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Option: Microbial Biotechnology

Title

Suppression of antibiotic resistance in multidrug-resistant *Klebsiella pneumoniae*: The synergistic effect between antibiotics and essential oils of *Thymus vulgaris* and *Origanum compactum*.

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Dedications

I dedicate this humble work,

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Å: Angstrom

Ag: Silver

API: Analytical Profile Index

ATB: Antibiotic

ATCC: American Type Culture Collection

Au: Gold

CFU: Colony Forming Unit

CHROMAgar: Brand name of a chromogenic agar medium

CLSI: Clinical and Laboratory Standards Institute

EDTA: Ethylenediaminetetraacetic acid

EOS: Essential Oils

EPS: Extracellular Polymeric Substances

ESBL: Extended-Spectrum Beta-Lactamase

FDA: Food and Drug Administration

FIC: Fractional Inhibitory Concentration Index

GC-MS: Gas Chromatography-Mass Spectrometry

H2S: Hydrogen sulfide

HCl: Hydrochloric acid

HEBBD: Botanically and Biochemically Defined Essential Oil

IgG: Immunoglobulin G

IgA: Immunoglobulin A

IgM: Immunoglobulin M

K.E.S: *Klebsiella*, *Enterobacter*, and *Serratia* (bacteria genders)

LDC: Lysine decarboxylase test

LPS: Lipopolysaccharide

MAE: Microwave-Assisted Extraction

MAbs: Monoclonal antibodies

MBC: Minimum Bactericidal Concentration

MDR: Multidrug-resistant

MIC: Minimum Inhibitory Concentration

mL: Milliliter

mm: Millimeter

MRSA: Methicillin-resistant Staphylococcus aureus

NaCl: Sodium chloride

NGS: Next Generation Sequencing

NMR: Nuclear Magnetic Resonance

ODC: Ornithine decarboxylase test

PCR: Polymerase Chain Reaction

PI: Propidium Iodide

QS: Quorum Sensing

QSI: Quorum-sensing inhibitors

SFE: Supercritical Fluid Extraction

TDA: Tryptophan deaminase test

UAE: Ultrasound-Assisted Extraction

URE: Urease test

UTI: Urinary Tract Infection

VRE: Vancomycin-resistant Enterococci

WHO: World Health Organization

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GENERAL INTRODUCTION

General Introduction

The introduction of antibiotics in the early 20 century marked a true revolution in global health. Discovered by Alexander Fleming in 1928 with penicillin, antibiotics provided a radical solution for treating bacterial infections that were often fatal until then **(**Tan et al., 2015**)**. They effectively eliminated a wide range of pathogens, drastically reducing mortality related to infectious diseases such as tuberculosis, syphilis, and postoperative infections. Thanks to these medications, medical interventions that were once risky, such as complex surgeries or transplants, became safer, thereby solidifying their essential role in modern healthcare **(**Cook et al., 2022**)**.

However, this golden age of antibiotics has quickly given way to a major issue: the emergence of antibiotic resistance. The intensive, and sometimes inappropriate, use of antibiotics has fostered the development of bacteria capable of surviving these treatments, leading to the appearance of multidrug-resistant strains, even those resistant to all available antibiotics. Their proliferation is responsible for numerous superinfections, therapeutic failures, and deaths, particularly in hospitals **(**Theuretzbacher, 2013**)**.

In response to this growing challenge, researchers are mobilizing to find effective alternatives to traditional antibiotics. Among these solutions, essential oils are generating increasing interest due to their natural antibacterial properties. Derived from aromatic plants, these volatile substances are rich in bioactive compounds such as carvacrol and thymol, which have demonstrated the ability to disrupt bacterial cell membranes and inhibit their growth. The use of essential oils, and more specifically certain types of them, could represent a promising approach to combat resistant infections while limiting the phenomenon of resistance **(**Chávez-González et al., 2016**)**.

This study aligns with this dynamic by exploring the potential of essential oils as a natural alternative to antibiotics. Through the study of a multidrug-resistant strain of *Klebsiella pneumoniae* isolated from a urinary infection, we evaluated the antimicrobial activity of two commonly used essential oils: thyme oil (*Thymus vulgaris*) and oregano oil (*Origanum compactum*). Additionally, we sought to determine whether a combination of these essential oils with conventional antibiotics could enhance their effectiveness and thus represent a promising new therapeutic approach.

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CHAPTER I

BACTERIAL RESISTANCE

Introduction

Antibiotic resistance poses a significant threat to global health. The emergence of multidrug-resistant bacteria has led to a dramatic increase in infections that are increasingly difficult to treat. These pathogens, once primarily confined to healthcare settings, have now spread widely in the community. Gram-positive bacteria such as *Staphylococcus aureus, Enterococcus faecium, Enterococcus faecalis, and Streptococcus pneumoniae* are among the most concerning MDR pathogens. Similarly, gram-negative bacteria like *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Acinetobacter baumannii* have become major public health challenges. The implications for public health are profound, as these infections can spread rapidly and result in severe illness or even death **(**Roberto et al., 2019**)**.

Accordingly, there is a critical global exigency to identify novel antibacterial therapies or alternative treatment modalities, concurrently with the implementation of rigorous infection prevention and control measures.

1.1. Antibacterial resistance

"It's not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them…there is the danger that the ignorant man may easily under-dose himself and, by exposing his microbes to non-lethal quantities of the drug, make them resistant." A. Fleming, Penicillin, Nobel Lecture, 11 December 1945[1]**.**

These words were declared by Alexander Fleming, the discoverer of penicillin, during his Nobel Prize acceptance speech for Medicine in December 1945. He highlighted the inevitable risk of antibiotic resistance, which was already evident in laboratory settings.

Fleming's predictions proved correct: Misuse and overuse of antibiotics accelerate the development and spread of antibiotic-resistant bacteria **(**Terreni et al., 2021**)**

According to the World Health Organization (WHO), antimicrobial resistance is the phenomenon where microorganisms such as bacteria, viruses, fungi, and parasites develop the ability to resist the effects of drugs that once effectively treated infections. Over time, these pathogens evolve mechanisms that render antimicrobial agents, including antibiotics, antivirals, antifungals, and antiparasitics, less effective. It arises from the overuse and misuse of these medications and directly affects the microorganism, not the humans or animals. Consequently,

[1] Source: MLA style: Sir Alexander Fleming – Nobel Lecture. NobelPrize.org. Nobel Prize Outreach AB 2024. Wed. 11 Sep 2024. https://www.nobelprize.org/prizes/medicine/1945/fleming/

infections become harder to treat, leading to more severe outcomes, increased spread of diseases, and higher mortality rates. The growing prevalence of antibacterial resistance (ABR) undermines the effectiveness of these vital medications, making infections more difficult, and sometimes impossible, to treat **(**WHO, 2021**).**

1.2. Origins of antibiotic resistance

Antibiotic resistance has existed for a long time, since the introduction of the first antimicrobials, sulfonamides, in 1937. Resistance mechanisms quickly compromised their effectiveness, as observed in Streptococcus pyogenes in military hospitals in the late 1930s. Alexander Fleming also described penicillin-resistant mutants shortly after its discovery. Today, some bacteria exhibit multidrug resistance, meaning resistance to several antibiotics (S. B. Levy & B. J. N. m. Marshall, 2004).

Antibiotic resistance in bacterial pathogens is an adaptive trait acquired after exposure to antibiotics, as pathogens are initially susceptible to these treatments. Two main mechanisms explain this resistance: mutations and horizontal gene transfer (HGT). Mutations usually affect genes encoding the antibiotic targets, transporters, or regulators of these elements, but only mutations in genes coding for efflux pumps or antibiotic-modifying enzymes confer true resistance. HGT suggests that resistance genes often come from environmental bacteria, rather than antibiotic producers, though the latter may also be sources. Understanding these mechanisms is crucial for accurately assessing the risk of resistance spread (Davies et al., 2010; Martinez, 2014).

1.3. Reasons behind antibiotic resistance

Microorganisms, such as bacteria are living organisms that adapt over time. Their main objective is to replicate, survive, and spread as rapidly as possible. As a result, microbes adjust to their surroundings and evolve in ways that guarantee their continued existence. If something stops their ability to grow, such as an antibiotic, genetic modifications may arise, making the bacteria immune to the medication and allowing them to survive. It is the natural process of bacteria to develop drug resistance (Uddin et al., 2021).

However, several elements remain currently at stake in the multifaceted etiology of antibiotic resistance. This involves antibiotic overuse and abuse, inexact diagnosis and improper antibiotic prescribing, patient sensitivity loss and self-medication, bad healthcare environments, poor personal hygiene, and widespread agricultural use **(**Uddin et al., 2021**).**

1.4. Modes of antibiotic resistance

Bacterial resistance is genetically based and can be either natural, existing from the start, or acquired through various mechanisms (Trieu-Cuot & Courvalin, 1987).

1.4.1. Intrinsic resistance

Intrinsic resistance is due to genes present in the bacterial chromosome that confer resistance to certain antibiotics. This type of resistance is permanent and shared by all bacteria of a given species. It is generally related to reduced permeability of the antibiotic or the absence of its target in the bacterium. For example, *Mycoplasma sp* is naturally resistant to beta-lactams because it lacks peptidoglycan. Natural resistance is also present in antibiotic-producing microorganisms like *Streptomyces sp* (Martinez, 2014; Normark & Normark, 2002; Van Duin & Paterson, 2016).

1.4.2. Adaptive resistance

Adaptive resistance is characterized as resistance to one or more antibiotics triggered by a specific environmental signal. This form of resistance appears to result from gene expression changes in response to environmental alterations (Sirot et al., 1987).

1.4.3. Acquired resistance

Bacteria can acquire resistance to an antibiotic to which they were initially sensitive. This type of resistance affects only certain strains of the species and can result from genetic mutations (mutational resistance) or the acquisition of external resistance genes (Munita & Arias, 2016).

1.4.3.1. Mutational resistance

Sensitive bacteria develop mutations in genes that affect the action of the antibiotic, allowing them to survive in its presence. Once these mutants emerge, the antibiotic eliminates the sensitive bacteria, leaving the resistant ones to dominate (Munita & Arias, 2016). This phenomenon is mainly influenced by the use of the antibiotic, which, although not directly mutagenic, promotes the selection of resistant bacteria. The mutations leading to this resistance typically affect three types of genes: those related to the antibiotic's target (reduced affinity), those involved in the transport of the antibiotic (decreased diffusion), and those regulating the expression of transporters or efflux pumps that remove the antibiotic (Martinez, 2014). Resistance to fluoroquinolones is often caused by mutations in the genes encoding the enzymes DNA gyrase and topoisomerase IV, which are targeted by these antibiotics to inhibit DNA replication (Hooper, 2002).

1.4.3.2. Resistance acquired via Horizontal Gene Transfer (HGT)

Horizontal gene transfer (HGT) is crucial for bacterial evolution and antibiotic resistance (Munita & Arias, 2016). It allows bacteria to acquire resistance genes through three main mechanisms:

- **Transformation:** Capture of free DNA by competent bacteria.
- **Transduction:** Transfer of bacterial DNA by bacteriophages.
- **Conjugation:** Transfer of plasmids between bacteria through direct cell contact.

Mobile genetic elements (MGEs) such as plasmids and transposons facilitate this transfer. Plasmids, often carrying resistance genes like those against beta-lactams, and transposons, which move resistance genes such as tet(M), play key roles. Integrons, containing mobile gene cassettes, also contribute to gene dissemination (Weingarten et al., 2018).

The variability of resistance genes in human pathogens is lower compared to that in natural environments, due to ecological connectivity and the founder effect. Once a resistance gene is established, there is little selective pressure to replace it, and bacteria must bear the costs associated with integrating the gene (Roberts, 1996; Weingarten et al., 2018).

1.5. Mechanisms of antimicrobial resistance in bacteria

The discovery of antibiotics at the beginning of the last century marked a turning point in medicine, drastically reducing bacterial infections and extending human life. However, the growing resistance of bacteria to antibiotics, whether intrinsic, acquired, or adaptive, has become a major concern. It is therefore essential to continue research on resistance mechanisms and to develop new antibiotics. Here, we will examine the molecular and biochemical mechanisms of bacterial resistance and the associated clinical challenges (Aghababa & Nadi, 2021).

1.5.1. Modification of the antimicrobial molecule

Bacteria modify antibiotics to make them ineffective. They use enzymes to destroy antibiotics or add chemical groups, reducing their affinity for their targets (Aghababa & Nadi, 2021). For example:

- **Aminoglycosides:** Aminoglycoside-modifying enzymes (AMEs) add chemical groups, decreasing their effectiveness (Benveniste & Davies, 1973).

- **Nitrofurantoin:** Bacterial reductases activate this molecule by producing toxic intermediates. Resistance is often due to mutations in the genes nfsA, nfsB, and ribE (Alonso et al., 2000).
- **Chloramphenicol:** Chloramphenicol acetyltransferases (CATs) inactivate this antibiotic and others like it, but florfenicol is resistant due to its modified structure (Aminov et al., 2001).,

1.5.2. Decreased permeability

It a bacterial resistance mechanism where bacteria reduce antibiotic entry by altering their outer membrane. Antibiotics must cross this membrane to reach their intracellular targets. Gram-negative bacteria, with their double membranes, use porins as the main entry routes for hydrophilic antibiotics like β-lactams and fluoroquinolones. Modifying or reducing these porins can prevent antibiotic entry, as seen with vancomycin, which cannot penetrate the outer membrane of gram-negative bacteria. This mechanism, combined with other resistance factors such as efflux pumps and degrading enzymes, results in high levels of resistance (Aghababa & Nadi, 2021).

1.5.3. Efflux pumps

Efflux pumps are a key mechanism of bacterial resistance that reduces intracellular accumulation of antibiotics by pumping them out of the cell. First identified in *Escherichia coli* in 1980, these pumps are found in many gram-positive and gram-negative bacteria. They impact a variety of antibiotics, including tetracyclines, fluoroquinolones, β-lactams, carbapenems, and polymyxins. The five major families of efflux pumps are: Major Facilitator Superfamily (MFS), Small Multidrug Resistance (SMR) family, Resistance-Nodulation-Division (RND) family, ATP-Binding Cassette (ABC) family, and Multidrug And Toxic compound Extrusion family. These families differ in their structure, energy source, substrates, and the types of bacteria they affect (Aghababa & Nadi, 2021).

1.5.4. Changes in target sites

A common strategy for developing antimicrobial resistance in bacteria is to obstruct the action of antibiotics by interfering with their target sites. Bacteria employ various methods for this, including target protection, which prevents the antibiotic from accessing its binding site, and target modification, which reduces the antibiotic's affinity for its target (Aghababa & Nadi, 2021; Aldred et al., 2014).

1.5.4.1. Target site protection

Ribosomal Protection Proteins (RPPs) are an example of antimicrobial resistance through target site protection. These proteins, found in both gram-positive and gram-negative bacteria, include Qnr proteins, which can be encoded by either chromosomal or plasmid DNA. Qnr proteins confer resistance to quinolones by mimicking DNA, thereby reducing the interaction between DNA and bacterial gyrase and topoisomerase IV. This action decreases the number of binding sites available for quinolones (Aghababa & Nadi, 2021).

1.5.4.2. Modification of the target site

Target site modification is a widespread mechanism of antibiotic resistance in bacteria, impacting nearly all classes of antimicrobial agents. This includes point mutations in the genes encoding the target, enzymatic changes like methylation, and replacement or bypass of the target site. Regardless of the specific modification, the result is a reduced affinity of the antibiotic for its target (Aghababa & Nadi, 2021).

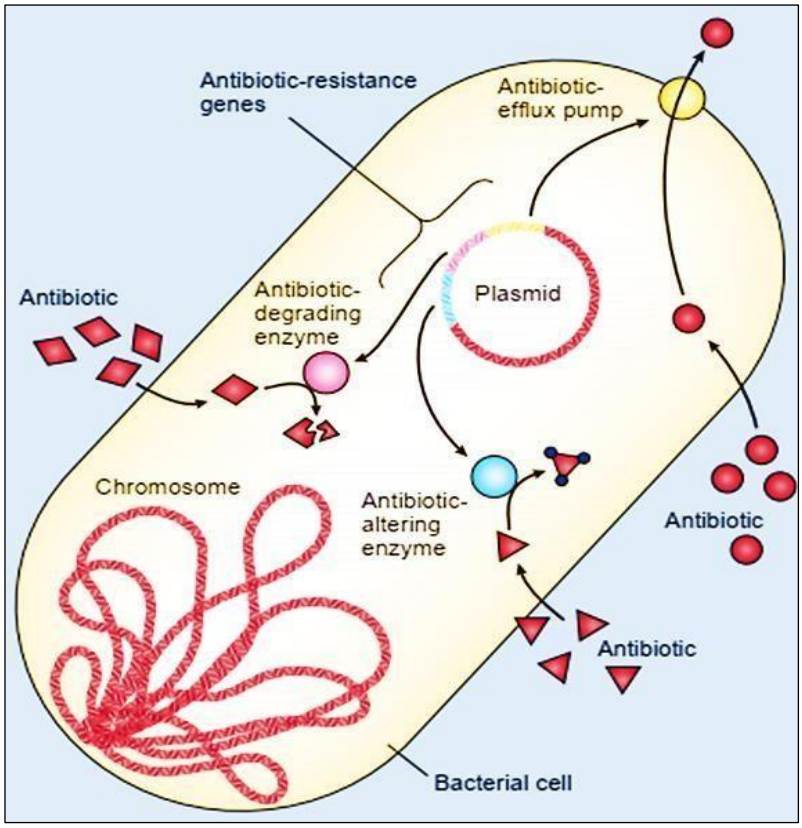

Figure 01: Biological mechanisms of resistance (S. B. Levy & B. Marshall, 2004).

1.6. Controls of antibiotic resistance

Antibiotics, safe water, hygiene, and vaccination have significantly improved well-being and survival in developed countries. The main challenge is to expand access to antibiotics in developing countries without fostering excessive antimicrobial resistance, which could have catastrophic consequences. Despite efforts to reduce antibiotic use, it is crucial to develop new antibiotics. At the same time, it is essential to improve diagnostics, vaccines, and infection prevention systems, particularly by innovating their formulation and distribution, to reduce the need for antibiotics (Gelband & Laxminarayan, 2015; Uddin et al., 2021).

AMR (antimicrobial resistance) is well known among doctors and researchers in many countries, but governments and policymakers have not yet prioritized it or developed a strategy to address it. It is crucial to integrate AMR into the national agenda, allowing specialists to come together around this issue. A reliable working group should be created, consisting of scientific professionals and partners from various fields (veterinary, agricultural, medical, industrial, academic, and governmental) (Gelband & Laxminarayan, 2015).

National strategies to address AMR should be tailored to specific country needs and include: improving the monitoring of resistant microorganisms, setting up antimicrobial stewardship and infection control programs for humans and animals, advancing research into new diagnostic and therapeutic methods, and developing educational initiatives for both healthcare professionals and the general public (Nguyen et al., 2013; Rahman et al., 2013).

According to Shahriar et al. (2019), to prevent and control antibiotic resistance, WHO recommends:

- **For individuals:** Use antibiotics only as prescribed, avoid unnecessary requests for them, practice good hygiene, don’t share antibiotics, wash hands regularly, stay up to date on vaccinations, and adopt safe behaviors.
- **For healthcare professionals:** Maintain hygiene, prescribe antibiotics only, when necessary, follow guidelines, track resistant diseases, and educate patients on proper antibiotic use.
- **For agricultural sectors:** Administer antibiotics under veterinary supervision and prioritize vaccination over antibiotic treatment.
- **For policymakers:** Strengthen policies, implement infection prevention strategies, and improve information accessibility to combat resistant microbes.

- **For the health sector:** Invest in research, vaccine production, diagnostics, and new treatments to prevent and monitor AMR.

1.7. Consequences of antibiotic resistance

Microbial resistance to antibiotics leads to several consequences. When infectious agents develop resistance to various antibiotics, the following may occur: treatment failure results in longer illnesses and an increased risk of death, extended hospital stays and prolonged illnesses increase the likelihood of transmission within the community. If a first-line antibiotic is no longer effective, second- or third-line antibiotics, which are often more expensive and sometimes riskier, must be used. In low-income countries, many second- and third-line treatments are unavailable, increasing dependence on first-line antibiotics. Medications become insufficient to treat microbial infections, and some crucial antibiotics for fighting resistant microorganisms are missing from the essential drug list. Finally, antibiotic resistance endangers the progress of modern medicine, making organ transplants, chemotherapy, and surgeries riskier without effective antibiotics (Chinemerem Nwobodo et al., 2022).

1.8. Alternative strategies for controlling multidrug-resistant bacteria

With the rise of multidrug-resistant (MDR) pathogens, various alternative control strategies have been explored by research groups. Among these, bacteriocins, essential oils (EOs), antibodies, and phage therapy have been highlighted as promising approaches. Additionally, attention has turned towards quorum-sensing inhibitors (QSI) and nanotherapy. Bacteriocins have been studied since the 1960s, while antibodies and bacteriophages have been explored since the 1970s. EOs have been investigated for controlling MDR pathogens since the 1990s, and more recently, from 2000 onwards, QSI and nanotherapy have emerged as potential methods to combat these resistant strains. The following sections provide a brief overview of these alternative approaches, outlining their significance and limitations (Vivas et al., 2019).

1.8.1. Bacteriocins

Bacteriocins are antimicrobial peptides produced by the ribosomes of most bacterial species, with different mechanisms of action and a wide range of activity. Snyder et al. (2014) Snyder et al. (2014) have provided a thorough review of their structure, classification, and general characteristics. Those produced by lactic acid bacteria are especially studied because they are safe and have many industrial uses, particularly in food production. More recently, their therapeutic potential has been explored, especially as anti-cancer agents (Kaur & Kaur, 2015),

and in treating skin infections and many other diseases (de Abreu et al., 2016). Additionally, they are being considered as a promising alternative to antibiotics, particularly for fighting drug-resistant pathogens (Vivas et al., 2019; Yang et al., 2014).

Phumisantiphong et al. (2017) isolated and characterized a novel bacteriocin produced by a strain of *Enterococcus faecalis* that showed high antibacterial activity against VRE (Vancomycin-resistant Enterococci) and MDRE (MDR enterococci). Shokri et al. (2014) demonstrated the potential of a bacteriocin produced by a strain of *E. faecium* in combating VRE. Okuda et al. (2013) showed that three bacteriocins effectively targeted MRSA (methicillin-resistant *Staphylococcus aureus*) biofilms by forming pores in cell membranes. Duracin 61A was effective against *Clostridium difficile*, VRE, and MRSA, and worked synergistically with reuterin or vancomycin, suggesting it could be a promising therapeutic option (Vivas et al., 2019).

Bacteriocins are promising alternatives to antibiotics due to their high stability, low toxicity, and varied spectrum of activity. Some, like lantibiotics, have a dual mechanism of action that reduces the risk of resistance. Synthesized by ribosomes, they allow for biological engineering to enhance their effectiveness and are sensitive to proteases in the stomach, which minimizes toxicity risks. Among lantibiotics, nisin is the most studied. It has been FDA-approved as a food additive since 1988 and also shows therapeutic potential. Nisin is effective against drug-resistant strains such as MRSA and *C. difficile*. Additionally, when combined with other antibiotics, it can also be effective against gram-negative pathogens (Vivas et al., 2019)

Bacteriocins have strong potential for controlling antibiotic-resistant pathogens, but several points need to be studied before their clinical use. First, resistance to bacteriocins, linked to physiological adaptation, remains a concern since they are not yet widely used. Combining them with antibiotics could help prevent the development of resistance. Second, better administration methods are needed, as gastrointestinal enzymes inactivate them. Alternatives like topical or intranasal administration have shown promising results (Vivas et al., 2019)

1.8.2. Bacteriophage therapy

The growing bacterial resistance and the lack of new antibiotics are driving the search for alternative treatment options, with phage therapy being one of the most promising. Phage, also known as bacteriophage, is a virus that infects bacteria. Their therapeutic potential in medicine to control MDR pathogens is due to their specificity and potency in inducing lethal effects in the host bacterium by cell lysis (Dufour & Debarbieux, 2017). Phage therapy, first used in the

1930s, lost interest with the advent of antibiotics. Although the FDA (Food and Drug Administration) granted phages "GRAS" (Generally Recognized as Safe) status in 2006, but their use in humans is still not approved (Cheng et al., 2017). However, the rise of antibiotic-resistant pathogens and the slowdown in new antibiotic development have reignited interest in this therapy, with several studies confirming its effectiveness against resistant pathogens (Roberto et al., 2019).

Chadha et al. (2016) highlighted the potential of a liposome-encapsulated phage cocktail for treating infections caused by *K. pneumoniae*. Rasool et al. (2016) and Jennes et al. (2017) each demonstrated the effectiveness of phage therapy. Rasool et al. (2016) highlighted the lytic activity of phages against MRSA, both in vitro and in vivo, while Jennes et al. (2017) showed its efficacy in treating colistin-resistant *Pseudomonas aeruginosa* septicaemia in a patient with acute kidney failure.

Phage therapy offers a promising alternative to antibiotics for resistant infections, with benefits such as lower costs, complete bacterial killing, high specificity, and often a single dose. It can be used alone or combined with traditional antibiotics to enhance effectiveness (Roberto et al., 2019). However, this method has failed due to adverse effects, particularly immunological ones. It could become promising if these effects are reduced. For now, it is better to avoid whole phages, which are quickly neutralized by resistant bacteria, and focus on developing phage-derived peptides that can kill bacteria (Lemaoui et al., 2017).

1.8.3. Antibodies

Antibodies appear to hold great promise as a new class of drugs against infectious diseases and treating antibiotic-resistant bacterial infections. The use of antibodies is a promising strategy to combat multidrug-resistant (MDR) bacterial strains by enhancing phagocytosis and complement protein activation. To maximize their effectiveness, a mixture of isotypes such as IgM, IgG, and IgA is often recommended. Several studies have confirmed the effectiveness of this approach, making it one of the most studied methods (Roberto et al., 2019). Monoclonal antibodies (MAbs) can neutralize bacterial toxins, trigger complement-mediated bacterial killing, promote phagocytosis, modulate the immune response, and directly target bacteria or their virulence factors (Chan et al., 2009).

Rossmann et al. (2015) demonstrated that IgGAM induces in vitro killing of MDR clinical isolates of *K. pneumoniae* through enhancement of phagocytosis. Results obtained by Giamarellos-Bourboulis et al. (2016) also showed that IgGAM was effective as an adjunct to

antimicrobial treatment for the management of septic shock caused by MDR gram-negative bacteria. Antibody-antimicrobial conjugates have proven effective against MDR strains. Lehar et al. (2015) demonstrated that an antibody-antibiotic conjugate eliminated intracellular S. aureus, outperforming vancomycin in treating bacteremia.

Recent advances in research and biopharmaceutical manufacturing have enabled the development of purified humanized antibodies against various pathogens. Monoclonal antibodies are generally safe and can complement antibiotic therapy, though identifying molecular targets in pathogens remains a challenge (Szijártó et al., 2015).

1.8.4. Nanotechnology

Nanomedicines offer an innovative approach to improving treatments for serious diseases. Using nanocarriers, they transport and release the active ingredient directly at the target, thereby increasing drug efficacy while reducing side effects by modulating their distribution and absorption in the body (Lemaoui et al., 2017).

Currently, several drug delivery systems, mainly based on liposomes or biocompatible nanoparticles, are used. In microbiology, nanoparticles help improve the effectiveness of antibiotics against bacterial resistance. Components such as silver, zinc oxide, copper oxide, and iron oxide have shown antibiotic properties against Gram-positive bacteria as *S. aureus*, *Bacillus subtilis* and Gram-negative bacteria like *P. aeruginosa*, *E. coli*. Additionally, nanoparticles made from plant extracts and silver (AgNO3) have proven effective against pathogens like *E. coli*, *Klebsiella spp*, and *Pseudomonas spp* (Franci et al., 2015; Lemaoui et al., 2017; Marslin et al., 2015).

A recent study showed that nanoparticles containing fluoroquinolones, such as ofloxacin, can overcome bacterial resistance by improving antibiotic penetration. These polyethylene glycol-based nanoparticles demonstrated better antimicrobial activity against strains like *E. coli, P. vulgaris, S. typhimurium, P. aeruginosa, K. pneumoniae, and S. aureus*. However, this strategy has mainly been tested in vitro, with few clinical trials conducted (Lemaoui et al., 2017; Marslin et al., 2015).

1.8.5. Quorum-sensing inhibitors

Quorum sensing inhibitors (QSI) are an emerging alternative method for controlling drug-resistant pathogens (MDR). Quorum sensing (QS) is an intercellular bacterial communication system that allows pathogens to coordinate their behavior based on cell density (Brackman &

Coenye, 2015). At high concentrations, this communication enables bacteria to alter their transcriptional profiles to become more invasive, involving genes related to antibiotic tolerance and virulence (Dickey et al., 2017). QSI work by blocking these cell-to-cell communications, which slows down disease progression and allows the immune system to better combat the infection by reducing virulence rather than directly killing the bacteria (Brackman & Coenye, 2015). Various compounds, such as solonamide B analogs and autoinducing peptides, have been tested to inhibit QS systems like AgrC and pqs, showing a reduction in virulence without significant adverse effects on immune functions (Brackman & Coenye, 2015). Other QSI derived from plants or microorganisms have also shown promising results in experimental models. However, their clinical application still requires further research and may have unfavourable interactions with host factors (Baldry et al., 2016)

1.8.6. Essential oils

Essential oils (EOs) are natural plant extracts that show promising antimicrobial activity against various MDR pathogens (Intorasoot et al., 2017). EOs are defined as volatile, natural, and fragrant liquids that can be extracted from different parts of the plants especially leaves and flowers, and are produced by plants to protect themselves from diverse pathogenic microbes. Due to their antimicrobial activities, EOs have been extensively studied to be used for the treatment of a wide range of microbial infections (Alwan et al., 2016).

They can inhibit biofilm-related infections and enhance the effectiveness of antibiotics when used in combination (Kot et al., 2018). Although their low water solubility and volatility limit commercial use, nanoemulsion formulations and vaporizers are being developed to address these challenges (Ghaderi et al., 2017).

Resistance to EO's could be difficult to arise. At the same time, EOs have not been used on the same scale as antibiotics, so an existing EO-resistant population does not exist. Therefore, to avoid emergence of resistant strains, the best way to use EOs in the control of MDR pathogens could be in combination with another antimicrobial agents (Roberto et al., 2019).

This section is a brief overview, as the second chapter will delve into the detailed use of essential oils, which is the primary focus of this study.

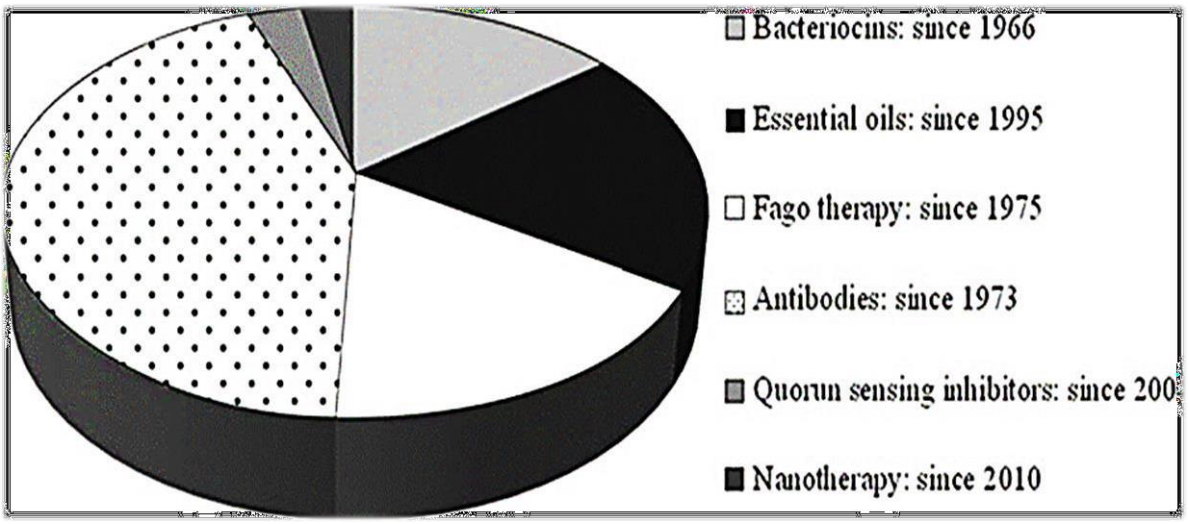

Figure 02: Published research on alternative solutions for the control of multidrug-resistant pathogens (Vivas et al., 2019).

CHAPTER II

ESSENTIAL OILS

Introduction

Some plants are remarkable for their ability to produce a wide variety of secondary metabolites with impressive biological and structural diversity. These compounds play a crucial role in protecting the plants from pathogenic microorganisms. Consequently, they represent a valuable and renewable source for exploring new antibiotics, which are essential in combating the growing resistance to existing drugs. Among these metabolites, EOs are particularly highlighted in research for their antibacterial properties (Chouhan et al., 2017).

It has been well-known since antiquity that EOs exhibit significant antiseptic activity. They are widely used in fields such as pharmacy, cosmetics, and the food industry. At the end of the 19th century and the beginning of the 20th century, several scientific studies reported on the antiseptic properties of various EOs. The term "aromatherapy" was introduced in 1937 (Gattefossé, 1937). The aromatogram is an in vitro method used to assess the antibacterial potential of EOs, comparable to an antibiogram, where antibiotics are replaced with EOs (Kaloustian et al., 2008).

Several EOs exhibit strong antibacterial properties, such as Spanish oregano, thyme (*Thymus vulgaris*), cinnamon (*Cinnamomum zeylanicum*), savory (*Satureia montana*), and clove (*Eugenia caryophyllata*). In comparison, others demonstrate moderate activity, including pine (*Pinus sylvestris*), cajeput (*Melaleuca leucadendron*), eucalyptus (*Eucalyptus globulus*), lavender (*Lavandula officinalis*), myrtle (*Myrtus communis*), and rose geranium (*Pelargonium graveolens*) (Kaloustian et al., 2008).

EOs are widely recognized for their antibacterial activity, particularly in the treatment of respiratory infections, as well as against pathogens such as *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella enterica*…etc (Prabuseenivasan et al., 2006). In addition to their antibacterial properties, some EOs also demonstrate significant effectiveness as antifungal and antimycotic agents, making them valuable in combating a variety of fungal and mycotic infections (Pellecuer et al., 1974). These multiple properties make EOs interesting alternatives to traditional treatments, with a wide range of applications in human and animal health. Specially to combat one of the major issues of our century, which is antibiotic resistance (Chouhan et al., 2017; Kaloustian et al., 2008).

2.1. Essential Oils

2.1.1. History of EOs

The term "essential oil" was introduced in the 16th century by Swiss reformer Paracelsus von Hohenheim, who referred to the active component of a medicine as Quinta essential (Guenther & Althausen, 1949). French chemist Gattefossé, often considered the father of aromatherapy, published a book in 1937 titled *"Aromatherapy, EOs, Plant Hormones"*, where he coined the term "aromatherapy" and established the first connections between the structure and activity of aromatic components (Gattefossé, 1937). Dr. Valnet continued his work by publishing reference books and experimenting with the effectiveness of EOs in military surgery due to a shortage of antiseptics, building on earlier research by Sévelinge, a Lyonnais pharmacist who demonstrated their antibacterial properties in veterinary medicine in 1929 (Valnet et al., 1978). The first medical aromatherapy congress took place in Paris in 1960, chaired by Dr. Valnet, and focused on the anti-infectious properties of EOs. In 1973, Valnet introduced the term "aromatogram " (Valnet et al., 1978). More recently, Pierre Franchomme improved the identification of active principles in essential oil extracts through the concept of chemotype. The use of chromatography has also advanced the analysis of the molecular components of EOs and the characterization of their modes of action (Martins, 2020; Valnet et al., 1978).

2.1.2. Definition

An essential oil is a concentrated plant extract produced through processes outlined by the European Pharmacopoeia and AFNOR (ISO 9235 Standard). It is defined as a fragrant substance, typically with a complex composition, derived from botanically defined plant materials. This extraction is achieved either through steam distillation, dry distillation, or an appropriate mechanical method that does not involve heating. EOs are generally separated from the aqueous phase through a mechanical process that maintains their original composition (Europe, 2004).

EOs are defined as volatile, natural, and fragrant liquids that can be extracted from different parts of the plants especially leaves and flowers, and are produced by plants to protect themselves from diverse pathogenic microbes. Due to their antimicrobial activities, EOs have been extensively studied to be used for the treatment of a wide range of microbial infections (Alwan et al., 2016). EOs are volatile due to their low molecular weight, which contributes to their characteristic scents. They are found in certain botanically defined plant species, are typically liquid and clear, and are rarely colored. EOs are hydrophobic, liposoluble, and are soluble only in alcohol and vegetable or mineral oils. They are flammable and do not contain

fatty substances. The components of EOs can be synthesized in various plant organs, such as buds, flowers, leaves, stems, branches, seeds, fruits, roots, wood, or bark, and are stored in secretory cells, cavities, channels, epidermal cells, or glandular trichomes. EOs are produced by aromatic plants as secondary metabolites. Their chemical composition mainly consists of terpenes and their oxygenated derivatives. Aromatherapy involves using these aromatic plant extracts for medical treatment purposes (Bakkali et al., 2008; Bonnafous, 2013; Martins, 2020).

2.1.3. Quality criteria for EOs

The HEBBD label (Botanically and Biochemically Defined Essential Oil) is one of the first quality labels for EOs, certified by the Scientific Aromatology Standardization Institute. According to Bonnafous (2013), it ensures several criteria:

- Exact botanical species (Latin name, genus, species, variety, chemotype).
- Producing organ of the essence (different plant parts can produce EOs with varying compositions).
- Geographical origin of the plant.
- Cultivation method (wild, organic), and harvest time (e.g., thyme harvested in the afternoon).
- Analysis of the essential oil using reference methods.
- Manufacturing process (distillation or cold pressing; oil must be raw, virgin, and unmodified).

The composition of EOs can vary depending on the plant's growth and flowering stages, with the highest production typically occurring during the flowering period (Jordán et al., 2006; Martins, 2020). The quality of EOs also varies depending on the extraction method, affecting their composition and properties. To ensure their authenticity and effectiveness, controls are carried out on organoleptic characteristics, physical measurements, and chemical analyses (Bonnafous, 2013; Martins, 2020).

2.2. Role of EOs

The role of EOs in plants is not yet fully understood, but studies suggest that they act as a defense mechanism by altering predator behavior. They may also protect the plant from oxidative damage caused by atmospheric agents. EOs have an allelopathic effect, inhibiting the germination and growth of nearby plants. Additionally, they attract pollinators, aid in seed dispersal, and repel herbivores. EOs are synthesized in specific plant structures, such as

glandular trichomes, secretory cavities, and ducts, which are found in various organs like flowers, leaves, and bark (Djilani et al., 2012; Martins, 2020).

2.3. Characteristics of EOs

2.3.1. Physical Properties

EOs are volatile, turning into liquid at room temperature. They can be colorless or vary in color from pale yellow to emerald green, and from blue to dark reddish-brown. They are generally less dense than water, except for sassafras and clove oils. EOs can be lipophilic or soluble in alcohol and organic solvents, but are only minimally soluble in water (Aljaafari et al., 2019).

2.3.2. Chemical Composition

The chemical composition of EOs is influenced by factors such as plant development stage, harvested plant parts, and geographic location. Typically, composition analysis is conducted using gas chromatography-mass spectrometry (GC-MS), though nuclear magnetic resonance (NMR) can also be utilized. EOs are complex mixtures containing about 20 to 60 different compounds, with two or three major components usually making up 20 to 70% of the oil. These major components largely determine the oil's biological properties and can vary even among oils from the same plant species, leading to different chemotypes. Essential oil constituents generally fall into two main biosynthetic groups: terpenes and terpenoids (more common) and aromatic compounds derived from phenylpropane (less common) (Djilani et al., 2012; Khoddami et al., 2019).

Table 01: Components and sources of some essential oils (Yangilar, 2021).

EOS	Plant piece	Raw material	Compounds	%
Angelica	Roots	*Angelica archangelica* L.	α-pinene	24.7
			δ-carene	10.5
			α-phellandrene+ myrcene	12.9
			limonene	10.4
			β-phellandrene	7.7
Bergamot	Fruits	*Citrus bergamia Risso*	Limonene + β-phellandrene - Terpinene	39.4
			Linalool	11.1
			Linalyl acetate	28
Cinnamon	Inner bark	*Cinnamomum zeylanicum*	(E)-cinnamaldehyde	77.1
Coriander	Seeds	*Coriandrum sativum* L.	Eugenol	7.2
			p-cymene	6.1

			Linalool	72
Dill (Indian)	Seeds	*Anethum sowa*	Limonene	50.9
			trans-dihydrocarvone	10.4
			Carvone	20.3
			Dillapiole	36.6
Eucalyptus	Leaves	*Eucalyptus citriodora*	Citronellal	72.8
Ginger	Roots	*Zingiber officinale*	Citronellol	14.5
			Camphene	14.1
			Neral	4.9

A. Terpenes and Terpenoids

Terpenes are a diverse class of hydrocarbons with varying functional and structural forms, formed by the combination of multiple five-carbon units called isoprene (C5H8). Their biosynthesis occurs in the cytoplasm, starting with the synthesis of the precursor isopentyl pyrophosphate (IPP) via the mevalonate pathway, which begins from acetyl-CoA. IPPs are then repetitively added together to form prenyl diphosphate, the precursor of various terpene classes. Allylic prenyl diphosphate undergoes rearrangements by specific synthases to form the terpene skeleton, and subsequent enzymatic modifications (redox reactions) assign functional properties to the different terpenes. The primary terpenes found in EOs are monoterpenes (C10) and sesquiterpenes (C15), but longer chains like diterpenes (C20) and triterpenes (C30) can also exist (Bakkali et al., 2008; Chouhan et al., 2017).

B. Phenylpropanoids

In plants, phenylpropanoids are synthesized from the amino acid phenylalanine, which is derived from the shikimic acid pathway. Phenylalanine serves as a precursor to a subclass of organic compounds called phenylpropanoids. EOs contain a relatively small proportion of phenylpropanoids, with the most studied being safrole, eugenol, isoeugenol, vanillin, and cinnamaldehyde. EOs may also include various degradation products from unsaturated fatty acids, lactones, glycosides, and compounds containing sulfur and nitrogen, such as allyl isothiocyanate and allicin, known for their antibacterial activity (Bakkali et al., 2008; Chouhan et al., 2017).

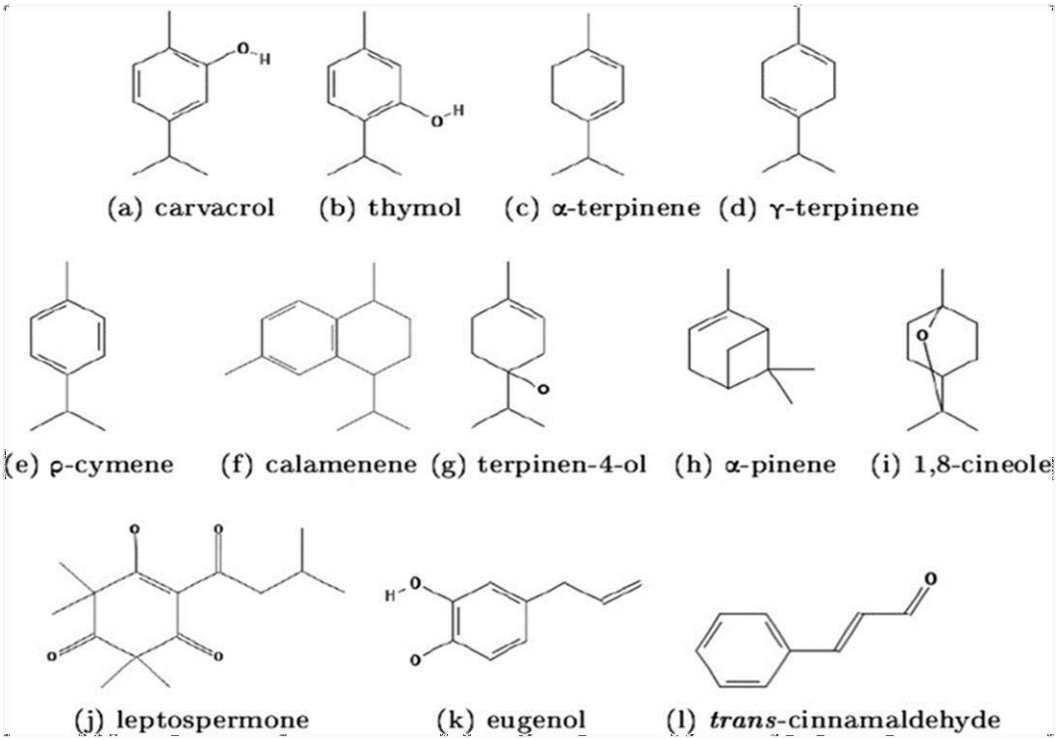

Figure 03: Some representative bioactive compounds present in EOs (Langeveld et al., 2014).

2.4. Essential Oil Extraction

Ancient civilizations utilized early machines for essential oil (EO) extraction over 5,000 years ago. Today, various methods have expanded, including hydro-distillation, steam distillation, expression, solvent extraction, and ultrasonic extraction and many others.

2.4.1. Classical Methods

2.4.1.1 Hydro-Distillation

Hydro-distillation is one of the oldest extraction methods, involving the complete immersion of plant material in water, which is then brought to a boil, typically at atmospheric pressure. The heat breaks down plant cells, releasing fragrant molecules that are carried away by steam and then condensed. Since EOs are not miscible with water, they are easily separated by decantation after condensation. This method provides some protection to the oils, as the surrounding water helps prevent overheating. Additionally, it allows for the recycling of condensates through a cohobation system. However, hydro-distillation has drawbacks, such as a lengthy extraction time (3 to 6 hours), potential degradation of certain terpenes due to prolonged contact with boiling water, and loss of some polar molecules into the water (El Asbahani et al., 2015).

2.4.1.2 Steam Distillation

Steam distillation is a standard method for obtaining EOs. Unlike hydro-distillation, this process does not directly contact water with the plant material. Instead, steam is generated by a boiler and passes through the plant material placed above a grid. As the steam moves through, it ruptures the plant cells, releasing essential oil, which evaporates and travels with the steam. The mixture of oil and water is then condensed in a cooling system. The resulting blend is separated in a separator into an aqueous phase and an organic phase, due to their different densities. This method reduces chemical alterations to essential oil molecules by avoiding direct contact with water and aromatic compounds, and by shortening the extraction time (El Asbahani et al., 2015). Hydrodiffusion is a variation of steam distillation where steam flows downward through the plant material. This method features a shorter processing time, higher oil yield, and uses less steam compared to traditional steam distillation (El Asbahani et al., 2015).

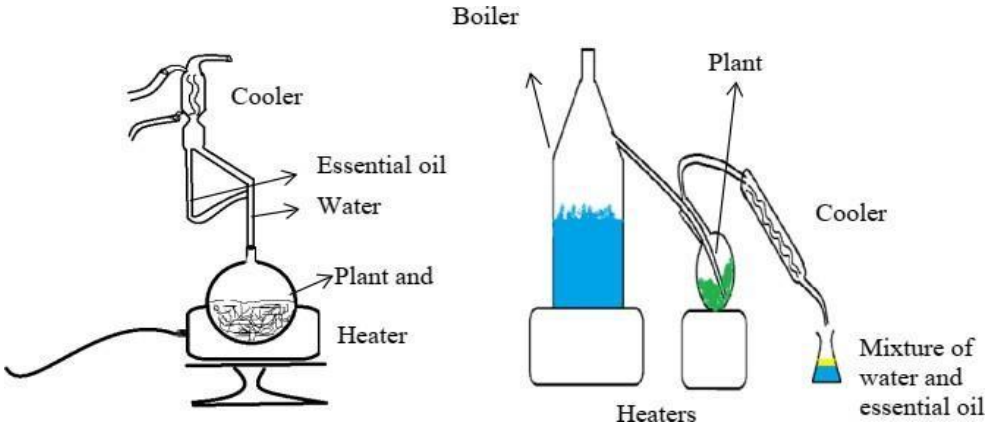

Figure 04: Water and steam distillation (Akdağ & Öztürk, 2019).

2.4.1.3 Solvent Extraction

Solvent extraction, also known as the liquid-liquid extraction method, separates compounds based on their solubility in two immiscible solvents, typically water and an organic solvent. In this process, plant material is ground with anhydrous hexane and then extracted multiple times with hexane to obtain a yellow extract. The extract is then purified using Norite A charcoal and centrifuged at low speed to remove the color. The solution is finally concentrated by evaporating the solvent. A modern variant, microwave-assisted simultaneous distillation-solvent extraction (MW-SDE), is more efficient and uses fewer solvents to extract volatile compounds compared to traditional methods (El Asbahani et al., 2015).

2.4.1.4 Cold Mechanical Expression

This unheated technique is used exclusively for extracting EOs from citrus peels. It is the simplest method but also the most limited, involving the mechanical rupture of oil sacs in citrus peels to collect the essential oil. This process produces an essence rather than an essential oil because no solvents or steam are involved. The essence is released by a stream of water and then separated by decantation (Bonnafous, 2013).

2.4.2. Innovative Methods

2.4.2.1 Ultrasound-Assisted Extraction (UAE)

Developed in the 1950s for laboratory use, UAE involves immersing plant material in water or another solvent while simultaneously applying ultrasonic waves with frequencies ranging from 20 kHz to 1 MHz. These waves cause mechanical vibrations in cell walls and membranes, facilitating the rapid release of essential oil droplets. The extraction process includes two key phenomena: diffusion through the cell wall and washing out the cellular content once the walls are disrupted. After extraction, the solvent is removed under vacuum. UAE is effective for extracting EOs from various sources, particularly seeds, and it enhances extraction efficiency, reduces extraction temperature, and broadens solvent selection. The equipment is relatively simple and less expensive compared to microwave-assisted extraction (MAE) (El Asbahani et al., 2015; Khan et al., 2010).

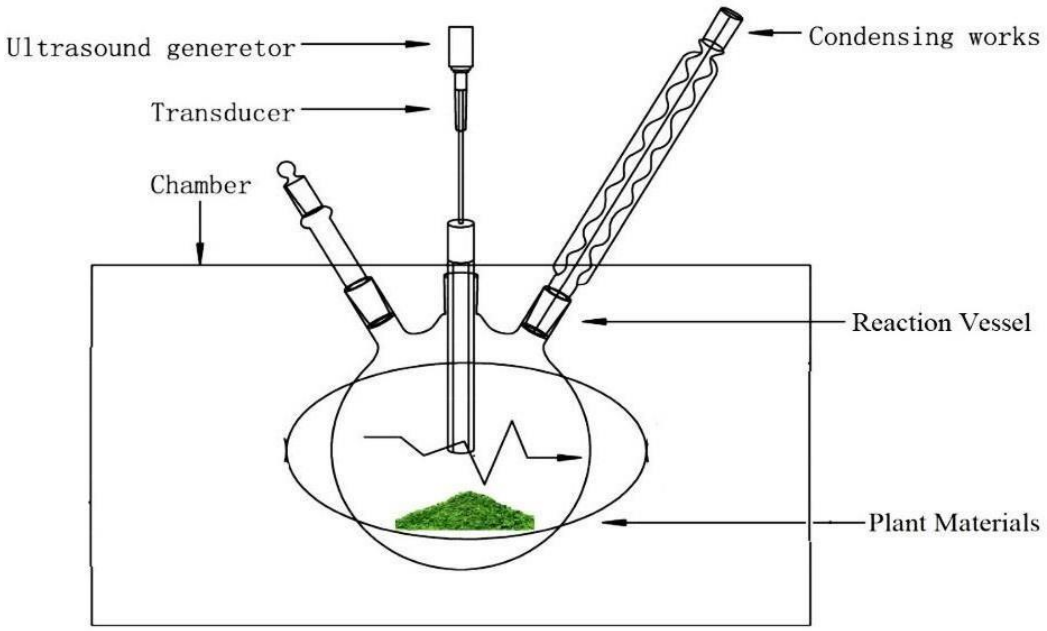

Figure 05: Ultrasound-assisted extraction (UAE) (Sahoo & Banik, 2023).

2.4.2.2 Microwave-Assisted Extraction (MAE)

Microwaves are electromagnetic waves with frequencies ranging from 300 MHz to 30 GHz and wavelengths from 1 mm to 1 m, typically operating at 2450 MHz (wavelength of 12.2 cm). Microwave technology has advanced with the shift towards green extraction methods and the need for energy-efficient processes. MAE includes various techniques such as solvent-free microwave extraction (SFME), microwave-assisted steam distillation, and microwave-assisted hydrodistillation (Benameur et al.). This method significantly reduces extraction time and increases essential oil yield while saving energy (El Asbahani et al., 2015).

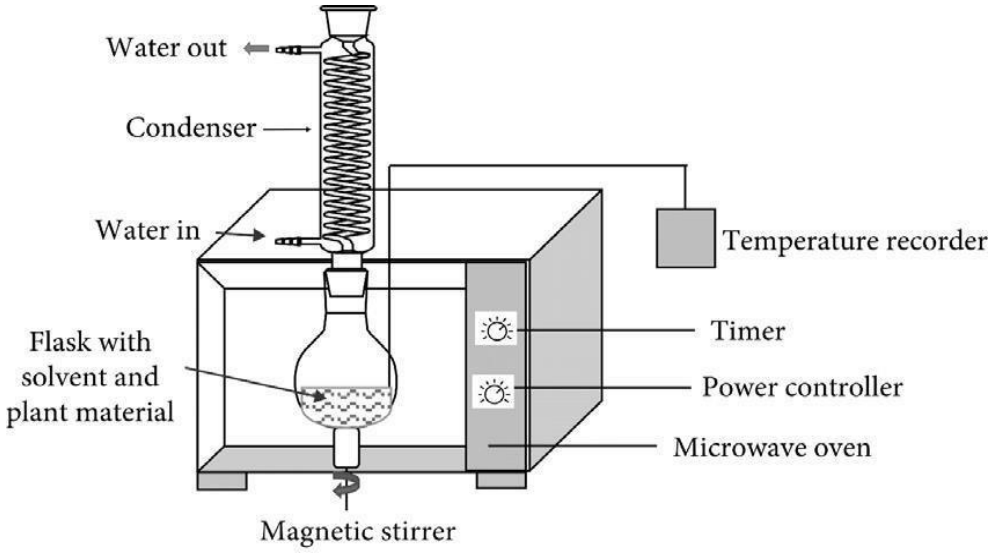

Figure 06: Schematic diagram of Microwave-assisted extraction (MAE) (Sahoo & Banik, 2023).

2.4.2.3 Supercritical Fluid Extraction (SFE)

Supercritical Fluid Extraction (SFE) is unique due to the use of solvents in their supercritical state, achieved by increasing temperature and pressure beyond a critical point. In this state, the solvent exhibits properties between liquid and gas phases, such as high density, low viscosity, and high diffusivity, along with enhanced solvating power. Carbon dioxide (CO_2) is the most commonly used solvent for essential oil extraction because it is easily obtained (with low critical temperature and pressure), highly pure, and inexpensive. Additionally, CO_2 is non-toxic, non-flammable, inert, and can be easily removed from the extract. However, its low polarity limits its ability to extract polar components, and the primary drawback of this method is the high cost of equipment, installation, and maintenance (Rassem et al., 2016).

2.5. Antibacterial Action of EOs

2.5.1. Evaluation of Antimicrobial Activity of EOs

The antimicrobial activity of EOs can be tested using in vitro methods, such as disk diffusion or broth/agar dilution, similar to antibiograms. Advanced techniques like flow cytometry provide a detailed analysis of the nature of the effect (bactericidal or bacteriostatic).

2.5.2. Solid Media Diffusion

This method measures the inhibition zone of bacterial growth around the EOs.

A. Disk Diffusion Method

The disk diffusion test on agar is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. A disk diffusion assay is used to determine the inhibition of bacterial growth by EOs. In this procedure, agar plates are inoculated with colonies of the test microorganism. Then, filter paper disks (approximately 6 mm in diameter), impregnated with EOs at the desired concentration, are placed on the surface of the agar. The Petri dishes are incubated at 37°C for 18 to 24 hours. The EO diffuses into the agar and inhibits the germination and growth of the test microorganism, after which the diameters of the inhibition zones are measured. The clear zone around the disks is proportional to the antibacterial activity of the essential oil. This provides qualitative results by classifying bacteria as sensitive, intermediate, or resistant. These results can help guide the appropriate selection of treatments for individual patients in specific situations due to the good correlation between in vitro data and in vivo outcomes. However, since the inhibition of bacterial growth does not necessarily mean bacterial death, this method cannot distinguish between bactericidal and bacteriostatic effects (Martins, 2020).

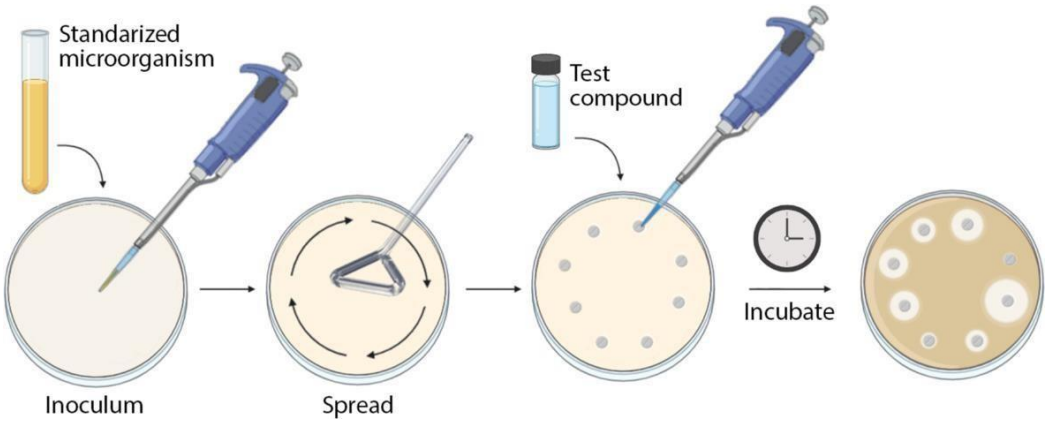

Figure 07: Scheme of the agar disk diffusion method (Guerrero Correa et al., 2020).

B. Agar Well Diffusion Method

Similar to the disk diffusion method, the agar well diffusion method involves inoculating the surface of an agar plate with a microbial suspension spread evenly. A well, approximately 6 mm in diameter, is then aseptically created in the agar, and a volume (20–100 µL) of the antimicrobial agent or extract solution at the desired concentration is introduced into the well. The agar plates are then incubated under conditions suitable for the tested microorganism. The antimicrobial agent diffuses into the agar and inhibits the growth of the test microorganism. The disk diffusion method is limited by the hydrophobic nature of EOs, which hinders their even diffusion through the agar medium. Consequently, most researchers prefer liquid medium methods for testing (Martins, 2020).

2.5.3. Liquid Media: Dilution Methods

Liquid dilution methods are used to determine the Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of EOs.

A. Broth Dilution Method

In this procedure, bacterial suspensions are diluted in a liquid growth medium and distributed into a 96-well microtiter plate (microdilution). EOs are added to create a range of concentrations. After thorough mixing, the microtiter plate is incubated under appropriate conditions for the test microorganism, typically at 37°C for 24 hours. Colorimetric indicators are used to determine growth in the wells and to ascertain the MIC based on color changes. The MIC is defined as the lowest concentration of the antimicrobial agent (EO) that inhibits visible growth of the microorganism under incubation conditions, usually expressed in µg/ml or mg/l. EOs with MIC values less than 0.50 mg/ml indicate "high activity against microorganisms." The MBC is the lowest concentration of the antimicrobial agent required to kill 99.9% of the microorganism. This is confirmed by re-cultivating the culture medium from wells with no visible growth onto agar plates, followed by incubation for 24 hours. Various factors such as inoculum size, type of growth medium, incubation time, and inoculum preparation method can affect MIC values. Guidelines from CLSI and EUCAST provide standardized procedures for testing (Martins, 2020).

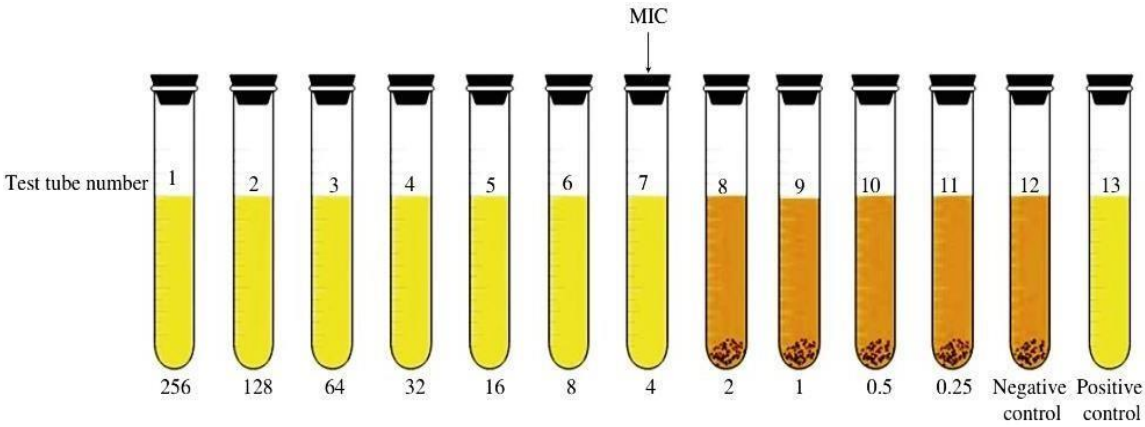

Figure 08: Schematic diagram of minimum inhibitory concentration determination (Chen et al., 2022).

B. Agar Dilution Method

This method involves incorporating different concentrations of the antimicrobial agent into an agar medium using serial dilutions, followed by inoculating a defined microbial inoculum on the surface of the agar plate. When testing multiple isolates against a single compound or when the compound's color interferes with microbial growth detection in liquid media, the agar dilution method is often preferred. It is particularly recommended for testing demanding organisms like anaerobes and Helicobacter species.

Dilution methods are best suited for determining MIC values and quantitatively measuring antimicrobial activity *in vitro* against bacteria (Martinez, 2014).

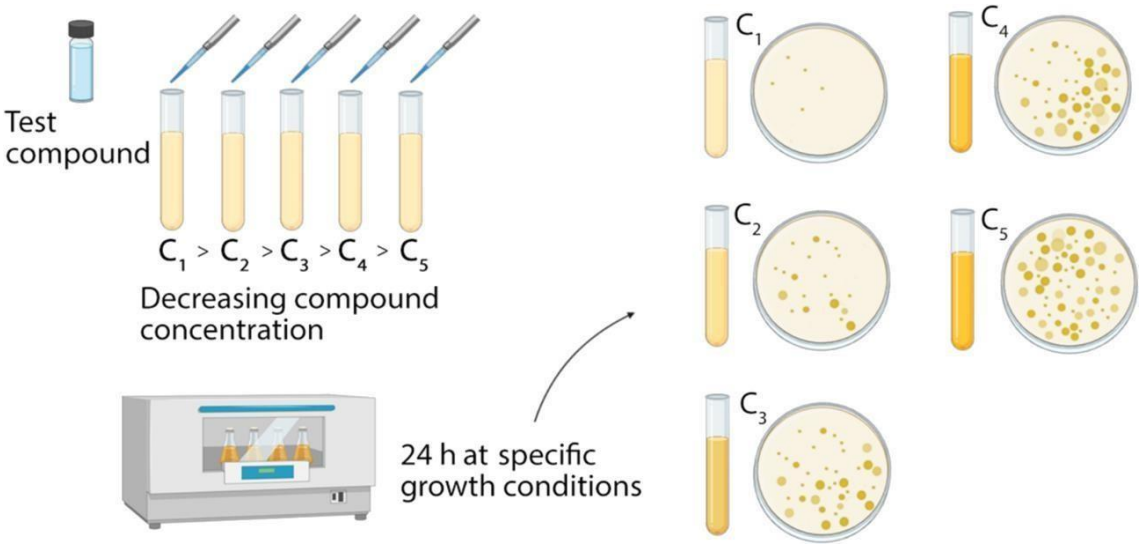

Figure 09: Scheme of the agar dilution method (Guerrero Correa et al., 2020).

2.5.4. Gas Phase

This method, though less commonly used, is useful for studying EOs in atmospheric diffusion. It analyzes the antibacterial activity of volatile components of EOs. In this method, an agar plate is inoculated, and a disk impregnated with EO is placed on the underside of the Petri dish lid, without direct contact with the agar. The sealed dish is incubated at 37°C for 24 hours. If the microorganism is sensitive, a translucent circular zone of inhibition will be observed in the agar (Martins, 2020).

2.5.5. Other Tests

According to Balouiri et al. (2016), these tests might be categorized as follows:

- **Time-Kill Test:** This method evaluates the bactericidal effect over time, using dyes like propidium iodide (PI) to assess cell damage and viability. It helps in understanding the dynamic interaction between the antimicrobial agent and the microorganism, and can distinguish between dead, viable, and damaged cells.
- **Synergy Test:** The checkerboard method is used to test antimicrobial synergy by combining different concentrations of substances in a microtiter plate. MICs and fractional inhibitory concentration indices (FIC) are calculated to determine the interaction between substances, identifying synergistic, additive, or antagonistic effects.
- **Chemical Composition Analysis:** EOs are analyzed using gas chromatography-mass spectrometry (GC-MS) to identify and quantify volatile compounds. This involves measuring peak heights and areas, and comparing data with reference libraries to determine the composition and structure of each compound.
- **ATP Bioluminescence Test:** This test measures the ATP produced by bacteria, which is indicative of microbial population. The amount of light emitted by the ATP-luciferase reaction is used to estimate cell viability and can be applied to various microbiological assessments.

Each method provides insights into different aspects of antimicrobial activity and essential oil composition (Martins, 2020).

2.6. Antibacterial EOs

EOs exhibit antibacterial properties largely due to their chemical composition. The effectiveness of EOs is influenced by their functional groups and structure (Martins, 2020). Among the most effective compounds are:

2.6.1. Phenols

These are powerful, broad-spectrum anti-infectives with antibacterial, antiviral, antifungal, antiparasitic, antioxidant, immunostimulant, and anti-inflammatory properties. Examples include carvacrol (oregano), thymol (thyme), and eugenol (cinnamon). They can be irritating and should be used with caution, particularly in diluted forms and for short periods (Helander et al., 1998; Saad et al., 2013).

2.6.2. Aromatic Aldehydes

These also offer broad-spectrum anti-infective action, including antibacterial, antiviral, antifungal, and antiparasitic properties, as well as immunostimulation and general tonics. Key examples are cinnamaldehyde (cinnamon) and cuminaldehyde (cumin). They can be dermocaustic and allergenic, so they should be used in lower concentrations and avoided in sensitive populations, including pregnant women and young children (Helander et al., 1998; Saad et al., 2013).

2.6.3. Monoterpenols

Known for their remarkable anti-infective properties and broader safety profile compared to phenols, these EOs are used for long-term treatments. They offer antibacterial, antiviral, antifungal, antiparasitic, immunomodulatory, and neurotonic effects. Examples include linalool (lavender), geraniol (palmarosa), terpinen-4-ol (tea tree), and menthol (peppermint). Generally well-tolerated, but caution is advised with menthol in children under 7 and pregnant women (Helander et al., 1998; Saad et al., 2013).

2.7. Antimicrobial mechanism of EOs on microbes

The antimicrobial activity of Essential oils has been recognized for many years. Due to their numerous constituents, EOs do not target a specific cellular site but act on various targets. Their bioactive components inhibit or slow microbial growth, primarily affecting the cell membrane and cytoplasm, and in some cases, altering cell morphology. EOs destabilize the cell structure, increase membrane permeability, disrupt energy production, nutrient processing, and

macromolecule synthesis, causing cellular leakage and ion loss. Some compounds also inhibit efflux pumps, contributing to antibiotic resistance. These processes can lead to cell coagulation and membrane breakdown (Martins, 2020).

2.7.1. Action on the bacterial cell membrane

EOs interact with the bacterial cell membrane, primarily due to their lipophilic nature, allowing them to penetrate the phospholipid bilayer. This leads to structural disruptions of the membrane, affecting nutrient transport, depolarizing mitochondrial membranes, and impacting ion channels. The membrane's fluidity is altered, causing leaks of intracellular materials, ultimately resulting in cell death through apoptosis and necrosis (Hajlaoui et al., 2010).

EOs can also inhibit toxin secretion. For example, carvacrol reduces diarrheic toxin production by *B. cereus*, and oregano inhibits *S. aureus* enterotoxins. Additionally, their effects differ between Gram-positive and Gram-negative bacteria. Gram-positives are more sensitive due to lipoteichoic acid, which facilitates the entry of EOs, while Gram-negatives are more resistant due to their outer membrane and lipopolysaccharide (LPS), which slow the diffusion of hydrophobic compounds (Martins, 2020).

2.7.2. Action on cell morphology

The activity of EOs and/or their components varies depending on the shape of the bacteria, with bacilli being more sensitive to EOs than cocci. *S. typhimurium* and *E. coli* (bacilli) have smooth surfaces, while *M. luteus* and *S. aureus* (cocci) have a round shape. After 24 hours of treatment with peppermint EO, cell damage was observed in bacilli, whereas cocci showed less damage. This suggests that the exopolysaccharide may have detached or the peptidoglycan and cytoplasmic membrane were disrupted (Hajlaoui et al., 2010).

Due to their lipophilic nature, cyclic monoterpenes preferentially accumulate in the aqueous phase of membrane structures, leading to membrane expansion, increased fluidity, and enzyme inhibition. Electron microscopy of *E. coli* cells exposed to tea tree oil revealed electron-dense material loss and cytoplasmic content coagulation, though these were secondary effects following cell death (Sikkema et al., 1995).

In another study, cinnamaldehyde altered the morphology of *B. cereus*, transforming well-separated rod-shaped cells into elongated filamentous structures. *S. typhimurium* cells treated with carvacrol and thymol exhibited swelling, suggesting these compounds penetrated and altered the cell envelope structure (Kwon et al., 2017; Sikkema et al., 1995).

Electron microscopy of *E. coli* treated with oregano EO (rich in thymol and carvacrol) showed cell collapse and holes or white spots on the cell wall. Morphological changes caused by carvacrol were similar to those seen with other antimicrobial agents, such as antimicrobial peptides. The presence of division septa in treated cells may be due to carvacrol's effect on proteins involved in cell division, as confirmed by proteomic studies on *Salmonella* treated with thymol (Kwon et al., 2017).

2.7.3. Action on membrane fatty acids

EOs impact bacterial membrane fatty acids by disrupting lipid biosynthesis, particularly of unsaturated fatty acids, which are crucial for membrane fluidity. Even at low concentrations, EOs reduce unsaturated fatty acid levels, leading to structural changes in the membrane. Compounds like thymol, carvacrol, and eugenol increase the amount of saturated fatty acids (C16, C18) while decreasing unsaturated C18 fatty acids. By increasing membrane permeability, EOs disperse desaturase enzymes, affecting fatty acid synthesis. However, they do not activate cis-trans isomerase, which usually helps cells adapt to environmental stress. The overall EO effect leads to a rise in cis-isomers, shorter fatty acid chains, and fewer unsaturated fatty acids. This causes the membrane to become less fluid and more rigid, eventually leading to cell lysis (Martins, 2020).

2.7.4. Action on Proteins

EOs can disrupt bacterial proteins, affecting cell division. For instance, thymol reduces the assembly of FtsZ, a key protein for bacterial cell division. In the presence of thymol, *Salmonella enterica* shows a different proteomic profile with proteins regulated up or down. Thiorodoxin-1, important for *E. coli*, is not expressed, and chaperone proteins are overexpressed to help bacteria adapt to adverse conditions. Thymol also upregulates certain membrane proteins, such as TolC, involved in bacterial resistance (Martins, 2020).

2.7.5. Action Against ATP

EOs impact ATP production in prokaryotes by disrupting the cell membrane and glycolysis. This disruption can imbalance intra- and extracellular ATP, affecting bacterial growth. Compounds like eugenol and carvacrol inhibit ATP generation and may affect ATPase activity, potentially through changes in membrane protein conformation, thus altering bacterial growth (Imelouane et al., 2009).

2.7.6. Action Against Quorum Sensing

Quorum sensing (QS) is a bacterial communication system that regulates functions like virulence and biofilm formation. EOs can inhibit QS by interfering with bacterial signaling molecules. This could help combat antibiotic resistance by preventing biofilm formation (Szabó et al., 2010; Trosko, 2016).

Table 02: The antibacterial mechanisms and components of some EOs (Yangilar, 2021).

EO or component	Mode of action	References
Oregano	Reduction in lipase and coagulase activity, enzyme inhibition	**(Carneiro de Barros et al., 2009)**
Carvacrol	Membrane disruption, leakage of cell ions, fluidization of membrane, destabilization, inhibition of ATPase activity, membrane lipid peroxidation, reduction of proton motive force	**(Di Pasqua et al., 2007); (Gill & Holley, 2006a,b); (Ultee et al., 2002)**
Thymol	Membrane disruption with potential intracellular targets, membrane metabolic pathway disruption	**(Di Pasqua et al., 2007); (Trombetta et al., 2005)**
p-Cymene	Membrane disruption	**(Ultee et al., 2002)**
Eugenol	Membrane disruption by inhibiting ATPase activity, possible efflux pump blocker, reduction of several virulence factors at sub-inhibitory concentrations	**(Bolla et al., 2011); (Gill & Holley, 2006a,b); (Hemalewarya & Doble, 2009; Qiu et al., 2010)**
γ-Terpinene	Membrane disruption	**(Oyedemi et al., 2009)**

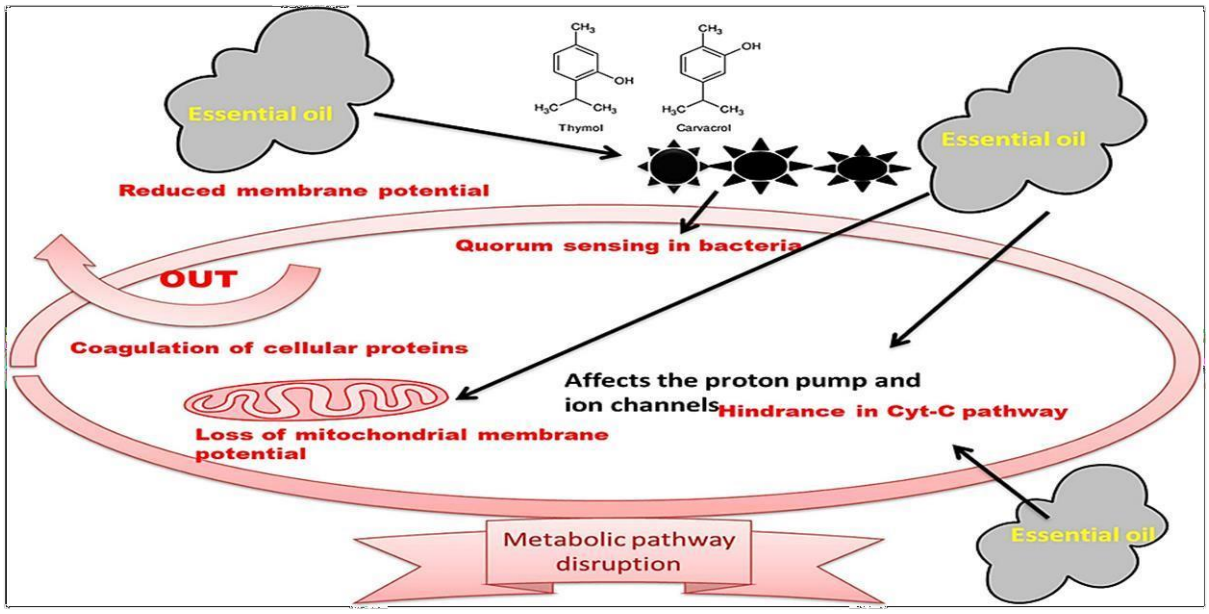

Figure 10: Antimicrobial mechanism of EOs on microbes (Tariq et al., 2019).

2.8. Study of Synergistic Antimicrobial Activity of EOs

With the rise of antibiotic-resistant bacteria and limited new antibiotics, alternative strategies are needed. Combining antibiotics with non-antibiotic drugs or natural antimicrobial agents, such as EOs, is one approach. These combinations can have synergistic, additive, or antagonistic effects. Synergy occurs when the combined effect is greater than the sum of individual effects, while an additive effect equals the sum, and an antagonistic effect is less effective than the individual components. The fractional inhibitory concentration (FIC) index is used to measure these interactions: an FIC index < 0.5 indicates synergy, 0.5 to 4 indicates additivity, and > 4 indicates antagonism. Synergistic effects may arise from targeting different bacterial mechanisms or improving drug solubility and bioavailability (Chouhan et al., 2017).

2.8.1. Synergism in EOs

2.8.1.1. Synergism Between Essential Oil Components

The antimicrobial activity of EOs often relies on their major constituents, but interactions with minor components are also crucial. For example, combinations of eugenol with linalool or menthol show high synergy. Binary and ternary mixtures, such as carvacrol with thymol or eugenol, have demonstrated enhanced antimicrobial effects (Langeveld et al., 2014).

2.8.1.2 Synergism Between Different EOs

Combining EOs like coriander with cumin or oregano with thyme can enhance antibacterial activity. Studies show that mixtures of oils such as peppermint and tea tree or oregano and thyme exhibit significant synergistic effects against various fungal strains (Langeveld et al., 2014).

2.8.1.3 Synergism Between EOs and Antibiotics

Essential oil components like thymol and carvacrol can enhance the efficacy of antibiotics such as penicillin against bacteria. Eugenol has shown synergy with ampicillin and gentamicin, while carvacrol improves the effectiveness of ampicillin and nitrofurantoin. Other combinations, like oregano oil with gentamicin, also demonstrate enhanced antimicrobial activity (Duarte et al., 2012; Langeveld et al., 2014). Combining EOs with antibiotics can improve treatment outcomes for resistant strains. For instance, coriander oil with various antibiotics shows promise, and EOs like those from Eucalyptus camaldulensis can resensitize multidrug-resistant strains. Such combinations could offer new therapeutic strategies against resistant bacteria (Langeveld et al., 2014).

EXPERIMENTAL PART

CHAPTER III

MATERIALS & METHODS

The objective of this study, divided into three phases, is to propose natural and effective alternatives to address the growing issue of antibacterial resistance (ABR).

1. **Isolation and identification of a multidrug-resistant bacterium:** This phase was conducted during an internship at the microbiology laboratory of University Hospital Nadir Mohammed in Tizi-Ouzou, between May 4th and June 4th. From a cytobacteriological examination of urine (CBEU), we isolated a strain of *Klebsiella pneumoniae*, identified as responsible for a urinary tract infection. The bacterium was characterized using phenotypic tests and an antibiogram, confirming its multidrug resistance to several classes of antibiotics. This initial phase is crucial for diagnosing resistance, a major challenge in treating nosocomial infections.
2. **Testing sensitivity to essential oils:** The second part of the study focused on evaluating the sensitivity of the isolated strain to two essential oils: thyme essential oil *(Thymus vulgaris*) and oregano essential oil (*Origanum compactum*). An aromatogram was performed to assess the antimicrobial efficacy of EOs against the strain. These oils were chosen due to their accessibility and well-documented antimicrobial properties, which have been empirically known for centuries, making them potential natural alternatives to antibiotics.
3. **Synergy test between essential oils and antibiotics:** The third and final phase of the study aimed to assess potential synergy between the essential oils and three commonly used antibiotics: chloramphenicol, cefotaxime, and piperacillin. This synergy test sought to determine whether the combination of essential oils with antibiotics could enhance therapeutic efficacy against the multidrug-resistant strain or even to suppress the antibio-resistance. If proven successful, this synergy could offer a novel therapeutic approach to overcome bacterial resistance.

In summary, this study explores an approach combining natural agents and antibiotics to propose a solution to the growing problem of bacterial resistance.

3.1. Materials

3.1.1. Biological Material

- **Essential oils:** The two essential oils employed in our study, procured from commercial sources, are thyme oil (*Thymus vulgaris*) and oregano oil (*Origanum compactum*).
- **Bacterial Strain:** In this study, the bacterial strain *Klebsiella pneumoniae* was isolated from a urine sample of a patient in the intensive care unit at CHU-Tizi-Ouzou.

3.1.2. Non-Biological Materials

The complete list of non-biological materials used in this study including equipment, culture media, reagents, antibiotics, disposable material, testing materials and other supplies is detailed in the appendices.

3.2. Methods

3.2.1. Isolation and Identification of a Multidrug-Resistant Bacterium

3.2.1.1. Collection and Transport of Urine Samples

To ensure accurate results in the diagnosis of urinary infections, it is crucial to follow a rigorous procedure for collecting and transporting urine samples (Berthélémy, 2016). Here are the steps for optimal collection:

a) Timing of Collection

Collect the sample in the morning, when the urine has been retained in the bladder long enough to accumulate a significant bacterial load, which is essential for a reliable culture (Berthélémy, 2016).

b) Collection Methods

According to Holliday (2014)**,** to minimize contamination in urinary samples, especially during urine collection for diagnostic purposes like a urinalysis or culture, the following techniques are essential:

- **For Men:** Collect a midstream urine sample in a sterile container after thoroughly cleaning the urinary meatus. This method reduces contamination from surrounding skin flora.
- **For Women:** Before the first morning urination, thoroughly clean the genital area with mild soap, rinsing from front to back. This step is crucial to avoid contamination from vaginal flora. The collection should be done outside of menstrual periods to avoid interference.
- **For Infants:** Use sterile pediatric urine collection bags available from the laboratory. Disinfect your hands, place the infant on their back, clean the urogenital area with soap and water, and dry thoroughly before applying the bag.
- **For Catheterized Patients:** Collect urine directly from the catheter by first purging a small volume to remove any stagnant urine. Use a sterile syringe or appropriate device to transfer the sample into a sterile collection bottle, avoiding contamination. Label the bottle

with the necessary information and transport it to the laboratory as quickly as possible, keeping it at 4°C if transport cannot be completed within 2 hours.

c) Handling the Samples

Transfer the urine into a sterile collection bottle without touching the inner rim of the container to avoid contamination. Label the bottle with the patient's name, surname, and the date of collection (Berthélémy, 2016).

d) Transport and Storage

To prevent bacterial growth, transport the sample to the laboratory as quickly as possible, ideally within 2 hours. If immediate transport is not possible, place the bottle in a container with ice to maintain a low temperature. Store the urine at 4°C for up to 24 hours if it cannot be analyzed on the same day (Forney, 1969).

3.2.1.2. Urinary Cytological Examination

a) Macroscopic Cytological Examination

The macroscopic cytological examination of urine begins with homogenization to ensure even distribution of elements. Next, the urine's color is observed, which can vary based on hydration levels or the presence of pathological substances; normal urine is generally clear or light yellow. The urine's appearance is also assessed, ranging from clear to turbid or milky, which can indicate the presence of cells, bacteria, or crystals. Finally, the urine's odor is noted, as an abnormal odor, such as a strong unpleasant smell, may suggest an infection or other pathologies (Koss & Hoda, 2012). These initial observations guide the more detailed microscopic analysis.

b) Microscopic Cytological Examination

According to Roxe (1990), the microscopic cytological examination involves preparing and analyzing a urine sample, which is collected using a micropipette and placed between a Malassez cell and a coverslip. The Malassez cell, which defines a volume of 1 mm^3 (or 1 µl), is used to count the formed elements in the urine, such as leukocytes, erythrocytes, bacteria, epithelial cells, and crystals. These elements are observed under an optical microscope with a 40x magnification. Counting is performed in 5 rectangles of the Malassez cell grid, and the results are multiplied to obtain a precise concentration. Microscopic analysis helps diagnose anomalies such as urinary infections, the presence of blood, or other pathologies based on the cells and crystals observed.

3.2.1.3 Bacteriological Culture of Urine

The bacteriological culture of urine is a crucial method for identifying pathogens responsible for urinary infections. This technique involves inoculating a urine sample onto an appropriate culture medium to promote the growth of present bacteria (Ouanes et al., 2013).

The choice of culture medium is based on the quality of the urine and the results of the microscopic examination. Typically, two media are used: Nutrient Agar (NA) and CHROMAgar (Manickam et al., 2020).

a) Protocol Used

- **Sample Collection:** A urine sample is collected aseptically.
- **Inoculation:** A drop of urine is placed on the surface of an agar plate (NA or CHROMAgar) and spread in streaks using a sterile platinum loop.
- **Incubation:** The Petri dishes are incubated at 37°C for 24 to 48 hours, optimal conditions for the growth of most bacteria responsible for urinary infections.

b) Criteria for Choosing the Culture Medium

- **Turbid Urine with High Leukocyturia (>10^4 CFU/mL):** Use CHROMAgar, which allows for rapid detection and preliminary identification of germs due to its colored colonies (D'Souza et al., 2004).
- **Clear Urine with Few or No Leukocytes:** Use Nutrient Agar, a more general culture medium that supports the growth of a broad spectrum of bacteria.

3.2.1.4 Colony Observation

a) Direct Examination

The wet mount of a colony, also known as a wet preparation or direct examination, is a microbiological technique that allows for real-time observation of bacterial motility, shape, and natural morphology. This method preserves the bacteria's true form and arrangement, providing insights into their movement, size, and cellular structure. It is a quick and straightforward approach that offers an initial, accurate assessment without the need for staining or complex procedures, making it useful for observing live bacteria in their natural state.

The direct examination is prepared following several steps. First, a small amount of the bacterial colony is picked up using a sterile pipette. This colony is then suspended in a drop of sterile water or saline on a microscope slide. The preparation is then covered with a coverslip to prevent evaporation and contamination. The preparation is observed under the microscope at

low and medium magnifications, typically 10x and 40x, with or without phase contrast, to examine the shape, size, and motility of the bacteria.

b) Isolation of the colony

After incubating the samples on culture media, we start by examining the bacterial colonies to note their key characteristics. We observe the shape of the colonies (round, irregular), their size (small or large), their color (white, yellow, red), their texture (smooth or rough), and their opacity (transparent, translucent, opaque). If different types of colonies are observed, they are isolated by transferring them to fresh media to obtain pure cultures, which facilitates their accurate identification. Selective or differential media such as CHROMAgar, Hektoen Enteric Agar, or Chapman Agar are often used for this purpose (Shapiro, 1995).

3.2.1.5 Identification of Bacterial Strains.

a) Identification using Hektoen Enteric Agar Orientation

The Hektoen medium is used to isolate pathogenic enteric bacteria, particularly *Salmonella* and *Shigella*. It inhibits the growth of Gram-positive bacteria due to bile salts and allows the growth of Gram-negative bacteria. It differentiates bacteria based on their ability to ferment sugars (fermenting colonies are yellow/orange, non-fermenting ones are green/blue). *Salmonella* also produces hydrogen sulfide (H_2S), visible as black precipitates in the colonies. This medium is used to isolate and differentiate *Salmonella* and *Shigella* from other enteric bacteria (Roxe, 1990).

b) Identification using CHROMAgar Orientation

CHROMAgar is a chromogenic, differential medium designed for the rapid identification and differentiation of bacterial species through specific enzymatic reactions. It contains chromogenic substrates that react with enzymes produced by various bacteria, resulting in distinct colony colors. For example, *Klebsiella pneumoniae* often appears as blue colonies, while other bacteria may show pink, green, or other colors. This medium is particularly useful for primary culture as it allows for the simultaneous isolation and identification of bacterial strains based on colony morphology and color, reducing the need for additional procedures like Gram staining or biochemical tests by up to 28**%** **(**Ouanes et al., 2013**)**. CHROMAgar is commonly used in clinical settings for the rapid detection of pathogens, especially those causing urinary tract infections, such as *E. coli*, *Klebsiella*, and *Proteus* (Perry, 2017).

c) Gram Staining

To perform Gram staining, we begin by preparing a slide by spreading a thin layer of the bacterial culture. We then fix the film by heating the slide or using a chemical fixative. We first apply crystal violet as the primary stain, followed by iodine to fix the dye in the cells. Next, we decolorize with alcohol or acetone and then apply safranin as a counterstain. Finally, we observe the slide under a microscope: Gram-positive bacteria will appear purple, while Gram-negative bacteria will be pink or red (Coico, 2006).

(The details of the Gram staining procedure are provided in the appendix.)

3.2.1.6. Biochemical Identification

After isolating bacterial strains on a CHROMAgar medium, it is crucial to perform biochemical tests to differentiate between species that may appear visually similar. Bacteria such as *Klebsiella*, *Enterobacter*, and *Serratia* can have similar appearances, making visual identification alone insufficient (Blok & Powers, 2009).

To refine identification, we used biochemical tests such as catalase, oxidase and coagulase tests, along with API 20E and VITEK® 2 COMPACT systems. These tools assess various biochemical characteristics, including urease-indole, allowing for more accurate species identification based on metabolic and chemical reactions, and reducing the risk of confusion between similar-looking strains.

- **Oxidase Test**

The oxidase test identifies the presence of the cytochrome oxidase enzyme in bacteria. To perform this test, place oxidase disks (containing N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride) on a slide. Using a Pasteur pipette, transfer a bacterial colony onto the disk. A positive result is indicated by the development of a purple color within a few seconds (Shields & Cathcart, 2010).

- **Catalase Test**

The catalase test detects the enzyme catalase in bacteria, which decomposes hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). To conduct this test, place a drop of hydrogen peroxide on a slide, then use a Pasteur pipette to transfer a colony from an 18 to 24-hour old pure culture into the hydrogen peroxide. A positive result is shown by the presence of bubbles due to oxygen release, indicating the bacteria produce catalase (Reiner, 2010).

- **Identification Using 20 E API System (Analytical Profile Index)**

The biochemical identification of the bacterial strain was performed using the API 20 E system from BioMérieux. The API 20E is a standardized system used for identifying Enterobacteriaceae and other non-fussy Gram-negative bacilli through 20 miniaturized biochemical tests. To use it, we first isolate a colony from an agar medium and prepare a bacterial suspension in sterile physiological water, corresponding to a 0.5 McFarland density. This suspension is then introduced into the microtubes of the API 20E gallery using a syringe, avoiding air bubbles. Tests CIT, VP, and GEL are filled directly, while for ADH, LDC, ODC, H2S, and URE tests, an anaerobic environment is created by adding mineral oil to the cupules. After incubation at 37°C for 18 to 24 hours, specific reagents are added to reveal the color changes produced by the bacteria, such as TDA reagent for the TDA test, Kovacs reagent for the IND test, and VP 1 and VP 2 reagents for the VP test. The results are read based on the observed color changes, and final identification is performed using the APIweb™ Biomerieux software or UPBM le LAB site (Holmes et al., 1978).

- **VITEK® 2 COMPACT Identification**

The VITEK® 2 COMPACT system provides rapid and precise bacterial identification. It uses specialized "VITEK cards" that contain various biochemical tests. Different card types are available for identifying a range of bacterial groups and species (Nakasone et al., 2007). This is how it works:

- For each bacterial strain, use two dry tubes (one for identification and one for the antibiogram). Add 3 mL of VITEK saline solution to each tube.
- Transfer a few colonies from a pure agar culture into each tube, mix thoroughly, and adjust the density to 0.5 McFarland with a densitometer.
- Place the appropriate identification card and the antibiogram card into the tubes (ensure the tab is positioned correctly).
- Insert the tubes into the VITEK rack. The suspension will transfer to the cards through the tab in about ten minutes in the first station.
- Enter patient information into the VITEK software. After receiving the signal, move the cassette to the second station for incubation, identification, and antibiogram testing.
- Results will be available after 24 hours and can be accessed through the VITEK software.

3.2.1.7 Antibiotic Susceptibility Test (Antibiogram)

Once a bacterium is isolated and identified, it is crucial to establish its sensitivity profile. This profile guides the physician in selecting the most effective antibiotics for treating the patient. There are several methods for performing an antibiogram, each with its specific features and applications (Akualing et al., 2016). Here are the two methods used in this work:

a) Disk Diffusion Method (Kirby-Bauer Method)

The Disk Diffusion Method, also known as the Kirby-Bauer Method, is a standardized procedure used in microbiology labs to determine the susceptibility of bacteria to various antimicrobial agents. This method assesses how effective specific antibiotics are against a particular bacterial isolate (Hudzicki, 2009).

- **Medium:** Pour the agar into Petri dishes to a thickness of 4 mm and let it dry.
- **Preparation of Inoculum:** Take colonies from an 18 to 24-hour culture, dilute in 5-10 ml of sterile saline or buffer, and homogenize to a 0.5 McFarland opacity.
- **Inoculation:** Inoculate the agar by making cross-streaks with a sterile swab. Reload the swab for each Petri dish if needed.
- **Application of Antibiotic Discs:** Do not exceed 6 discs per 90 mm dish. Use sterile forceps to place the discs without moving them.
- **Incubation:** Follow the specific conditions for temperature, atmosphere, and duration.
- **Reading:** Measure the inhibition zones with a caliper, compare to the critical values, and classify as Resistant (R), Sensitive (S), or Intermediate (I).

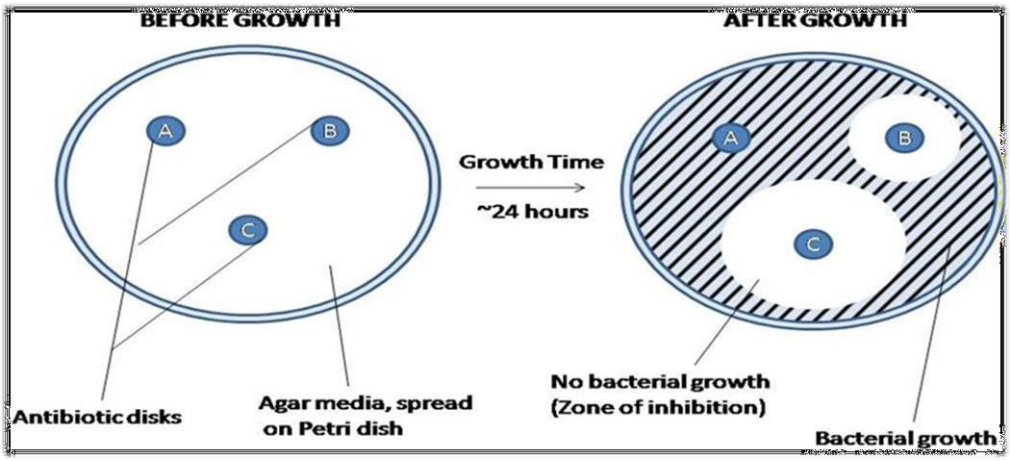

Figure 11: Disk diffusion method (Kirby-Bauer Method) (Brown & Kothari, 1975).

Table 03: List of tested antibiotics.

Abbreviation	Antibiotic Name	Family	Concentration (µg)
COT	Co-trimoxazole (trimethoprim/sulfamethoxazole combination)	Sulfamides and diaminopyrimidines	25
AMC	Amoxicillin + clavulanic acid	Beta-lactams (penicillin/inhibitor combination)	30
F	Flucloxacillin	Beta-lactams (penicillins)	300
CTX	Cefotaxime	Beta-lactams (3rd generation cephalosporins)	30
C	Chloramphenicol	Phenicol	30
CL	Colistin	Polymyxins	10
AMP	Ampicillin	Beta-lactams (penicillins)	10
CZ	Cefazolin	Beta-lactams (1st generation cephalosporins)	30
CRO	Ceftriaxone	Beta-lactams (3rd generation cephalosporins)	30
NA	Nalidixic acid	Quinolones	30
AX	Amoxicillin	Beta-lactams (penicillins)	25
CN	Gentamicin	Aminoglycosides	10
ETP	Ertapenem	Carbapenems	10
TI	Ticarcillin	Beta-lactams (penicillins)	75
LEV	Levofloxacin	Fluoroquinolones	5

Antibiogram results can also be classified according to critical values such as critical concentration and critical diameter, which are arbitrarily chosen thresholds for specific antibiotic-bacteria pairs. According to Cavallo et al. (2006), the strains are classified as follows:

- **Sensitive (S):** When the minimum inhibitory concentration (MIC) of the tested antibiotic is less than or equal to the lower critical concentration (C), which corresponds to an inhibition zone diameter equal to or greater than the critical diameter D.
- **Resistant (R):** When the MIC is greater than or equal to the higher critical concentration (C), which corresponds to an inhibition zone diameter less than the critical diameter D.
- **Intermediate (I):** When the MIC of the tested antibiotic and the corresponding inhibition zone diameter fall between the two critical concentrations and the two critical diameters.

b) Automated Method (VITEK® 2 COMPACT)

The VITEK® 2 COMPACT system is an advanced, automated technology used for bacterial identification and antibiotic susceptibility testing. It enables rapid and precise analysis of bacterial strains using pre-filled cards with biochemical tests. The process begins with preparing a bacterial suspension standardized to 0.5 McFarland, which is then inoculated into sterile VITEK® 2 COMPACT tubes. This suspension is transferred to VITEK® 2 COMPACT cards containing specific reagents for assessing antibiotic susceptibility. The cards are placed in the VITEK® 2 COMPACT system, where they are automatically incubated and analyzed. The associated software then provides a detailed report on the bacterial strain's sensitivity or resistance to the tested antibiotics (Quentin-Noury, 2016).

3.2.2. Testing sensitivity to essential oils

To evaluate the antibacterial efficacy of the two essential oils in our study, we employed a two-step approach. First, we prepared a series of dilutions of the essential oils to obtain a range of concentrations. Then, we conducted an aromagram to assess the impact of these concentrations on the bacterial growth of the tested strain. This method allows us to determine the antibacterial activity of the essential oils and their potential as alternatives to traditional antibiotic treatments.

3.2.2.1 Preparation of Solutions

To evaluate the antibacterial activity of essential oils, we employed the serial dilution method, also known as "two-fold dilution." This method is designed to prepare a series of solutions with decreasing concentrations by successively diluting a stock solution in an appropriate solvent.

- **Preparation of the Stock Solution:** The stock solution was prepared by combining 500 µL of sterile distilled water with 500 µL of DMSO (dimethyl sulfoxide), which acts as a solvent

to dissolve the essential oils. To this mixture, we added 200 µL of essential oil, creating a concentrated stock solution.

- **Serial Dilution:** From the stock solution, we performed successive two-fold dilutions. In each step, a portion of the resulting solution was mixed with an equal volume of solvent to halve the concentration compared to the previous solution. For instance, in the first dilution, 500 µL of the stock solution was mixed with 500 µL of solvent (DMSO and distilled water in a 1:1 ratio), resulting in a concentration of 100 µL/mL of essential oil. This process was repeated to obtain a series of solutions with decreasing concentrations. The successive dilutions yielded the following concentrations: 200 µL/mL, 100 µL/mL, 50 µL/mL, 25 µL/mL, 12.5 µL/mL, and 6.25 µL/mL. Each dilution was prepared by combining a portion of the previous solution with an equal volume of solvent, thus halving the concentration at each step.
- **Control Solution:** In addition to the solutions containing essential oils, a control solution was also prepared. This control solution did not contain any essential oil and was used to ensure that any observed effects in the tests were due to the essential oils and not to other factors.

Table 04: Summary Table of Dilutions and Concentrations of the Two Essential Oils Used.

EOs Dilutions	Negative Control	Stock Solution	1/2	1/4	1/8	1/16	1/32
Concentration (µL /mL)	0	200	100	50	25	12.5	6.25

To ensure precise and uniform concentrations in each dilution, it is crucial to homogenize each solution thoroughly. This involves mixing the solutions carefully after each dilution step to ensure even distribution of the essential oils in the solvent. Effective homogenization is essential to achieve correct concentrations and prevent variations that could affect the test results.

This method allows for the creation of a range of essential oil concentrations, facilitating the evaluation of their antibacterial activity at various levels.

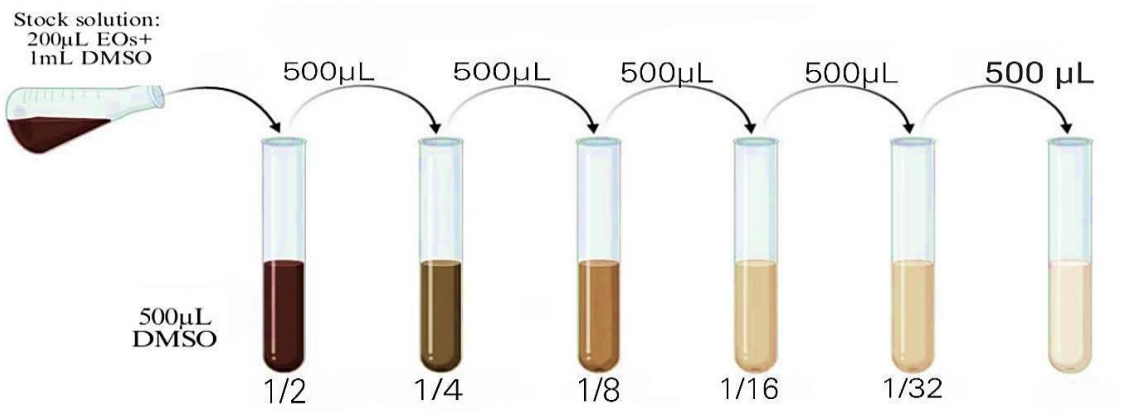

Figure 12: Scheme of the two-fold serial dilution method.

3.2.2.2 Aromatogram Test

The aromatogram is an in vitro method used to measure the antibacterial activity of essential oils, analogous to an antibiogram but with essential oils replacing antibiotics. The detailed steps of this method are as follows:

- **Preparation of Cultures:** From a 24-hour pure and young culture of the isolated microbial strain, select a few identical and well-isolated colonies. Transfer them into 5 mL of sterile physiological water and thoroughly homogenize the bacterial suspension until its opacity matches 0.5 McFarland (10^8 CFU/mL).
- **Inoculation of Plates:** Dip a sterile swab into the bacterial suspension, then press it firmly against the inside wall of the tube to remove excess liquid. Rub the swab over the entire surface of the Mueller Hinton Agar plate in a top-to-bottom, overlapping streaking pattern. Repeat the process three times, rotating the plate 60° each time to ensure even inoculation.
- **Application of Discs:** Under aseptic conditions, sterilized 6 mm diameter discs (Whatman) are impregnated with 10 µL of each essential oil concentration prepared by dilution. These discs are then placed on the surface of the inoculated agar.
- **Incubation and Measurement:** The inoculated plates are incubated at 37°C for 18 to 24 hours. After incubation, antimicrobial activity is evaluated by measuring the diameter of the inhibition zone around the discs, expressed in millimeters (mm). Each test is performed in triplicate to ensure accuracy and reproducibility of the results.

This method allows for the assessment of the effectiveness of essential oils by measuring their ability to inhibit the growth of the tested microorganism, providing a detailed evaluation of its antibacterial potential.

Table 05: Bacterial Resistance and Sensitivity to Essential Oils (Ponce et al., 2003).

Sensitivity of the Strain	Diameter of the Inhibition Zone
Extremely sensitive (+++)	Greater than 20 mm
Very sensitive (++)	Between 15- 19 mm
Sensitive (+)	Between 9- 14 mm
Intermediate (+/-)	Between 6- 8 mm
Resistant (-)	Less than 6 mm / None

3.2.2.3. Determination of the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) is determined after the aromatogram by examining the inhibition zones around the discs impregnated with different concentrations of essential oils. The diameters of the inhibition zones are measured, and the MIC is identified as the lowest concentration that produces a visible inhibition zone, indicating the cessation of bacterial growth. This concentration represents the threshold at which the essential oil effectively inhibits the microorganism's development, thus reflecting its minimum antibacterial potential (Andrews, 2001).

3.2.3. Synergy test between essential oils and antibiotics

The synergy test between essential oils and antibiotics aims to evaluate the combined effect of these two agents against bacterial growth (Amassmoud et al., 2023). The detailed steps of this method are as follows:

- **Preparation of Bacterial Cultures:** A suspension of the bacterial strain is adjusted to a density of 0.5 McFarland (10^8 CFU/mL) and used to uniformly inoculate a Mueller Hinton agar plate using a swab.
- **Application of Antibiotic Discs and Essential Oil:** Once the plate is inoculated, antibiotic discs (chloramphenicol, cefotaxime, and piperacillin) are placed on the agar. Next, 10 µL of the essential oil dilution corresponding to the minimum inhibitory concentration (MIC), determined during the aromatogram (thyme or oregano), are added

to each disc using a micropipette. This step assesses the effect of the combination between antibiotics and essential oils. The plates are incubated at 37°C for 18 to 24 hours.

- **Results Evaluation:** After incubation, the inhibition zones around the discs are measured. If an extension or overlap of the inhibition zones is observed, it indicates a positive synergy, enhancing the antibacterial action of both agents. In the absence of changes, it indicates no synergy.
  - **Positive Synergy:** If the inhibition zones around the antibiotic and essential oil discs overlap or extend towards each other, it suggests positive synergy. This means the combination of the two agents has a greater antibacterial effect compared to their individual actions.
  - **No Synergy:** If the inhibition zones remain separate or if there is no significant difference in the size of the inhibition zones compared to controls, it indicates no synergy. This suggests that the combination of agents does not improve the antibacterial effect compared to their individual actions.
  - **Antagonism:** The presence of essential oils seems to reduce the effectiveness of the antibiotic. Rare, but may indicate that the combination of the two agents is counterproductive.

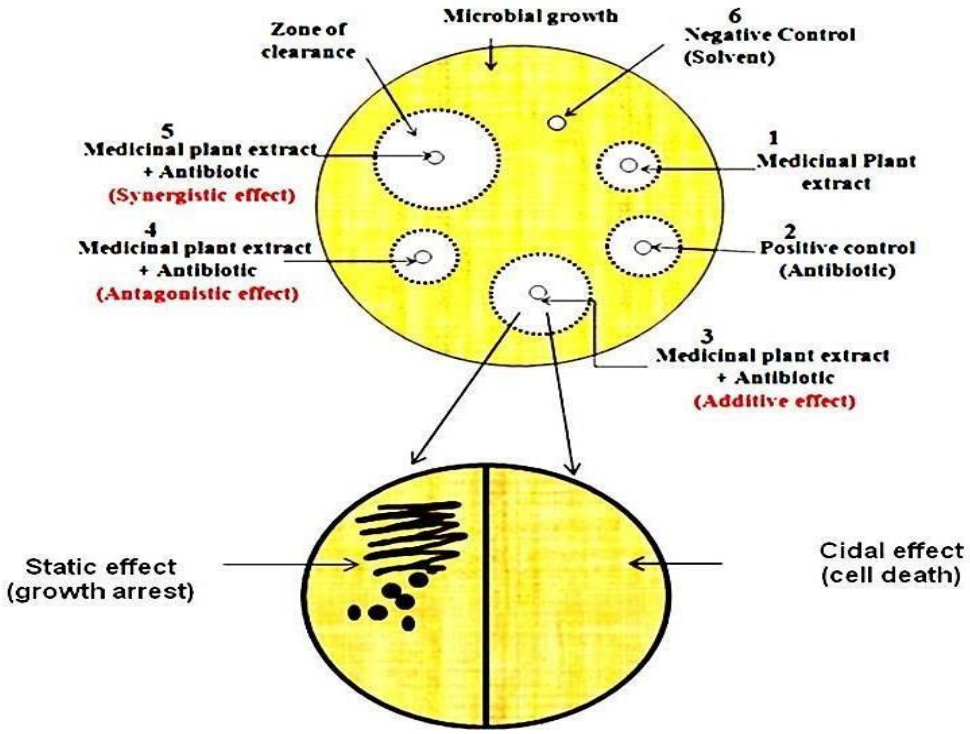

Figure 13: Agar well diffusion method for synergistic activity (Rolta et al., 2018).

CHAPTER IV

RESULTS & DISCUSSION

1. Results of the identification of the bacterial strain

1.1. Urinary Cytological Examination Results

I.1.1 Macroscopic Cytological Examination

The macroscopic analysis of the urine sample reveals several significant features. The dark yellow color suggests concentrated urine, often associated with dehydration or an ongoing infection (Queremel Milani & Jialal, 2024). Additionally, the cloudy appearance likely indicates the presence of leukocytes, bacteria, crystals, or mucus, all of which are typical signs of a urinary tract infection (Elsamra, 2021).

Figure 14: Macroscopic analysis of urine sample: visual examination and observations (Original).

A cytobacteriological examination of the urine (ECBU), accompanied by an antibiogram, plays a crucial role in diagnosing urinary tract infections. This examination is essential for confirming the presence and type of infection by identifying the bacteria responsible.

Additionally, the antibiogram provides valuable information regarding the sensitivity of the identified bacteria to different antibiotics, allowing healthcare providers to determine the most effective treatment. By guiding the choice of antibiotic, this combined approach helps ensure that the prescribed medication targets the specific pathogen, reducing the risk of treatment failure and minimizing the development of antibiotic resistance (Klinker et al., 2021).

1.1.2. Microscopic Cytological Examination

The microscopic observation of the urine sample on the Malassez cell, reveals the presence of epithelial cells, likely from the urinary tract, along with a few red blood cells, suggesting possible hematuria. Leukocyturia, with more than 1000 leukocytes/mm³, indicates an infection or inflammation of the urinary tract, often associated with cystitis or pyelonephritis. The presence of higher amount of mesophilic flora, indicating significant bacterial colonization, supports the hypothesis of a bacterial urinary infection. Thus, the observed elements point towards a urinary infection, requiring further analysis, such as a bacterial culture and antibiotic sensitivity testing, to accurately identify the pathogen and tailor the treatment (Mancuso et al., 2023).

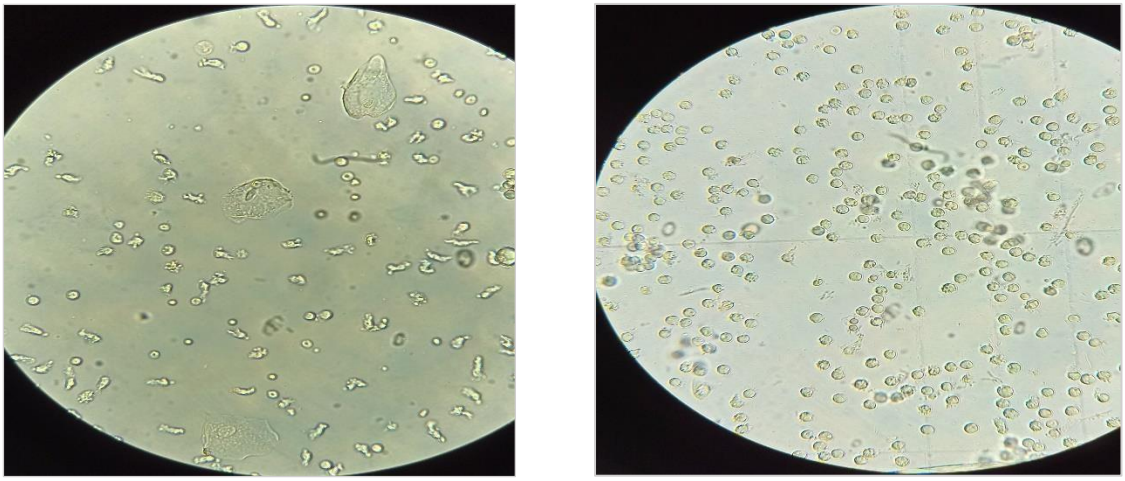

Figure 15: Cytology on Malassez cells of a Urine sample (Original).

Table 06: Interpretation of Urine Cytology Examination Results (Bonacorsi, 2016)

Bacteria	Leukocytes	Red blood cells	Interprétation
$<10^3$UFC/ml	$< 10^4$ / ml	< 1000 / ml	Absence d'IU
$<10^3$UFC/ml	$> 10^4$ / ml	< 1000 / ml	Leukocyturia
$>10^3$à 10^5UFC/ml	$> 10^4$ / ml	> 1000 / ml	Présence d'IU
$>10^3$à 10^5UFC/ml	$< 10^4$ / ml	< 1000 / ml	Early infection or contamination
$<10^3$UFC/ml	$< 10^4$ / ml	> 10 000 / ml	Macroscopic hematuria

1.2. Results of Urine Bacteriological Culture

Observing the cultural characteristics of the grown colony on the Petri dish, after culturing on nutrient medium agar and incubating for 24 hours at 37°C, allowed us to examine the shape and appearance of the colonies. At first glance, we observe that these colonies closely resemble those of *Klebsiella pneumoniae*.

According to Mitrea and Vodnar (2019), on nutrient agar, it produces distinctive colonies that serve as reliable indicators of the bacterium's presence, characterized by these unique features:

- After 72 hours of incubation, the colonies typically grow to a size of 2 to 3 mm in diameter and display a cream or pale gray color.
- Their surface is smooth, shiny, and may appear slightly moist or mucoid due to a prominent polysaccharide capsule.
- They are generally circular with well-defined edges and are slightly raised or convex.
- These colonies are often thick and can be filamentous when lifted with a platinum loop.
- These morphological characteristics are indicative of *Klebsiella pneumoniae*, though precise identification requires further testing (figure 16).

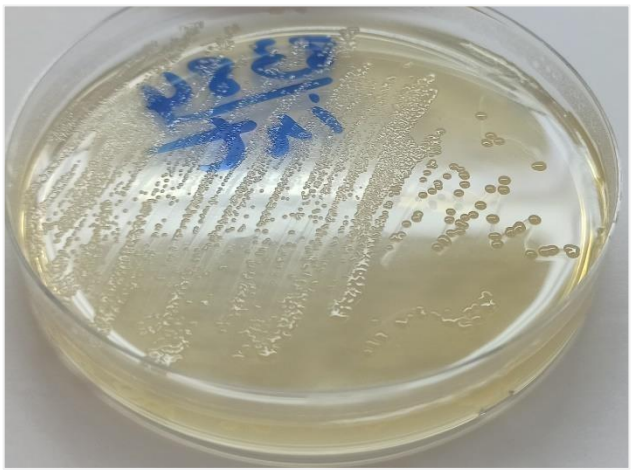

Figure 16: Macroscopic characteristics of the colony grown on nutrient agar.

1.2.1. Colony observation and isolation

A. Results of the direct examination

A wet mount is prepared after 24 hours of bacterial culture from the urine sample to assess the morphology, motility, and size of the bacterium (Palma et al., 2022). This microscopic examination provides valuable information that will further guide the identification process and

aid in selecting the most suitable media for subsequent isolation and cultivation of the bacterial strain.

The bacterium appears as bacilli (small rods), with its cell wall not highlighted since no specific staining is used. It is also characterized by its immobility, which is easily observed due to the lack of active movement, unlike some motile species. These signs are typically indicative of *Klebsiella pneumoniae* (Figure 17).

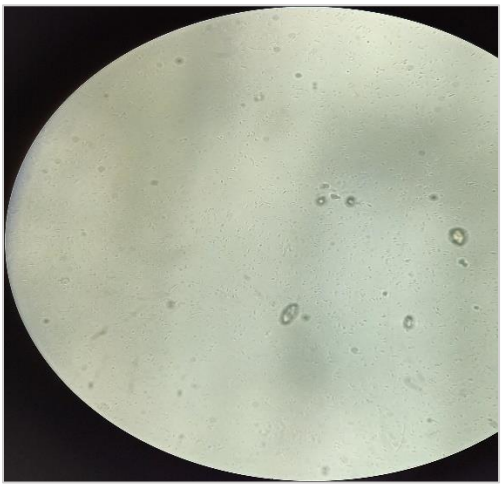

Figure 17: Microscopic Observation of a Fresh Bacterial Colony (Original).

B. Gram staining results

Microscopic examination at 1000x magnification revealed bacilli, which are small rod-shaped bacteria arranged either singly or in pairs. These Gram-negative bacteria appeared pink in color, as shown in (Figure 18).

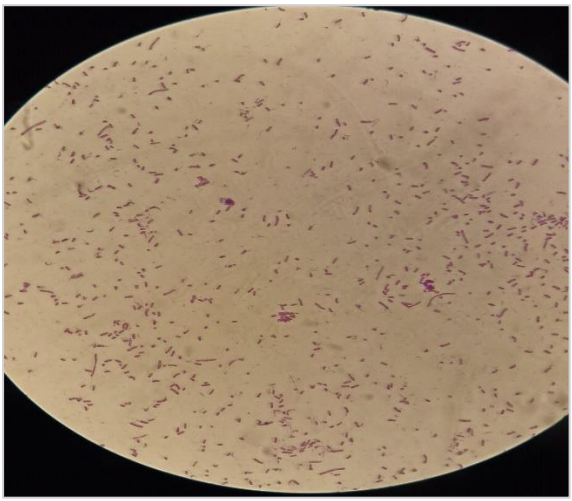

Figure 18: Microscopic Observation of Gram-Stained Sample at 100x Magnification.

C. Identification of Bacterial Strains (based on medium orientation)

Using CHROMAgar Orientation

As described in the previous chapter, we used CHROMAgar medium to identify bacterial colonies. The metallic blue color observed on this medium is a distinctive hue produced specifically by certain bacteria, namely those in the (K.E.S) family: *Klebsiella*, *Enterobacter*, and *Serratia*. CHROMAgar medium is designed to differentiate these species using specific dyes that react with enzymes produced by these bacteria. In this case, the metallic blue color indicates the presence of one of these three species, as they are the only ones that produce this color on this type of medium (Validi et al., 2016).

By observing this color, we were able to narrow our search to the genera *Klebsiella*, *Enterobacter*, and *Serratia*. However, to identify the exact strain among these three, further biochemical analysis is required. This step involves using specific biochemical tests to examine the metabolic and enzymatic characteristics of the bacterial colonies, thus allowing for precise strain identification.

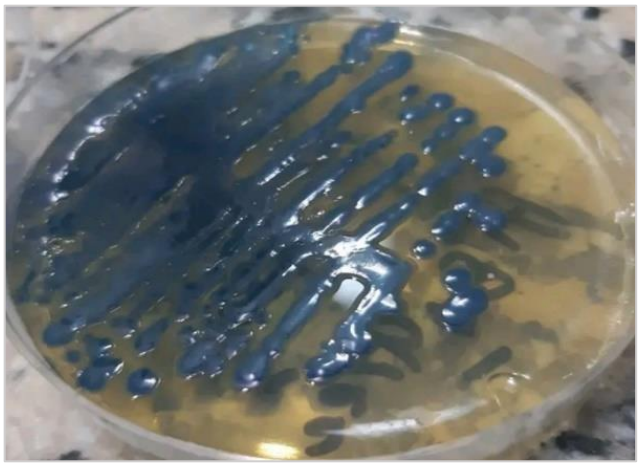

Figure 19: Colonies of (K.E.S) on CHROM agar Orientation medium (metallic blue).

Using Hektoen Enteric Agar Orientation

On Hektoen agar, the colonies appear bright orange, indicating that the bacterial strain ferments lactose. This coloration results from the acid produced during lactose fermentation, which changes the color of the medium from its usual green. Hektoen enteric agar is designed to differentiate bacteria based on their ability to ferment lactose and produce hydrogen sulfide (H_2S) (Hudzicki, 2010). In this case, the isolated bacterial strain

is lactose-positive, meaning it has the necessary enzymes to ferment lactose and produce acid, leading to the color change of the colonies. Additionally, this medium isolates Gram-negative bacilli.

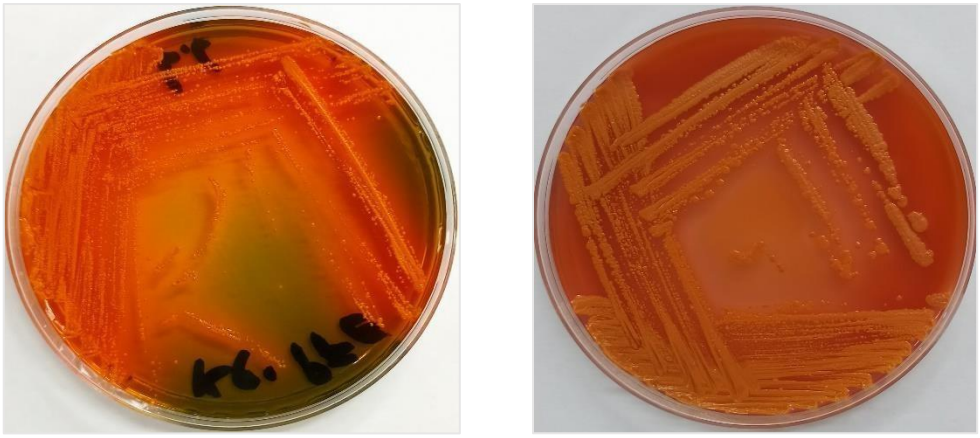

Figure 20: Macroscopic appearance of the bacterium colony on Hektoen Enteric medium (Original).

1.3. Biochemical Identification Results

- **Oxidase test:** The oxidase test performed on the bacterial strain yielded a negative result. This means that the strain does not possess the oxidase enzyme, which excludes certain bacterial species that are typically oxidase-positive.
- **Catalase test:** The bacterial strain, identified as a Gram-negative and oxidase negative but showing a positive result in the catalase test, suggests several possible bacterial genera. This profile points to genera such as *Escherichia*, *Klebsiella*, *Enterobacter*, and sometimes *Proteus*, all characterized by their catalase positivity and oxidase negativity. This information guides towards these groups for more precise identification using additional tests.
- **API 20E Results:** The following figure (figure 21) illustrates the color changes of the reagents in the API 20 E test after incubation for 18 to 24 hours. These color changes enable the identification and characterization of the tested bacterial species based on the observed reactions.

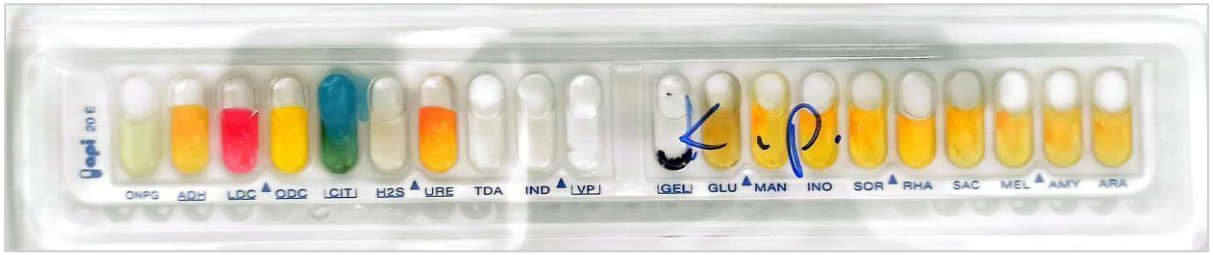

Figure 21: Identification of *Klebsiella pneumoniae* by API 20E system (Original).

After incubation, reagents were added to IND, TDA, and VP capsules, and results were read. Tests are grouped into 7 sets, with numbers assigned for positive or negative results. The 7-digit profile is compared to the analytical index to identify the bacterium.

The API 20E analysis revealed a biochemical profile characteristic of *Klebsiella pneumoniae*. Each microtube corresponds to a test of the bacterium's substrate metabolism, producing color changes. The resulting profile was compared to a reference database, confirming the identification of *Klebsiella pneumoniae*, a Gram-negative bacterium often responsible for nosocomial infections, especially UTIs.

The test conducted according Atlas et al. (1995) in conformation with the French manufactured company (BioMarieux).

Table 07: Summary table of positive and negative biochemical reactions *for Klebsiella pneumoniae (*API 20E test).

Test	Code	Negative Results	Positive Results	Results
β-galactosidase	ONPG	Colorless	Yellow	-
Arginine Dihydrolysis	ADH	Yellow	Red-orange	-
Lysine Decarboxylase	LDC	Yellow	Red-orange	+
Ornithine Decarboxylase	ODC	Yellow	Red-orange	-
Citrate Utilization	CIT	Yellow	Green-blue	+
Hydrogen Sulfide	H2S	Colorless	Black sediment	-
Urease Production	URE	Yellow	Red-orange	+
Tryptophan Deaminase	TDA	Yellow	Dark brown	-
Indole Production	IND	Yellow ring	Red ring	-
Acetone Production	VP	Colorless	Pink-red	-
Gel Hydrolysis	GEL	No pigments	Black pigments	-
Glucose	GLU	Blue	Yellow	+
Mannitol	MAN	Blue	Yellow	+
Inositol	INO	Blue	Yellow	+
Sorbitol	SOR	Blue	Yellow	+
Rhamnose	RHA	Blue	Yellow	+
Sucrose	SAC	Blue	Yellow	+
Melibiose	MEL	Blue	Yellow	+
Amygdalin	AMY	Blue	Yellow	+
Arabinose	ARA	Blue	Yellow	+

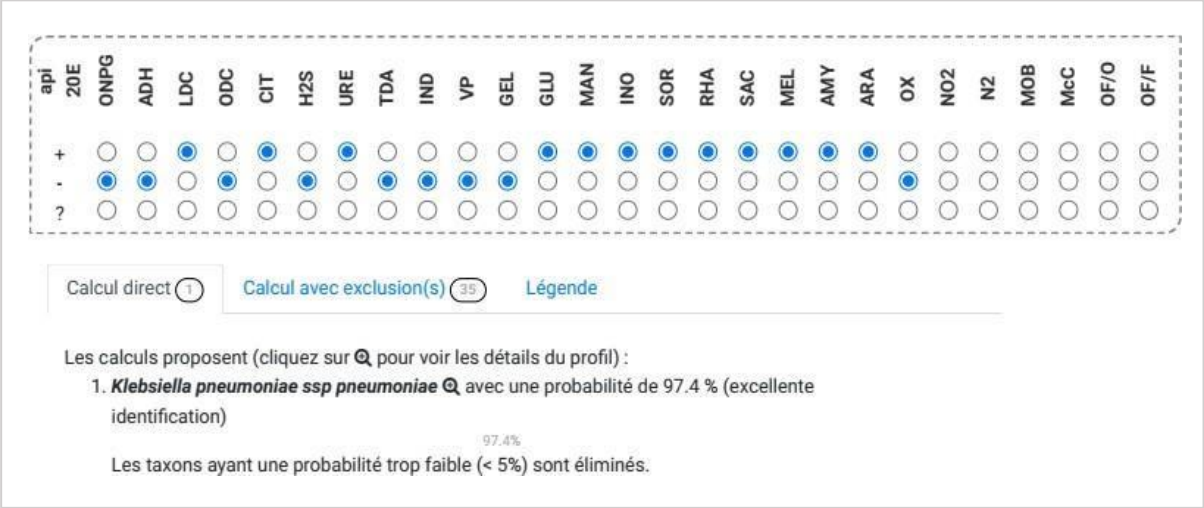

Figure 22: Bacterial identification by API 20E using UPBM Le Lab' software: *Klebsiella pneumoniae subsp. pneumoniae* with a probability of 97,4%.

Following the analysis of the API 20E using UPBM Le Lab' software:

- **Bacterial identification:** The software identified the bacterium with a very high probability (97.4%) as *Klebsiella pneumoniae subsp. pneumoniae*.
- **Biochemical profile:** The obtained biochemical profile corresponds to the positive and negative reactions of the various tests performed on the bacterium. Filled circles indicate a positive reaction, empty circles indicate a negative reaction, and question marks indicate uncertain or irrelevant results.
- **Reliability of results:** The probability of 97.4% indicates excellent reliability of the identification. This means that the obtained biochemical profile corresponds very well to that expected for *Klebsiella pneumoniae subsp. pneumoniae* in the software's database.

The analysis of the API 20E allowed the very probable identification of the bacterium as *Klebsiella pneumoniae subsp. pneumoniae*. This result is based on the comparison of the obtained biochemical profile with a reference database.

VITEK® 2 COMPACT Identification Results

The results of the VITEK® 2 COMPACT automated analysis indicate a very high probability (98%) that the bacterium isolated from the urine culture (Urine Cytobacteriological Examination) of patient M.F. is *Klebsiella pneumoniae subsp. pneumoniae* **(see appendix).**

1.4. Antibiogram results

The antibiograms performed on both MH (Mueller-Hinton) and cooked blood agar or chocolate agar media show a complete resistance of the *Klebsiella pneumoniae* strain to all the antibiotics tested (list of the antibiotics is detailed in the "materials and methods" section). This total resistance indicates that the strain is multidrug-resistant, meaning it can withstand many classes of antibiotics.

Figure 23: Antibiogram results of the *Klebsiella pneumoniae* (Mueller-Hinton medium).

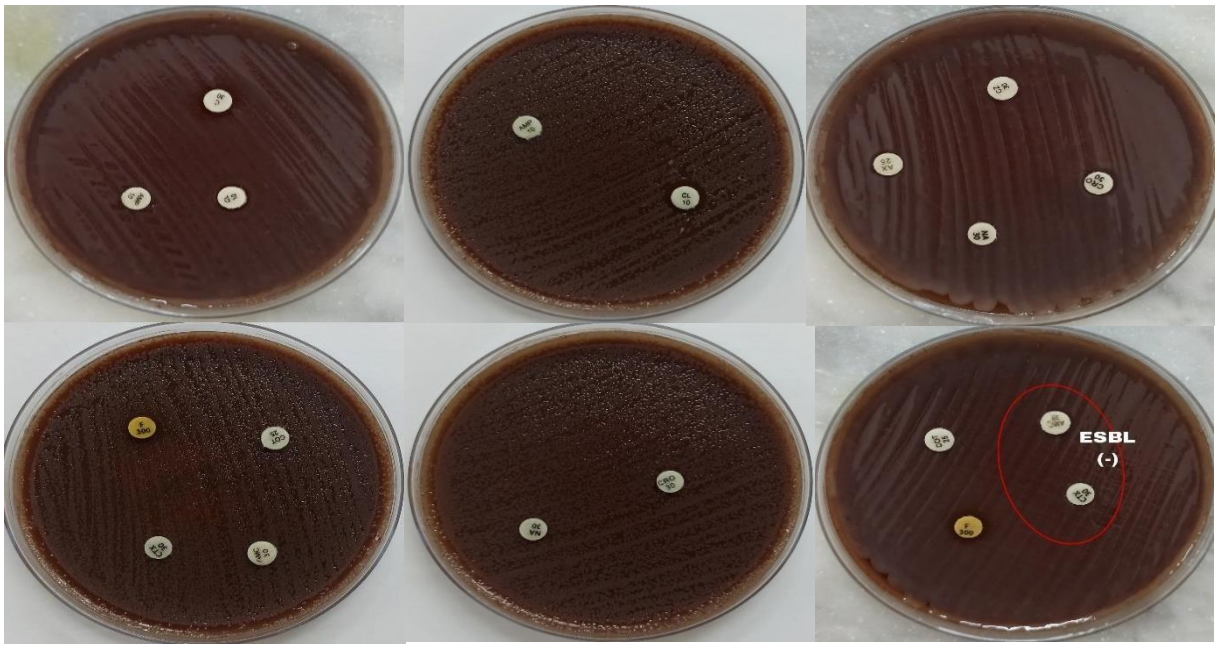

Figure 24: Antibiogram results of the *Klebsiella pneumoniae* (chocolate medium agar).

Additionally, the test for ESBL (Extended-Spectrum Beta-Lactamase) production is negative, meaning this enzyme, which often contributes to resistance against several beta-lactam antibiotics, is not present in this strain. Therefore, other resistance mechanisms, such as efflux pumps or enzymes that modify antibiotics, might be responsible for this multidrug resistance.

These results suggest that this *Klebsiella pneumoniae* strain is particularly difficult to treat due to its widespread resistance.

Table 08: Summary Table of Antibiotic Sensitivity Test Results.

Antibiotics	COT	AMC	F	CTX	C	CL	AMP	CZ	CRO	NA	AX	CN	ETP	TI	LEV
Susceptibility Test Results	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S: Sensitive; I: Intermediate; R: Resistant															

In summary, after analyzing the inhibition zones, it has been demonstrated that the strain is classified as multidrug-resistant, in accordance with CLSI (2022) recommendations (see appendix).

The results obtained with the VITEK® 2 COMPACT automated system confirmed the same findings: the strain exhibited complete resistance to all tested antibiotics, without exception. The VITEK® 2 COMPACT results will be included in the appendix to avoid repetition of the data.

2. Results of Essential Oil Sensitivity Testing

2.1. Sensitivity Testing with *Thymus vulgaris* essential oil

The two essential oils used, namely thyme essential oil (*Thymus vulgaris*) and oregano essential oil (*Origanum compactum*), exhibited significant antibacterial activity against the multi-resistant *Klebsiella pneumoniae* strain tested **(Figure 25** and **Table 09)**.

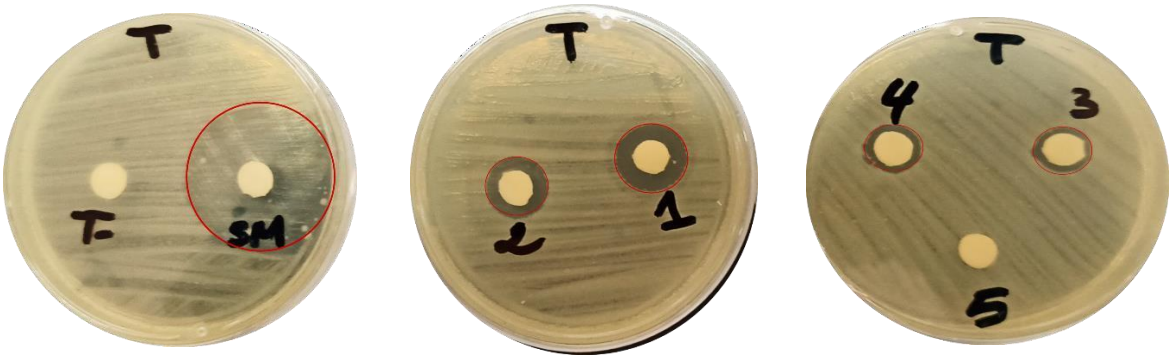

Figure 25: Antimicrobial activities of thyme essential oil (*Thymus vulgaris*).

Table 09: Antibacterial activity of the *Thymus vulgaris* essential oil (10µl/disc.) against the bacterial strain *Klebsiella pneumoniae* tested using disc-diffusion method, inhibition zones in mm.

Thymus vulagris	***Klebsiella pneumoniae***						
	T-	**SM**	**1**	**2**	**3**	**4**	**5**
Oil Dilutions	**Negative control**	**Stock solution**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**
Concentrations (µL /mL)	**0**	**200**	**100**	**50**	**25**	**12,5**	**6,25**
Inhibition Zone (mm)	6	22	12	10	8	7	6
Sensitivity Results	(-)	(+++)	(+)	(+)	(±)	(±)	(-)
Resistant: (-); Intermediate: (±); Sensitive: (+); Very sensitive: (++); Extremely sensitive: (+++).							

The results showed significant antibacterial activity of *Thymus vulgaris* essential oil against *Klebsiella pneumoniae*. The highest concentration tested (200 µL/mL) produced the largest zone of inhibition (22 mm), indicating a strong bactericidal effect. As the concentration decreased, the antibacterial activity reduced, with a zone of inhibition of 10 mm at 50 µL/mL, and no measurable inhibition at 6.25 µL/mL.

These findings suggest a typical dose-response relationship, where the antibacterial efficacy increases with the concentration of the essential oil. Furthermore, the minimum inhibitory concentration (MIC) of thyme essential oil was estimated to be between 50 and 100 µL/mL, indicating that this concentration range is required to completely inhibit the growth of *Klebsiella pneumoniae* under the conditions tested.

2.2. Sensitivity Testing with *Origanum compactum* essential oil

The study revealed significant antibacterial activity of oregano essential oil (*Origanum compactum*) against *Klebsiella pneumoniae*. A concentration of 200 µL/mL produced the largest inhibition zone, reaching 30 mm, indicating a strong bactericidal effect. Interestingly, inhibitory activity was observed down to a concentration of 50 µL/mL, with an inhibition zone of 14 mm. These results demonstrate a marked sensitivity of *Klebsiella pneumoniae* to oregano essential oil **(Figure 26** and **Table 10).**

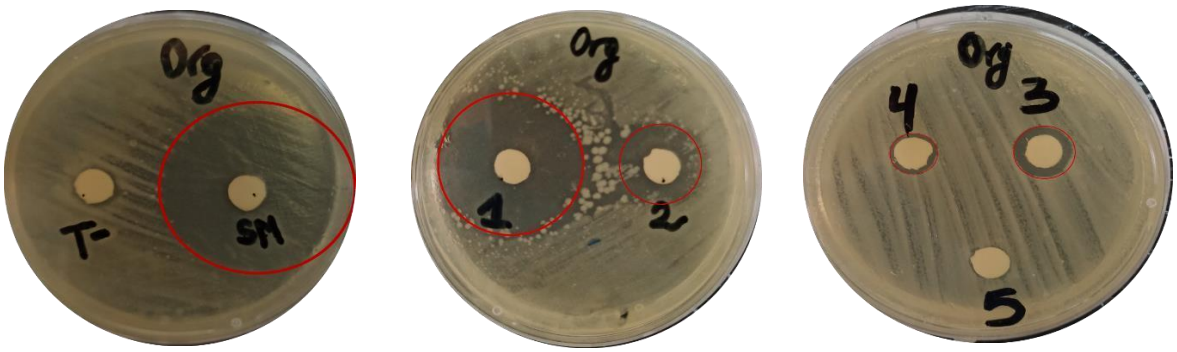

Figure 26: Antimicrobial activities of oregano essential oil (*Origanum compactum*).

Table 10: Antibacterial activity of the *Origanum compactum* essential oil (10µl/disc.) against the bacterial strain *Klebsiella pneumoniae* tested using disc-diffusion method, inhibition zones in mm.

Origanum compactum	***Klebsiella pneumoniae***						
	T-	**SM**	**1**	**2**	**3**	**4**	**5**
Oil Dilutions	**Negative control**	**Stock solution**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**
Concentrations (µL /mL)	0	200	100	50	25	12,5	6,25
Inhibition Zone (mm)	6	30	25	14	8	7	6
Sensitivity Results	(-)	(+++)	(+++)	(+)	(±)	(±)	(-)
Resistant: (-); Intermediate: (±); Sensitive: (+); Very sensitive: (++); Extremely sensitive: (+++).							

Based on this data, the minimum inhibitory concentration (MIC) of oregano essential oil against this bacterium is estimated to be 50 µL/mL, representing the concentration required to completely inhibit the growth of *Klebsiella pneumoniae* under the experimental conditions.

3. Results of the Synergy Test Between Essential Oils and Antibiotics

As part of our study, we evaluated the synergistic effect of thyme essential oil (*Thymus vulgaris*) in combination with three classes of antibiotics: cefotaxime, a third-generation cephalosporin, piperacillin and chloramphenicol. The objective was to determine whether this synergy could enhance efficacy against the multidrug-resistant strain *Klebsiella pneumoniae* (**Figure 27** and **Table 11**).

Figure 27: Results of synergy tests between thyme essential oil and the tested antibiotics (chloramphenicol, piperacillin, and cefotaxime) at concentrations of 50, 100, and 200 µL/mL against multidrug-resistant *Klebsiella pneumoniae*.

Table 11: Summary of the results from synergy tests between thyme essential oil and the antibiotics tested against *Klebsiella pneumoniae*.

EOs Concentrations (µL/mL)	Inhibition zones diameter Eos + ATB (mm)		
	Antibiotics		
	Chloramphenicol (C30)	Piperacillin (PRL30)	Cefotaxime (CTX30)
200	>30	>30	>30
100	20	20	25
50	12±1	12±1	12±1

The diameters of the inhibition zones were measured and compared to those produced by the essential oil alone. The test results demonstrated a clear synergy between thyme essential oil and the antibiotics tested. At a concentration of 50 µL/mL, the inhibition zone increased by approximately 3 mm compared to the essential oil alone. With 100 µL/mL, the inhibition zone expanded by 8 mm. At 200 µL/mL, the entire 6 cm Petri dish was fully inhibited, emphasizing the strong synergy between thyme essential oil and the antibiotics, as illustrated in **(Figure 27)**.

The combination of thyme essential oil with chloramphenicol, piperacillin, and cefotaxime shows promising potential to enhance the effectiveness of antimicrobial treatments against *Klebsiella pneumoniae*. This positive synergy can be explained by several potential mechanisms. First, thyme essential oil may increase the permeability of the bacterial cell membrane, facilitating the entry of antibiotics and boosting their efficacy. Second, it could inhibit bacterial resistance mechanisms, such as beta-lactamases, which neutralize the effects

of antibiotics. Finally, the active compounds in thyme essential oil may act synergistically with the antibiotics, amplifying their antimicrobial activity (Benameur et al., 2019; Fahimi et al., 2015; Marchese et al., 2016; Martins, 2020).

We also evaluated the synergistic effect of oregano essential oil (*Origanum compactum*) in combination with the same antibiotics: cefotaxime, piperacillin, and chloramphenicol. The objective was to determine whether this synergy could enhance efficacy against the multidrug-resistant strain *Klebsiella pneumoniae* (**Figure 28** and **Table 12**).

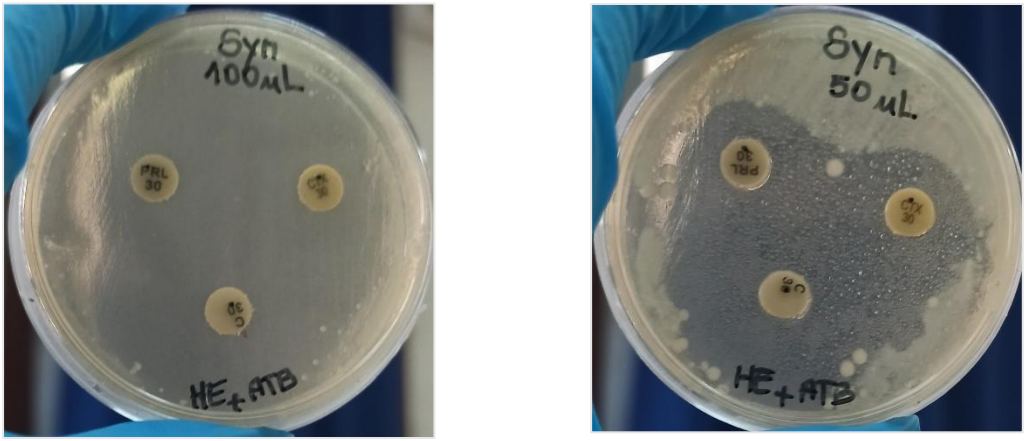

Figure 28: Results of synergy tests between oregano essential oil and the tested antibiotics (chloramphenicol, piperacillin, and cefotaxime) at concentrations of 50 and 100 µL/mL against multidrug-resistant *Klebsiella pneumoniae.*

Table 12: Summary of the results from synergy tests between oregano essential oil and the antibiotics tested against *Klebsiella pneumoniae*.

EOs Concentrations (µL/mL)	**Inhibition zones diameter EOs+ ATB (mm)**		
	Antibiotics		
	Chloramphenicol (C30)	**Piperacillin (PRL30)**	**Cefotaxime (CTX30)**
100	>30	>30	>30
50	27±2	20±1	22±1

After incubation, the diameters of the inhibition zones were measured and compared to those obtained with the essential oil alone and the antibiotics individually. The results showed a significant increase in inhibition zones at concentrations of 100 µL and 50 µL of essential oil, clearly indicating a positive synergy. Notably, at the concentration of 100 µL, total inhibition

of bacterial growth was observed across the entire surface of the 6 cm diameter Petri dish. These results confirm the enhanced efficacy of the combination of oregano essential oil with the tested antibiotics against multidrug-resistant *Klebsiella pneumoniae*.

Klebsiella is a prominent member of the *Klebsiella* genus within the *Enterobacteriaceae* family, primarily known for causing pneumonia. It can also lead to infections in the urinary tract and lower biliary tract. *Klebsiella* species are frequently isolated as Gram-negative bacteria in cases of primary bacteremia, ranking as the second most common pathogen in urinary tract infections after *E. coli* (Cross et al., 1983). This bacterium predominantly affects individuals with compromised immune systems, including hospital patients, those with diabetes, and individuals with chronic lung conditions (Sikarwar & Batra, 2011). Alcoholics are also at risk of *Klebsiella pneumoniae* infections, which can be acquired both in hospital settings and within the community (Al-Agha et al., 2017; Sikarwar & Batra, 2011).

Extensive research underscores the powerful antibacterial and antimicrobial properties of essential oils from *Thymus vulgaris* and *Origanum compactum*, primarily attributed to their key compounds, thymol and carvacrol (Chroho et al., 2024). The findings reveal that these essential oils exhibit strong antimicrobial effects, surpassing those of many synthetic antibiotics (Memar et al., 2017). Thymol and carvacrol are particularly effective against bacterial pathogens, including *Klebsiella pneumoniae* (Köse, 2022).

Thymol and carvacrol, present in the tested essential oils, demonstrate their antibacterial effects through multiple mechanisms (Gavaric et al., 2015). They disrupt bacterial cell membranes, increasing their permeability and causing leakage of crucial cellular components. This membrane disruption facilitates the penetration of antibiotics into the bacteria, helping to overcome resistance mechanisms that often reduce antibiotic effectiveness. Additionally, these compounds hinder bacterial biofilm formation, a common defense strategy employed by pathogens to evade immune responses and antimicrobial treatments (A. Drioiche et al., 2024; Gavaric et al., 2015).

Thymus vulgaris essential oil exhibits notable antibacterial activity, largely due to the high concentration of thymol, its primary compound. Thymol, a phenolic compound known for its broad-spectrum antibacterial properties, significantly contributes to this effectiveness. It is naturally present in the essential oils of many thyme species, enhancing the antibacterial action of this essential oil (Basch et al., 2004). Thyme EO has the highest percentage yield and

antibacterial potential from all different tested EOs, it is therefore recommended to be used alone as the antimicrobial agent (Shabnum & Wagay, 2011).

Oregano essential oil, particularly through its main component carvacrol, demonstrates significant antibacterial effects by disrupting the bacterial cell membrane, leading to leakage of essential cellular components. It inhibits the tricarboxylic acid cycle and key enzymes involved in energy metabolism, impairs bacterial growth, and interferes with DNA by causing structural damage. It also reduces the expression of the pvl gene, which is linked to increased virulence, thereby enhancing its effectiveness in combating MRSA (Cui et al., 2019).

The antimicrobial activity of *Origanum compactum* is largely attributed to its high concentrations of carvacrol (30.53%) and thymol (27.5%). These phenolic compounds are among the most potent plant-based antibacterial agents. Carvacrol disrupts the bacterial cytoplasmic membrane, acting as a proton exchanger and collapsing the proton motive force, which leads to cell death. Thymol also contributes significantly to the oil's antibacterial properties. Additionally, while carvacrol and thymol are major contributors, other components present in smaller amounts, such as p-cymene, linalool, and β-pinene, also play a role in the oil's overall antimicrobial activity (Bouhdid et al., 2008).

Antibiotic resistance in bacterial pathogens has made most existing antibiotics ineffective (Prestinaci et al., 2015). As a result, alternative approaches are needed to fight drug-resistant infections.

The synergistic interaction between essential oils and antibiotics represents a promising new strategy to combat bacterial resistance (Cheesman et al., 2017). Synergy occurs when two substances work together more effectively than they do individually, resulting in a combined effect that exceeds the sum of their individual actions. In this case, the enhanced antibacterial activity is due to the presence of multiple active compounds in both the essential oils and antibiotics. These interactions can disrupt bacterial membranes, increasing permeability, inhibiting motility, blocking ATPase activity, or inhibiting efflux pumps. Additionally, they may enhance the solubility or availability of certain components or target different bacterial pathways, leading to a more potent antibacterial effect (Hyldgaard et al., 2012). Combined therapies between EOs and conventional antibiotics to enhance their effectiveness appear to be the most effective solution (Langeveld et al., 2014).

Oregano species' essential oils have shown significant antibacterial activity against all tested bacteria. The works of Prakash et al. (2020) on the use of Origanum essential oils in

combination with antibiotics have shown significant synergistic interactions. Also Aziz Drioiche et al. (2024) in his study has revealed synergistic interactions between oregano essential oils and selected antibiotics. The results support these studies by showing that Origanum essential oils have a synergistic effect on antibiotic-resistant bacteria, such as *Klebsiella pneumoniae*. Indeed, oregano species' essential oils have shown significant antibacterial activity against all tested bacteria. It is noteworthy that *O. compactum* essential oil exhibited the highest antibacterial efficacy, primarily attributable to the high levels of its bioactive constituents, including Carvacrol, γ-Terpinene, p-Cymene, Thymol, and (E)-Caryophyllene. These substances have been praised for their antibacterial properties because of their distinct chemical composition and strong synergy when combined (Aziz Drioiche et al., 2024).

Romo-Castillo et al. (2023) demonstrated the synergistic effect between thyme essential oil and antibiotics in combating *Klebsiella pneumoniae*. While antibiotics are the standard treatment for bacterial infections like those caused by *K. pneumoniae*, the rapid rise in resistance necessitates new therapeutic approaches. Essential oils, including thyme, have shown promise as tools for developing synergistic therapies. This study reveals that thyme essential oil alters the hyper-muco-viscosity phenotype of the strains, breaking the lipid-soluble barrier that prevents antibiotics from entering. Once this barrier is disrupted, both the antibiotics and the essential oil can penetrate the pathogen and induce its destruction. These findings pave the way for effective combined therapies against *Klebsiella pneumoniae*, regardless of its virulence, resistance level, or hyper-muco-viscosity phenotype.

A relevant study carried out by Köse (2022), examined the synergistic effects of thymol and carvacrol, phenolic compounds found in *Thymus vulgaris* essential oil, when combined with various antibiotics against drug-resistant strains of *Klebsiella pneumoniae*. The study showed that adding thymol and carvacrol to antibiotics significantly enhanced their effectiveness by promoting the penetration of antibiotics into bacterial cells and increasing their antibacterial action against resistant strains.

CONCLUSION

Conclusion and Perspectives

In conclusion, our research highlights the urgent need to address the rising threat of multidrug-resistant bacteria, particularly strains such as *Klebsiella pneumoniae*, which pose significant challenges in clinical settings. The findings from our study demonstrate that essential oils, specifically thyme (*Thymus vulgaris*) and oregano (*Origanum compactum*), exhibit promising antibacterial activity against resistant strains. Key bioactive compounds, including thymol and carvacrol, are primarily responsible for these antimicrobial properties. These natural compounds not only offer a potential alternative to conventional antibiotics but may also enhance treatment efficacy when used in combination with traditional therapies.

As the landscape of antibiotic resistance continues to evolve, integrating essential oils rich in thymol and carvacrol into infection control strategies could represent a critical advancement in public health. However, further research is necessary to fully understand the mechanisms underlying their antimicrobial effects and to optimize their application in clinical settings. Studies should focus on exploring the optimal concentrations, combinations, and delivery methods of these essential oils, as well as their safety and efficacy in diverse patient populations.

Future investigations could also expand to include other essential oils and their synergistic effects with a wider range of antibiotics. This exploration may lead to the development of novel therapeutic protocols that effectively combat resistant infections. Additionally, understanding the phytochemical profiles of these oils, particularly the roles of thymol and carvacrol, could enhance our knowledge of their specific modes of action.

While essential oils show great promise as viable adjuncts to traditional antibiotics, continued research and clinical validation are essential to establish their role in modern antimicrobial therapies. By advancing our understanding and application of these natural compounds, we can better equip ourselves to tackle the ongoing challenges posed by antibiotic resistance and safeguard global health.

ANNEXES

Annexe 01

Non-Biological Materials

- **Equipment**
  - Bunsen burner
  - Incubator
  - Electronic balance
  - Refrigerator
  - Optical microscope
  - Vortex mixer
  - Water bath
  - Jar
  - Autoclave
  - Densitometer
  - Anaerobic jar
- **Glassware and Tools**
  - Pasteur pipettes
  - Inoculating loop
  - Syringes
  - Slides and cover slips
  - Hemolysis tubes
  - Sterile gloves
  - Petri dishes
  - Bacteriological forceps
  - Pipette bulb
  - Racks
- **Culture Media**
  - Hektoen agar
  - Nutrient agar
  - Mueller-Hinton agar
- **Stains**
  - Lugol's iodine
  - Fuchsin
  - Gentian violet
- **Indicators**
  - Kovac's reagent
  - Voges-Proskauer reagents (VP1 and VP2)
  - Nitrate reductase reagents (NR1 and NR2)
  - Urease Indole medium
  - Oxidase disks
  - Vaseline oil
- **Diluting Agents**
  - Sterile physiological water
  - Distilled water
- **Disinfectant Agent**
  - Bleach
- **Antiseptics**
  - Alcohol

Annexe 02

Biological Materials

Figure: Thyme essential oil and oregano essential oil used in the study **(AROMA ZONE).**

Table: *Origanum compactum*

Compound	Approximate Percentage	Main Properties	Example of Use
Carvacrol	High (varies depending on quality)	Powerful antiseptic, antibacterial, antifungal	Disinfection of minor wounds (diluted in a carrier oil)
Thymol	Present in varying quantities	Antiseptic, antibacterial, expectorant	Relief of cold symptoms (diffused in the air)
Other phenols		Antiseptics, anti-inflammatory	Skin care for problematic skin (acne, fungal infections)
Monoterpenes		Antiseptics, antispasmodics	Muscle relaxation (in massage)

Table: *Thymus vulagris*

Compound	Approximate Percentage	Main Properties	Example of Use
Thymol	50-65%	Powerful antiseptic, antibacterial, antifungal	Wound disinfection (diluted in a carrier oil)
Carvacrol	5-15%	Antiseptic, anti-inflammatory	Relief of muscle pain (in a diluted massage oil)
Paracymene	8-28%	Antispasmodic, expectorant	Facilitates breathing in case of cold (diffused in the air)
Gamma-terpinène	5-15%	Anti-inflammatory, antioxidant	Care for irritated skin (diluted in a cream)

Annexe 03

➢ **CHROMagar Composition**

- 33g/L total components
- 15g agar
- 17g peptone and yeast extract
- 1g chromogenic mix
- pH of 7.0
- Store at 15-30°C
- Shelf life greater than 18 months

➢ **Nutrient agar Composition**

- 1g meat extract
- 2g yeast extract
- 5g tryptone
- 5g sodium chloride
- 12g bacteriological agar
- pH of 7.4

➢ **Mueller-Hinton agar Composition**

- 300ml meat infusion
- 17.5g casein peptone
- 1.5g corn starch
- 10g agar
- pH of 7.4

➢ **Hektoen Agar Composition**

- Peptone: 12 g
- Yeast extract: 3 g
- Sodium chloride (NaCl): 5 g
- Bile salts: 9 g
- Sodium thiosulfate: 5 g
- Ferric ammonium citrate: 1.5 g
- Lactose: 12 g
- Salicin: 2 g
- Sucrose: 12 g
- Bromothymol blue (BTB): 0.002 g
- Acid fuchsin: 0.1 g
- Agar: 14 g
- pH: 7.5 ± 0.2

Annexe 04

Table: Summary Table of VITEK® 2 COMPACT Results for *Klebsiella pneumoniae ssp. pneumoniae*

Catégorie	Test	Résultat	Signification potentielle
Identification	Probabilité	98%	Très haute probabilité d'identification de la bactérie
	Espèce	Klebsiella pneumoniae ssp pneumoniae	Espèce bactérienne identifiée
Profil biochimique	Code	6.60773E+15	Code unique identifiant le profil biochimique
	Heure de l'analyse	5.82 heures	Heure à laquelle l'analyse a été réalisée
	État	Final	Statut de l'analyse (terminée)
Fermentations et autres réactions	APPA	+	Capacité à fermenter l'acide pyruvique
	ADO	+	Capacité à utiliser l'adénosine
	PyrA	+	Présence d'une pyrazinamidase
	IARL	+	Capacité à utiliser l'inositol
	...	...	...
	ILATa	+	Capacité à utiliser l'i-leucine
Autres tests	H2S	+	Production d'hydrogène sulfuré
	URE	+	Production d'uréase

Annexe 05

Table: Comparative Table of Antibiogram VITEK® 2 COMPACT Results

Antibiotic	MIC	Interpretation (English)
Ampicillin	>32	R
Amoxicillin/clavulanic acid	>=32	R
Piperacillin/tazobactam	>=128	R
Cefazolin	64	R
Cefotaxime	>=64	R
Ceftazidime	>=64	R
Ertapenem	>=8	R
Imipenem	>=16	R
Amikacin	>=64	R
Gentamicin	>-16	R
Ciprofloxacin	>=4	R
Fosfomycin	128	R
Nitrofurantoin	256	R
Chloramphenicol	>64	R
Colistin		
Trimethoprim/sulfamethoxazole	>=320	R

Antibiotic	MIC	Interpretation	Antibiotic	MIC	Interpretation
BLSE	NEG		BLSE	**NEG**	
Témocilline	>=32	R	Ceftolozane/tazobactam	**>=32**	**R**
Ticarcilline acide clavulanique	>=128	R	Céfépime	**>=32**	**R**
Piperacilline	>=128	R	Aztréonam	**>64**	**R**
Céfazoline	64	R	Méropénéme	**>16**	**R**
Céfoxime	>64	R	Lévofloxacine	**>8**	**R**
Céfuroxime axetil	>64	R	Tigecycline	**<0.5**	**S**
Céfixime	>=4		Chloramphenicol	**>=64**	**R**
Ceftriaxone	>64	R	Triméthoprime	**>=16**	**R**

Annexe 06

Figure: VITEK® 2 COMPACT System

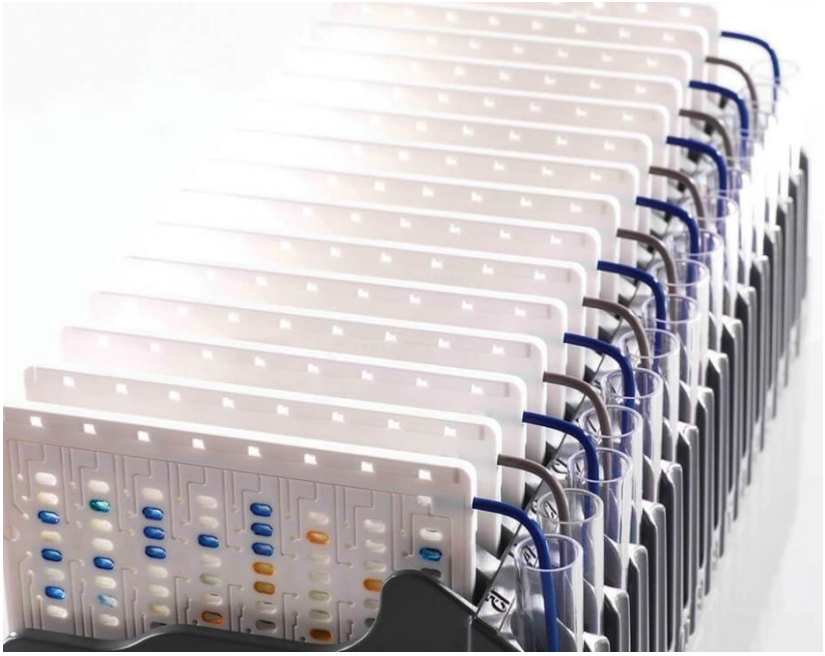

Figure: VITEK® 2 COMPACT Cards

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Abstract : The growing problem of multidrug-resistant bacteria poses a serious public health challenge. This study explores the potential of natural compounds, specifically essential oils, as alternatives to fight resistant pathogens. By analyzing a multidrug-resistant strain of *Klebsiella pneumoniae* from a urinary infection, we assessed the antimicrobial effects of thyme (*Thymus vulgaris*) and oregano (*Origanum compactum*) essential oils. Both oils showed strong antibacterial activity through standard testing methods, suggesting their promise as alternatives to conventional antibiotics. Additionally, combining these essential oils with traditional antibiotics indicated a potential synergistic effect, improving antibiotic effectiveness and addressing resistance. This research supports the use of essential oils as complementary treatments to help control infections amid the rising threat of antibiotic resistance.

Keywords: Antibiotic resistance, *Klebsiella pneumoniae*, essential oils, *Thymus vulgaris*, *Origanum compactum*, synergy.

Résumé : Le problème croissant des bactéries multirésistantes représente un défi majeur pour la santé publique. Cette étude explore le potentiel des composés naturels, en particulier des huiles essentielles, comme alternatives pour lutter contre les pathogènes résistants. En analysant une souche multirésistante de *Klebsiella pneumoniae* issue d'une infection urinaire, nous avons évalué les effets antimicrobiens des huiles essentielles de thym (*Thymus vulgaris*) et d'origan (*Origanum compactum*). Les deux huiles ont montré une forte activité antibactérienne selon les méthodes de test standard, suggérant leur potentiel en tant qu'alternatives aux antibiotiques conventionnels. De plus, la combinaison de ces huiles essentielles avec des antibiotiques traditionnels a montré un effet synergique potentiel, améliorant l'efficacité des antibiotiques et traitant la résistance. Cette recherche soutient l'utilisation des huiles essentielles comme traitements complémentaires pour aider à contrôler les infections face à la menace croissante de la résistance aux antibiotiques.

Mots clés : Résistance aux antibiotiques, *Klebsiella pneumoniae*, huiles essentielles, *Thymus vulgaris, Origanum compactum*, synergie.

Asbter : Ugur ay la yettimɣuren n tbaktiriyin ay izemren ad ɣelvent atas n lizuntibyutik d ayen ay d-yesskanayen aɣilif ameqran i tdawsa taɣelnawt. Tazrawt-a tettnadi ɣef tzemmar n yiferdisen inagmayen, ladɣa zzit n zaâtar ak d win n z3itra, d tifrat i lmend n yimenɣi mgal yiferdisen n tdawsa ay izemren ad ttwaḥebsen. S usexdem n yiwen n ṣṣenf n *Klebsiella pneumoniae* i d-yekkan seg waman n tasa, nessemres azal n tmeẓdiyin n zzit n Thymus vulgaris d zzit n *Origanum compactum*. Sin-a n zzit sseknen-d tigawt ijehden mgal tbaktiriyin s ttawilat n uɛeyyen n tmenḍawt, dɣa aya yesskanay-d tazmert-nsen d tifrat n yimuḍinen n tmeẓdiyin tiqburin. Rnu ɣer waya, asdukkel n zzit-a tazegzawt d lizuntibyutik yesskanay-d azal n tdukli ay izemren ad d-yili, yerna yesselhu-d lfayda n yimuḍinen yerna yesseḥbiber ɣef tmeẓdiyin. Tazrawt-a tettqadar aseqdec n zzitat agi am yisufar n tkemmelt i lmend n uɛiwen n uḥezzeb mgal Aɣbel n lizuntibyutik.

Awalen igejdanen: Aɣbel n lizuntibyutik, *Klebsiella pneumoniae*, zzit n, *Thymus vulgaris, Origanum compactum*, Tadukli n tigawin.

تمثل مشكلة البكتيريا المقاومة للأدوية المتعددة تحديًا خطيرًا للصحة العامة. تستكشف هذه الدراسة إمكانيات المركبات الطبيعية، وخاصة الزيوت الأساسية، كبدائل لمكافحة العوامل الممرضة المقاومة. من خلال تحليل سلالة مقاومة للأدوية المتعددة من بكتيريا **Klebsiella pneumoniae** المعزولة من عدوى بولية، قمنا بتقييم التأثيرات المضادة للميكروبات لزيوت الزعتر الأساسية (Thymus vulgaris) والأوريجانو (Origanum compactum). أظهرت كلتا الزيتتين نشاطًا قويًا مضادًا للبكتيريا باستخدام طرق الاختبار القياسية، مما يشير إلى إمكانية استخدامهما كبدائل للمضادات الحيوية التقليدية. بالإضافة إلى ذلك، أظهرت تركيبة هذه الزيوت الأساسية مع المضادات الحيوية التقليدية تأثيرًا تآزريًا محتملاً، مما يعزز فعالية المضادات الحيوية ويعالج مشكلة المقاومة. تدعم هذه الدراسة استخدام الزيوت الأساسية كعلاجات مكملة للمساعدة في السيطرة على العدوى في ظل التهديد المتزايد لمقاومة المضادات الحيوية.

الكلمات المفتاحية : مقاومة المضادات الحيوية، كليبسيل نيومونيا، الزيوت الأساسية، ، التآزر