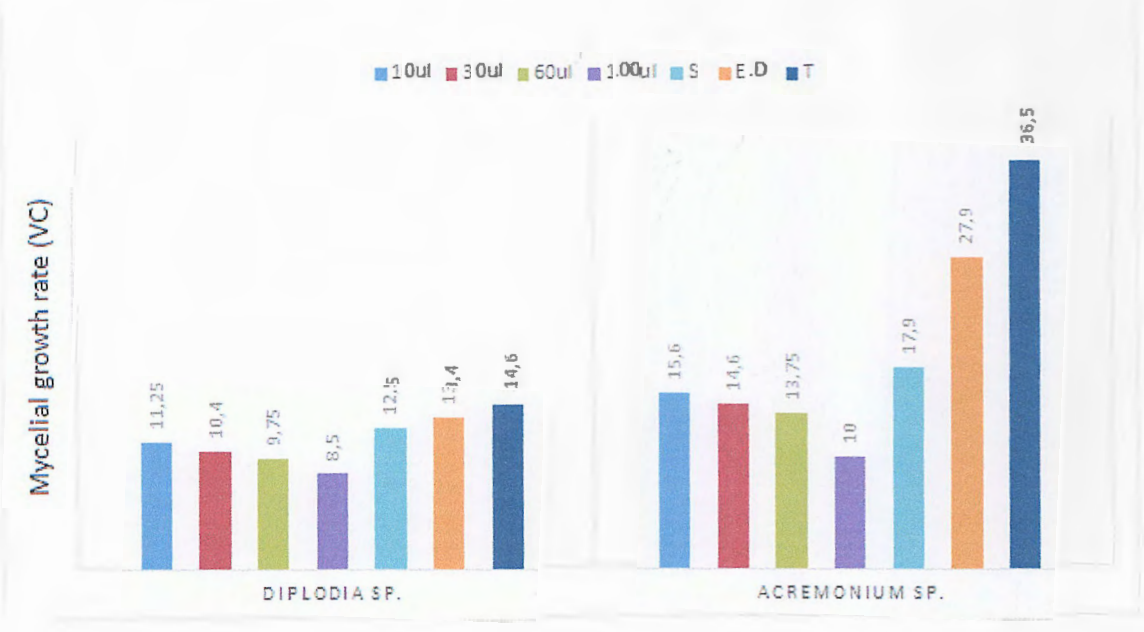

Figure 24: Mycelial Growth Rate (VC).

The essential oil was found to have significant antifungal activity against the two tested fungi. These results are in agreement with the data reported by **Giamperi et al., 2018**.

4.3.3 Insecticidal Activity

4.3.3.1 Average Mortalities Observed Following Treatment With EO of *A. ligustica*

The (**table** 13) and (**Figure** 25) represent the average observed mortalities and the standard deviations, following the treatment of the *Corn aphids* populations at the EOs of *A. ligustica* as a function of concentration and time.

Compared to witnesses, the different concentrations affected the insects for the first three hours.

This effect increased over time, as after six hours it was observed that 9 insects died under the influence of the concentration of 100 ul. Not only that, even the lowest concentration (10 ul) caused the death of five insects. This indicates the significant toxicity of *Achillea* essential oils. The work reported by **(Laater and Bousmaha, 2012)** confirms the result found for the essential oil of *Achillea* against the Aphids insects where small concentrations can cause a remarkable effect.

Table 13: Average Mortalities.

Concentrations	Average mortalities		
	3H	6H	24H
10ul	0	2,5	5
30ul	1	4,5	6,5
60ul	1	6	8
100ul	1,5	9	10
S	0	3	5,5
E.D	0	0,5	2,5
T	0	0,5	1,5

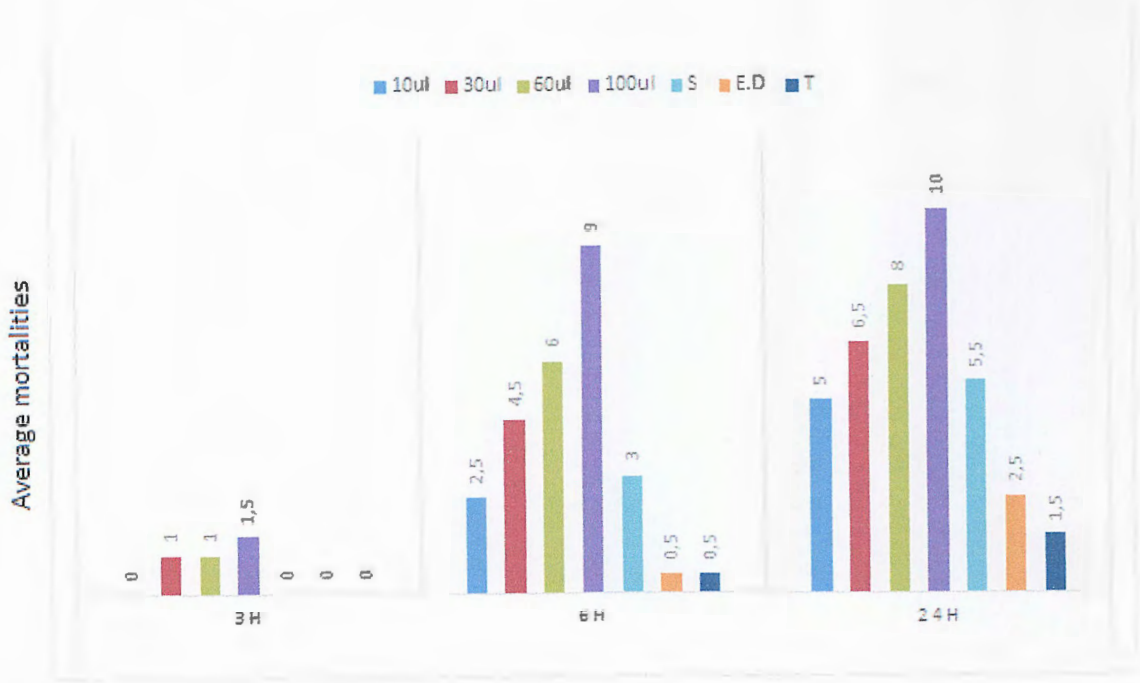

Figure 25: Average Mortalities Observed Following Treatment With EO of *A. ligustica* .

4.3.3.2 Mortality Correction

The **Figure 4.15** and **(Table 4.8)** displays the corrected and cumulative mortality rates, recorded following the treatment of *Corn aphids* populations with EOs of *A. ligustica* as a function of concentration and time.

Table 4.8: Mortality Correction (percent).

	Mortality correction		
Concentrations	3h	6h	24h
10ul	0	21	11,8
30ul	10	42	58
60ul	10	57,9	76,5
100ul	15,0	89,5	100
S	0,0	26,3	47
E.D	0	0	11,8

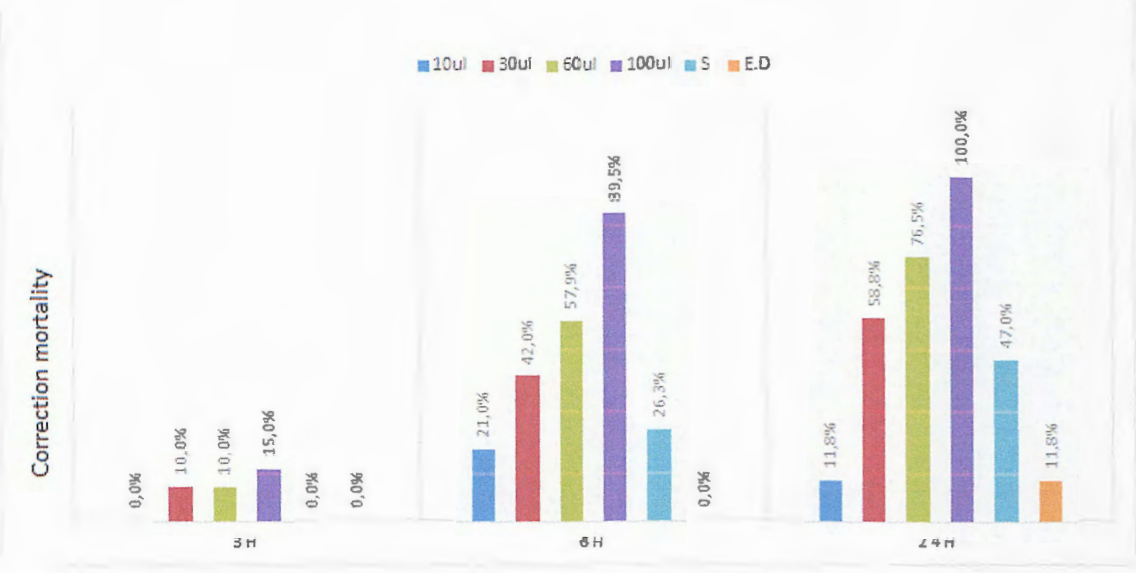

Figure 4.15: Corrected Mortality Rate.

A change in the mortality rate is observed with the concentration of essential oil tested and time. The highest concentration (100 ul/ml) causes total mortality, 100 percent, of *Corn aphids* after 24 hours of treatment. The lowest concentration (10 ul/ml) causes 12 percent mortality at the same time interval, which confirms the high degree of toxicity of this essential oil to these insect.

For the 30 and 60 ul concentrations, the insects showed a slight resistance which did not last more than 3 hours since it was possible to achieve significant mortalities after 6 hours. Therefore, we can conclude that the EOs of this plant does not require a long time to manifest, this indicates the existence of acute toxicity causing very high mortality during the first six hours post-treatment.

4.3.3.2 Mortality Correction

The **Figure** 26 and (**Table** 14) displays the corrected and cumulative mortality rates, recorded following the treatment of *Corn aphids* populations with EOs of *A. ligustica* as a function of concentration and time.

Table 14: Mortality Correction (percent).

	Mortality correction		
Concentrations	3h	6h	24h
10ul	0	21	11,8
30ul	10	42	58
60ul	10	57,9	76,5
100ul	15,0	89,5	100
S	0,0	26,3	47
E.D	0	0	11,8

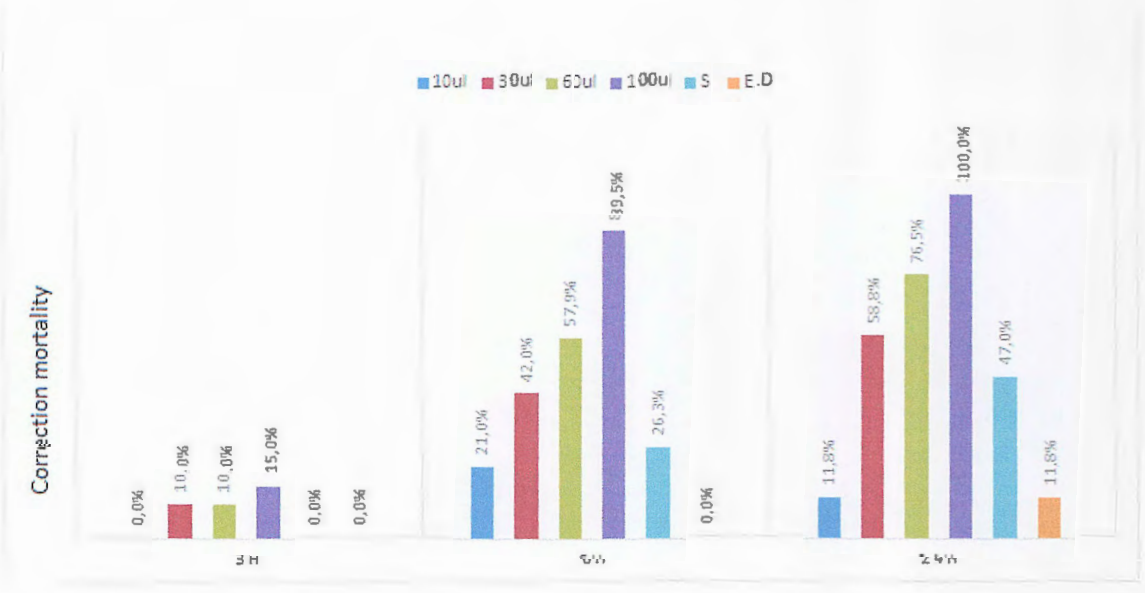

Figure 26: Corrected Mortality Rate.

A change in the mortality rate is observed with the concentration of essential oil tested and time. The highest concentration (100 ul/ml) causes total mortality, 100 percent, of *Corn aphids* after 24 hours of treatment. The lowest concentration (10 ul/ml) causes 12 percent mortality at the same time interval, which confirms the high degree of toxicity of this essential oil to these insect.

For the 30 and 60 ul concentrations, the insects showed a slight resistance which did not last more than 3 hours since it was possible to achieve significant mortalities after 6 hours. Therefore, we can conclude that the EOs of this plant does not require a long time to manifest, this indicates the existence of acute toxicity causing very high mortality during the first six hours post-treatment.

4.3.3.3 DL50 Determination

Table 15: Percentage of Dead Insects Under the Influence of the Concentrations of Essential Oil.

Concentrations	Insect mortality percent
10ul	11,8
30ul	58,8
60ul	76,5
100ul	100

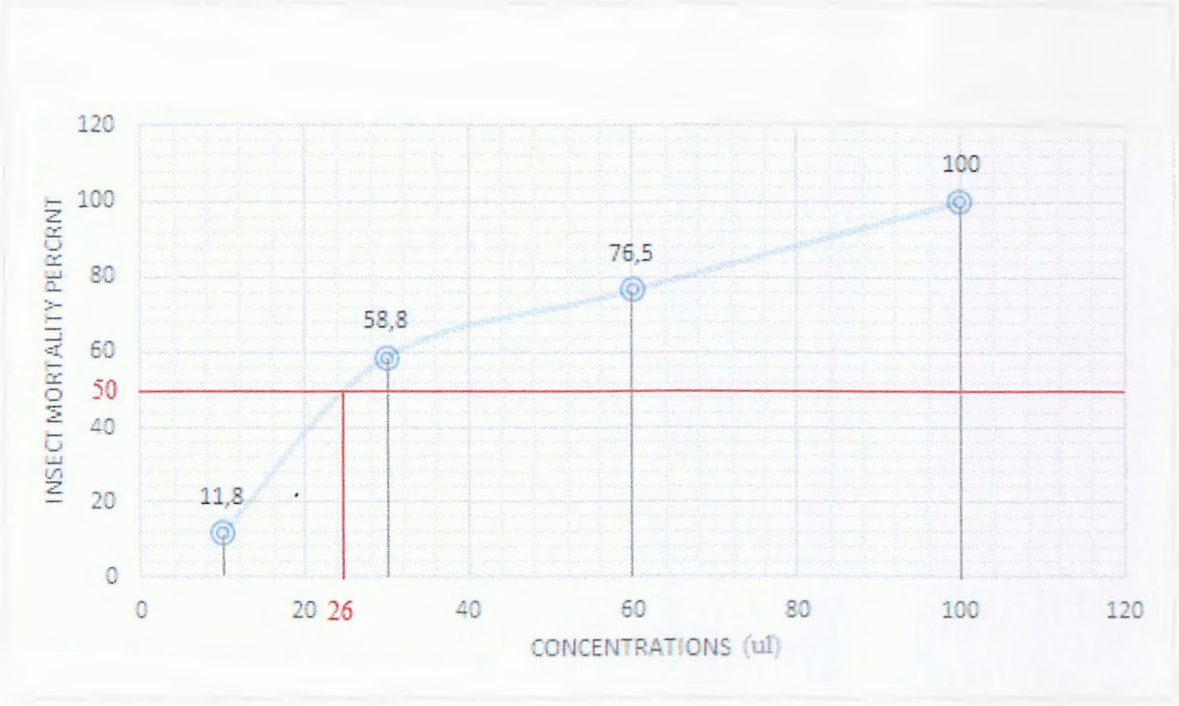

Figure 27: Percentage of Dead Insects Under the Influence of the Concentrations of Essential Oil.

According to **(Figure 27)**, *Achillea ligustica* essential oil is very effective, with an LD50 of 26ul calculated after 24 hours of treatment.

Conclusion

In this work, we are interested in making a synthesis of the effect of essential oils vis à vis bacteria, fungal isolates and on the insect tested, based on results obtained in experiments already done.

The extraction of EOs from the leaves and flowers of *Achillea ligustica* was carried out by hydrodistillation, which gave a high yield (0.75 percent).

Achillea essential oils express inhibition on Gram-negative bacteria such as *E. coli*, and Gram-positive bacteria such as *Staphylococcus sp.*, on other hand, on two fungal isolates as *Acremonium sp.*, and *Diplodia sp.*. Therefore, The essential oils showed a remarkable antifungal, antibacterial, and anti-insect activity, proportional to the concentration. Indeed, the higher the dose of essential oil, the lower the mycelial growth, and the bigger the bacteria inhibition zone. However, the results obtained indicate proved to be very effective, especially against *Corn aphids.*

Following these results, it would be interesting to complete this study with other species of plants and research the antibacterial, antifungal, and insecticidal activity of EOs. to develop a means of biological control based on natural substances.

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Appendix

Appendix N°1:

- **Preparation of PDA culture medium:** We prepared it according to the following protocol:
  - Cook 200 g of peeled, washed and thinly sliced potatoes in a 1L of water for 20 minutes.
  - Filter the liquid with a sieve into a glass bottle and add 20 g of glucose and 17 g of agar agar and top up to 1L if necessary.
  - One must autoclave for 30 min at 110°C.
  - Dispense 20 ml of medium in each sterile Petri dish, Once the medium is solidified, the boxes are placed in an oven set at a temperature of 25°C.

- **Nutrient Agar Composition:**
  Peptone: Peptone is the principal source of organic nitrogen for the growing bacteria.
  Beef extract/yeast extract: It is the water-soluble substances which aid in bacterial growth, such as vitamins, carbohydrates, organic nitrogen compounds and salts.
  Agar: It is the solidifying agent.
  NaCl: The presence of sodium chloride in nutrient agar maintains a salt concentration in the medium that is similar to the cytoplasm of the microorganisms.
  Distilled water.

- **Mueller Hinton Agar Composition:**
  It contains Beef Extract, Acid Hydrolysate of Casein, Starch, and Agar. Beef Extract and Acid Hydrolysate of Casein provide nitrogen, vitamins, carbon, amino acids, sulfur, and other essential nutrients.

Appendix N°2:

Escherichia coli (*E. coli*) is a Gram-negative,it can be found in the environment, food, and the intestines of both humans and animals. A large and diverse group of bacteria are *E. coli* . While the majority of *E. coli* strains aren't harmful, some of them can make you sick. Some *E. coli* strains can cause diarrhea, while others can cause infections, respiratory infections, and other problems.

Staphylococcus sp. are gram-positive aerobic organisms.*Staphylococcus sp* . is one of the most dangerous bacteria that could cause food poisoning. It is a pathogenic bacterium which is able to produce enterotoxin in foods.

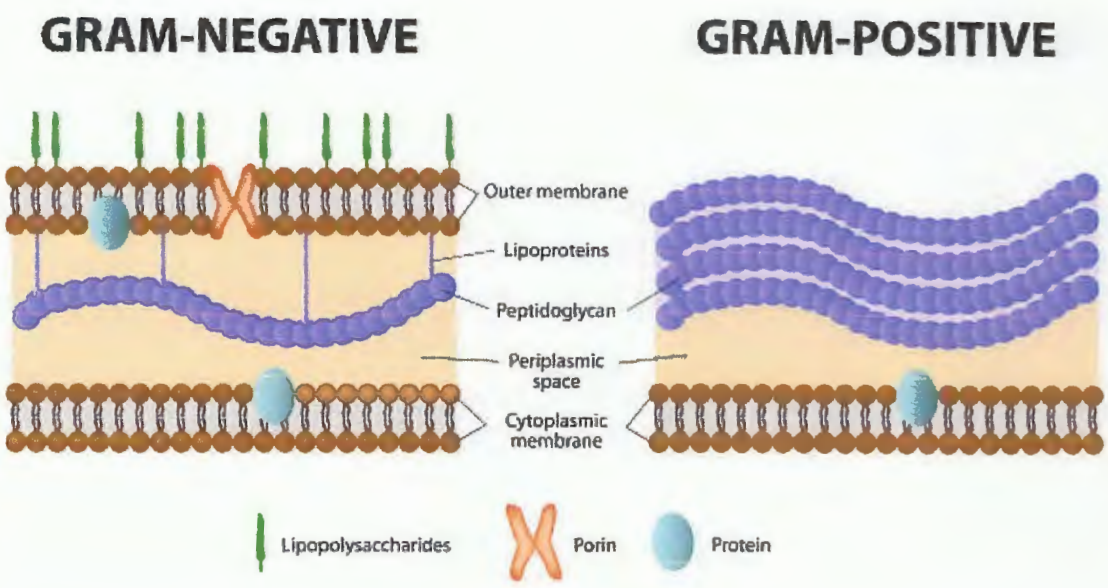

Figure 4.17: The Difference Between Gram-negative and Gram-positive Bacteria.

Gram positive bacteria have lots of peptidoglycan in their cell wall. but Gram negative bacteria have less peptidoglycan.

Appendix N°3:

The corn leaf aphid is a blue-green or gray. Most corn leaf aphids are wingless. However, as populations increase, some develop delicate, filmy wings. These wings enable them to fly to uninfested plants to start new colonies. Corn leaf aphids do not generally appear until mid-June or early July.

Effect of Essential Oil of Achillea Ligustica to Inhibit Growth and Infestation of Certain Fungi, Bacteria and Insect.

Presented by:
Belharda Rima

Session: September 2022

Abstract:

This work aims to study the effect of *Achillea ligustica* essential oils and know if this oil can inhibit the growth of bacteria, and fungi, and stop insect infestation.

In this study, hydrodistillation was used to obtain essential oils (EOs) from *Achillea ligustica*. The antifungal, antibacterial, and anti-insect activities of *Achillea* essential oils at various concentrations (10- 30 - 60 and 100) ul were evaluated in vitro against *E. coli, Staphyloccocus sp.*, and on two fungal strains isolated from Branches, stems, and leaves of *Cock oak* tree, and *Corn leaf aphid.*

The results show that most doses of essential oils of *A. ligustica* were active, and the dose of 100 ul is the most effective.

The essential oil of *Achillea ligustica* shows remarkable antibacterial, antifungal, and anti-insect effects. This is a recognized leap for the future of natural therapy.

Key words: *Achillea Ligustica, Corn Leaf Aphid, Escherichia coli, Staphylococcus sp, Essential Oil.*

Résumé:

Ce travail a pour objectif d'étudier l'effet des huiles essentielles d'*Achillea ligustica* et de savoir si cette huile peut inhiber la croissance des bactéries et des champignons, et stopper l'infestation par les insectes.

Dans cette étude, l'hydrodistillation a été utilisée pour obtenir des huiles essentielles (HE) à partir de *Achillea ligustica.* Les activités antifongiques, antibactériennes et anti-insectes des huiles essentielles de *Achillea* à différentes concentrations (10- 30 - 60 et 100) ul ont été évaluées in vitro contre *E. coli, Staphyloccocus sp.*, et sur deux souches fongiques isolées de Branches, tiges et feuilles de *chêne liège* arbre, et *Puceron du maïs.*

Les résultats montrent que la plupart des doses d'huiles essentielles *d'A. ligustica* étaient actives, et la dose de 100 ul est la plus efficace.

L'huile essentielle d'*Achillea ligustica* présente des effets antibactériens, antifongiques et anti-insectes remarquables. Il s'agit d'un saut reconnu pour l'avenir de la thérapie naturelle.

Mots clés : *Achillea Ligustica, Puceron du Maïs, Escherichia coli, Staphylococcus sp, Huile Essentielle.*

ملخص:

يهدف هذا العمل إلى دراسة تأثير الزيوت العطرية لنبات الأخيليا ومعرفة ما إذا كان هذا الزيت يمكن أن يثبط نمو البكتيريا والفطريات ويوقف الإصابة بالحشرات.

في هذه الدراسة، تم استخدام التقطير المائي للحصول على الزيوت الأساسية من نبات الأخيليا. تم تقييم الأنشطة المضادة للفطريات والبكتيريا والحشرات لزيوت الأساسية بتركيزات مختلفة (١٠ ، ٣٠ ، ٦٠ و ١٠٠) ميكرليتر في المختبر ضد الإشريكية القولونية والمكورات العنقودية، وعلى سلالتين فطريتين معزولتين من فروع وسيقان وأوراق شجر الفلين، كما تم تقييم نشاط الزيوت على حشرة من أوراق الذرة. أظهرت النتائج أن معظم جرعات الزيوت العطرية لنبات الأخيليا كانت نشطة ، والجرعة ١٠٠ ميكرولتر هي الأكثر فعالية.

يُظهر الزيت العطري لنبات الأخيليا تأثيرات ملحوظة كمضاد للبكتيريا، الفطريات والحشرات. وهذه قفزة معترف بها لمستقبل العلاج باستخدام المواد ذات الأصل الطبيعي.

الكلمات المفتاحية: الأخيليا ، من أوراق الذرة ، الإشريكية القولونية ، المكورات العنقودية ،الزيت العطري.