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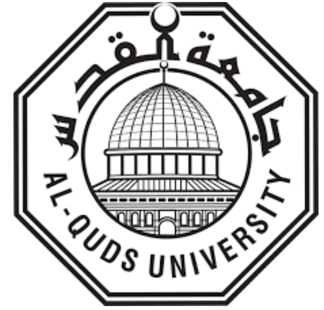

The use of thyme and eucalyptus essential oils as preservatives and antioxidants in pharmaceutical preparations

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The use of thyme and eucalyptus essential oils as preservatives and antioxidants in pharmaceutical preparations

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A thesis submitted in partial fulfillment of the requirements for the Master's degree in Applied and Industrial Technology at Al-Quds University.

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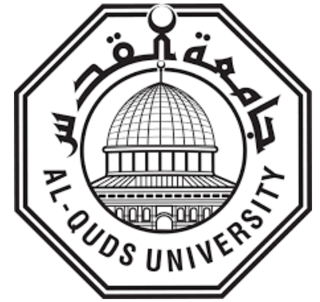

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Jerusalem-Palestine

1447-2025

Dedication:

This thesis is dedicated, first and foremost, to God, my Creator and source of strength, and to my great Teacher and Savior, Jesus Christ, who has revealed to us the true purpose of life. I also dedicate it to my beloved parents, whose unconditional love and unwavering support form the foundation of all that I am; to my brothers, my sister, and precious family, whose encouragement has always served as my guiding light; and to my dearest friends, who have stood by me with love and loyalty.

I extend my deepest gratitude to my supervisor, Dr. Fuad Al-Rimawi, and to all my teachers in the College of Science and Technology, as well as my respected colleagues, for their invaluable guidance and support throughout my academic journey.

This work is also dedicated to the Palestinian martyrs and prisoners, whose sacrifices inspire resilience and hope, to my cherished university, Al-Quds University, and, above all, to my beloved homeland, Palestine.

Declaration:

I hereby declare that this thesis, presented in partial fulfillment of the requirements for the Master's degree, is the product of my dedicated independent research, investigation, and analysis, except where explicit references and acknowledgments have been made. To the best of my knowledge and belief, this work has not been submitted previously, either in whole or in part, for any academic degree or qualification at this or any other university or institution.

Signed

Johnny Yacoub Abdallah Khair

Date: 10/1/2026

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Abstract:

Thyme and eucalyptus essential oils are gaining popularity for their potent therapeutic benefits. With antibacterial, antioxidant, and preservative properties, they play a key role in natural health and wellness pursuits. This study evaluated their potential as natural alternatives to synthetic preservatives and antioxidants in skin creams and vitamin D3 supplements, aiming to enhance product safety, stability, and efficacy. The oils were incorporated at varying concentrations into antibacterial/anti-infective creams and vitamin D3 oral drops. Antimicrobial efficacy was assessed against Gram-positive bacteria (*S. aureus, S. epidermidis*), Gram-negative bacteria (*E. coli, P. aeruginosa*), yeast (*C. albicans*), and mold (*A. niger*). Antioxidant activity was measured via the DPPH free radical scavenging assay.

The study assessed essential oils' impact on the chemical stability of three active pharmaceutical ingredients: fusidic acid, mafenide acetate, and crystalline vitamin D3, over 180 days. pH levels were monitored to evaluate formulation compatibility. Microbial tests confirmed sterility, with all samples (oils, finished products, and preservative/antioxidant-free negative control) showing <10 CFU/mL of microorganisms, including bacteria, yeast, and mold; broth media remained clear without turbidity. Eucalyptus oil displayed strong, concentration-dependent antibacterial activity: complete inhibition of *S. aureus* and *S. epidermidis* at all concentrations (0.5–4.0% v/v), *E. coli* at ≥2.0% v/v, and P. aeruginosa at ≥3.0% v/v. Thyme oil inhibited *S. epidermidis* and *E. coli* at all concentrations, *S. aureus* at ≥1.0% v/v, while *P. aeruginosa* remained resistant.

In the DPPH assay, thyme oil showed superior free radical scavenging with 62.3% inhibition at 4.0% w/v, outperforming the 4.0% w/v ascorbic acid reference (38.9% inhibition). Eucalyptus oil exhibited lower activity at 40.1% inhibition. The antibacterial cream with 1.0% w/w thyme/eucalyptus oils inhibited *S. aureus*, *S. epidermidis*, and *As. Niger* growth at all observed time points. Initial *P. aeruginosa, E. coli*, and *C. albicans* were eliminated by day 7; ≥2-log reduction by day 14; no regrowth by day 28, meeting pharmacopeial standards. The anti-infective cream-maintained sterility for 28 days, fully inhibiting *S. aureus* and *A. niger*, and clearing *P. aeruginosa*, *E. coli*, and *C. albicans* post-day 7. Control samples (creams without chemical/natural preservatives; bacterial/yeast/mold suspensions in Letheen broth without product) showed increased bacterial/fungal colony counts throughout testing.

This indicates that the essential oil mixtures effectively act as natural preservatives. Active pharmaceutical ingredient tests showed that the chemical stability is very good. The fusidic acid in the anti-bacterial cream stayed between 96.0% and 101.0% of its starting amount. The cream with 1.0% w/w oil showed very little breakdown, dropping from 101.1% on day 0 to 99.8% after 180 days. The amount of mafenide acetate in the anti-infective cream was between 93.4% and 103%. The 1.5% w/w oil mix stayed the most stable. However, the higher concentration of 3.0% w/w showed a little more breakdown. The vitamin D3 oral drops were very consistent, staying strong with a potency level between 96.63% and 101.1%. Formulas containing 2.0% w/v eucalyptus or thyme oil had very little decrease, meaning they were effective at shielding against

free radicals. The pH levels were good for using the products and keeping them stable over time. The antibacterial cream had a pH range of 4.40 to 4.55, the anti-infective cream ranged from 6.50 to 6.62, and the vitamin D3 drops ranged from 2.90 to 3.05.

Thyme and eucalyptus essential oils work well as natural preservatives, show a strong ability to kill bacteria, and offer better protection against oxidation. This helps keep products safe from harmful microbes, maintains their chemical structure stability, and enhances their effectiveness when used in medicines applied to the skin or taken orally. The research shows that these oils can work as good, environmentally friendly options instead of man-made preservatives and antioxidants, which help create safer, better quality, and more sustainable medicines.

Keywords: thyme oil, eucalyptus oil, preservatives, antioxidants, DPPH, MLT, MIC, thymol, 1,8-cineol.

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List of Abbreviations

DPPH	2,2-Diphenyl-1-picrylhydrazyl-hydrate
SOP	Standard operating procedure
HPLC	High Performance liquid chromatography
MIC	Minimum inhibitory concentration
MLC	Microbial limit test
SAB	Sabouaraud dextrose broth
SABS	Sabouaraud dextrose agar
TSB	Tryptic soy broth
TSA	Tryptic soy agar
SCD	Soybean-Casein Digest Medium
SCDA	Soybean-Casein Digest Agar
LFC	Laminar flow cabinet
PET	Preservative efficacy test
CFU	Colony-forming unit
ROS	Reactive oxygen species
COX-2	Cyclooxygenase-2
iNOS	inducible nitric oxide synthase
AGEs	Advanced glycation end-products
APIs	Active pharmaceutical ingredients
SOD	Superoxide dismutase
BHT	Butylated hydroxytoluene
BHA	Butylated hydroxyanisole
WHO	World Health Organization
ATP	Adenosine Triphosphate
FRAP	Ferric Reducing Antioxidant Power

Chapter One:

Introduction

1.1 Analysis of Natural Essential Oils as Safer Pharmaceutical Additives

The use of essential oils has increased in several industries, including pharmaceuticals, food, and cosmetics, particularly thyme and eucalyptus essential oils. Eucalyptus essential oil, which is extracted from the leaves of eucalyptus trees such as *Eucalyptus globulus*, has a long history in both traditional medicine and modern wellness practices. According to its fresh, power scent, this oil contains up to 90 % 1,8-cineol, esteemed for its antimicrobial, antioxidant, and anti-inflammatory properties. As a result, eucalyptus essential oil is a popular choice in aromatherapy, as well as in pharmaceutical formulations such as creams, gels, solutions, and natural cleaning products (Sagaste et al., 2024). Moreover, the other essential oil, which is thyme oil, is extracted from the leaves and flowers of various species within the *Thymus genus*, particularly *Thymus vulgaris*. Since ancient times, people have utilized it for its therapeutic benefits. This essential oil is a rich blend containing over 70 distinct compounds, including thymol, carvacrol, and p-cymene, which are the most common and studied. These powerful compounds show a broad spectrum of biological activities, including antimicrobial, antifungal, antiviral, antioxidative, and anti-inflammatory effects. This makes thyme oil an invaluable asset in both traditional and contemporary medicine, showcasing its potential to enhance health and well-being effectively. (Nazzaro et al., 2013)

The therapeutic potential of thyme oil is significantly influenced by various factors, including the specific thyme species, the extraction method used, and the plant's growth stage during oil extraction. Recent research reveals that the concentration of bioactive chemicals in thyme oil fluctuates with the plant's growth stages, significantly enhancing its remarkable anti-inflammatory

and antibacterial properties. With its diverse applications and considerable health benefits, there is a strong case for continued research to refine thyme oil formulations, ensuring maximum therapeutic effectiveness and unlocking its full potential for health and wellness. Because of their superior qualities, eucalyptus and thyme essential oils will be used in medicinal formulations in different quantities as antioxidants and antibacterial. In two different cream formulations, these oils will be incorporated in varying concentrations as substitutes for the antibacterial agent or preservative currently used, specifically methylparaben. Additionally, in the vitamin D3 product, the essential oils will be added in different concentrations as an alternative to the chemical antioxidant, hydroxybutyl toluene. These experiments aim to replace chemical agents with natural essential oils. In order to provide high-quality medications that are safer and more effective, there is currently a need to design pharmaceutical preparations with fewer hazardous and toxic components. Additionally, ensuring the safety and efficacy of pharmaceutical products is critical, and one common method to prevent pathogenic and spoilage microorganisms is the use of chemical preservatives or antibacterial agents. However, it's important to recognize that these preservatives can pose significant toxicity risks. It is imperative to utilize preservatives in packaged goods at safe concentrations, ensuring they remain well below levels that could pose any risk to human health. (Pandur et al., 2022)

1.2 Comparative Assessment of Natural and Synthetic Antimicrobial Agents

Recent research has highlighted the intriguing potential of natural antibacterial agents as safer and more effective alternatives to conventional chemical preservatives in the pharmaceutical industry. This change not only demonstrates an increasing commitment to health and safety, but it also acknowledges the power of nature in improving product integrity. Chemical preservatives and antibacterial agents are often linked to carcinogenic properties and residual toxicity, especially in topical formulations, due to their potential side effects on humans. This has sparked a global trend toward researching natural compounds derived from plants, animals, and microorganisms that exhibit antibacterial properties without the harmful side effects associated with manmade medications. In order to effectively inhibit bacterial growth in a variety of dosage forms, including topical and oral treatments, the researchers advise implementing natural antimicrobials, such as essential oils and plant extracts. The WHO has recognized the need for these alternatives and encourages the use of medicinal herbs and bioactive compounds that demonstrate lower toxicity and improved safety profiles compared to conventional preservatives. As the problem of antibiotic resistance grows stronger, it is critical to investigate the extraordinary potential of novel antibacterial chemicals derived from nature. By doing so, we can forge effective treatments against resistant organisms while safeguarding consumer safety. This embraces the opportunity to innovate and protect human health for the future. (Nainu et al., 2021)

In addition, research on medicinal plants as natural antimicrobial agents has primarily focused on essential oils and crude extracts. Despite their potential, the low antibacterial activity of these natural components in the specific microenvironments of pharmaceutical formulations poses challenges to their application. When incorporating these substances into pharmaceutical

packaging, it is essential to evaluate their antibacterial properties and assess any possible negative effects to enhance their performance. This focus on the antimicrobial activity of various plant extracts is an important area of research, as it seeks to overcome the limitations of existing chemical preservatives and addresses the increasing demand for safer alternatives. (Gonelimali et al., 2018)

1.3 Natural Antioxidants as Safe Replacements for Synthetic Compounds

Antioxidant activity is the capacity of a substance to neutralize free radicals, which are unstable molecules that can lead to oxidative stress and cellular damage. Both synthetic (chemical) antioxidants and natural antioxidants, like those found in eucalyptus and thyme essential oils, are important for protecting against oxidative damage (Pham-Huy et al., 2008). The most common synthetic antioxidants used in food preservation and pharmaceutical formulations include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate. These compounds are effective in preventing oxidation. Emerging research has sparked increased worry about the safety of synthetic antioxidants like BHA and BHT, which have been linked to probable carcinogenic consequences in animals. This crucial discovery has prompted regulatory authorities to become more careful about their usage. Natural antioxidants are becoming more and more popular as a superior substitute in light of these concerning findings. Recent research has shown that adding natural antioxidants to pharmaceutical formulations is not only more effective but also more appealing due to their remarkable safety profile and intrinsic benefits. Furthermore, natural antioxidants can work synergistically, meaning that combining eucalyptus oil with other natural extracts such as thyme oil can enhance their antioxidant and antimicrobial properties. This concept of synergistic activity refers to the idea that when two substances are mixed, their combined effect becomes greater than the sum of their individual effects. Therefore, when eucalyptus and thyme oils are used together, their antimicrobial, antioxidant, and antibacterial properties are significantly strengthened compared to when each is used alone. This powerful interaction not only improves their effectiveness in pharmaceutical applications but also provides additional health benefits, boosting the overall ability of the combination to combat harmful microorganisms. (Sharma et al., 2020)

1.4 Chemical structures and major components of thyme oil

Among the many chemical components found in thyme oil, the most common ones are:

1. Thymol: it constitutes 25.30% of the oil, a monoterpenoid phenol known for its strong antifungal and antibacterial properties.

2. Carvacrol: It is another phenolic substance that has 8.43 % of the oil and has strong antibacterial properties, and can break down bacterial cell walls.

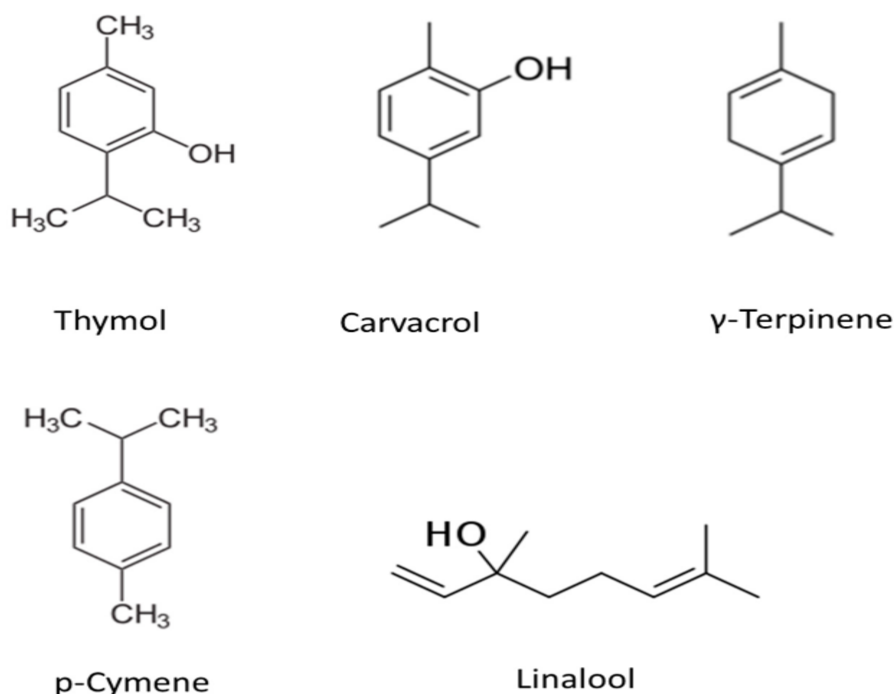
3. *P*-cymene: This remarkable monoterpene boasts powerful anti-inflammatory and antioxidant properties, making it a valuable ally for promoting overall well-being. It accounts for a small percentage of the oil, approximately 1.38%.

4. One monoterpene that adds to the oil's antibacterial properties is ***γ*-terpinene**, which is found in a percentage of 2.0%.

5. Linalool: An analgesic and anti-inflammatory terpene alcohol that has been demonstrated to have these qualities and found in a percent of 1.54% of the oil.

The Chemical structures of major thyme oil components are shown in **Figure 1.5**

Figure 1.5: retrieved from Vassiliou, E., et., al. (2023).



These components support thyme oil's antibacterial, antifungal, and anti-inflammatory properties, among other medicinal benefits. The highest percent of the oil is thymol and carvacrol, which are the two most common active substances found in thyme oil. (Vassiliou et al., 2023)

1.6 Chemical structures and major components of eucalyptus oil

Among the many chemical components found in eucalyptus oil are the following:

1. The primary constituent of the oil, **1,8-cineole (eucalyptol)**, which comprises 44% to 84% of the total, is widely recognized for its antimicrobial properties and distinct camphor-like aroma. (Čmiková et al., 2023)

2. Eucalyptus oil contains approximately 7% to 26.35% ***α*-pinene**. The oil gets its fresh aroma from this ingredient. Its well-known bronchodilator and anti-inflammatory properties are good for breathing problems.

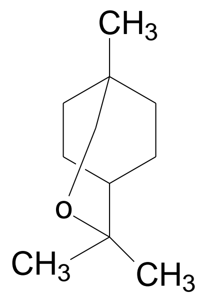
3. *P*-Cymene: It is present in amounts ranging from 2% to 42.1% and is known for its excellent antioxidant activity, and is found in many other essential oils.

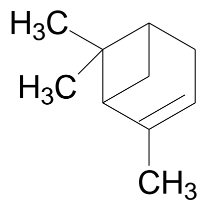
4. *α*-Limonene: It is found in a percent of 6.9% of the oil and is well known for its pleasant citrus fragrance and potential health benefits, including anti-inflammatory effects.

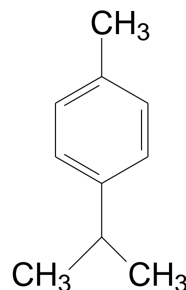
5. Terpinen-4-ol: It is found in smaller amounts in the eucalyptus oil, and it is associated with antimicrobial activity. (Dogan et al., 2017)

The Chemical structures of major eucalyptus oil components are shown in **Figure 1.7**

Figure 1.7



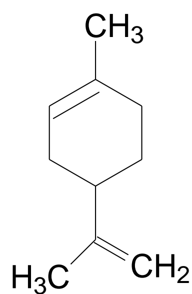


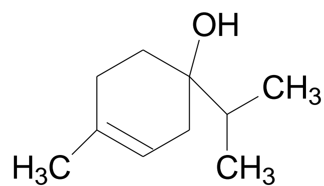


1,8 Cineole

a- pinene

P- Cymene





a- Limenone

Terpinen-4-ol

Burt (2004) found that these are the most prevalent chemicals in eucalyptus essential oil, though it may contain additional substances as well. 1,8-cineole is the most important component, followed by α-pinene and p-cymene. These significant components contribute to the oil's numerous medicinal uses in addition to defining its properties. Moreover, the combined properties of eucalyptus oil facilitate its extensive use in products for respiratory health, such as inhalants and cough syrups, as well as in anti-infective and anti-inflammatory applications. The synergy between the major and minor components of eucalyptus oil is crucial: research indicates that its therapeutic effects often result from the collaborative action of its entire chemical profile, rather than from a single dominant molecule.

1.8 Mechanism of action of thyme oil

1.8.1 Vassiliou et al., (2023) found that the compound's potent active ingredients, carvacrol and thymol, which have potent antibacterial and antioxidant properties, are primarily responsible for its remarkable effects. These compounds work via various mechanisms, including:

1. **Antimicrobial action:** Thymol and carvacrol are powerful antimicrobials that effectively disrupt the structure of fungal and bacterial cell membranes. This disruption results in cell lysis and ultimately leads to the demise of these microorganisms, showcasing the potent efficacy of these natural compounds**.** They can also prevent the growth of infections like Pseudomonas aeruginosa and Staphylococcus aureus by interfering with the creation of vital cellular components.

2. **Breaking the Microbial Cell Membrane:** Thymol and carvacrol, the standout active ingredients in thyme oil, possess a remarkable ability to blend readily with fats. This property empowers them to penetrate the protective outer layers of bacteria and fungi, which are predominantly composed of lipid membranes. This unique characteristic makes thyme oil an intriguing and potent ally in the battle against microbial threats. Once these compounds penetrate the membrane, they compromise its structure, rendering it less stable and more permeable. Consequently, essential components inside the cell, including genetic material, protein, and potassium ions, start to leak out, indicating a significant disturbance in cellular integrity. Once this leakage occurs, the cell cannot survive and ultimately dies.

3. **Disrupting Cell Functions:** These two compounds also interfere with the internal processes of the cell. For example, they can block bacteria from generating energy (ATP) or from creating vital structural components, like the cell wall and DNA. In the absence of these essential processes, the microbes cannot grow or replicate

Specific Effects:

1. **Damaging the Cell Membrane:** Thymol and carvacrol penetrate the membrane, causing it to destabilize.
2. **Causing Cell Leakage:** This fluctuation prompts the discharge of crucial ions, especially potassium, alongside other necessary internal substances, ultimately causing the cell to break apart.
3. **Blocking Enzymes:** These compounds may bind to enzymes on the cell membrane, interfering with energy production and other vital functions.
4. **Spectrum of Activity:** These mechanisms are effective against a broad range of bacteria and fungi, including Staphylococcus aureus, Escherichia coli, and pathogenic fungi like Candida albicans.

1.8.2 Antioxidant properties: Thymol and carvacrol exhibit remarkable antioxidant properties. They help neutralize free radicals and reduce oxidative stress within cells by donating electrons to these free radicals. This process stabilizes the free radicals and helps prevent damage to essential cellular components, including DNA, proteins, and lipids. By neutralizing free radicals, thyme oil can help in protecting cells from damage, support cellular health, and improve immune function.

Furthermore, thymol and carvacrol, the primary active constituents found in thyme oil, are recognized for their effectiveness in fighting oxidative stress within the body. Their antioxidant properties primarily stem from their chemical structure, particularly the hydroxyl group (-OH) attached to a phenol ring. This segment of the molecule has the remarkable ability to donate a hydrogen atom or electron to reactive oxygen species (ROS) and unstable free radicals, including the notorious hydroxyl radicals and superoxide. They protect human cells from possible harm by expertly neutralizing these dangerous radicals and turning them into harmless compounds.

Another reason why thymol and carvacrol work effectively is because of how their electrons are structured. When a hydrogen atom is given to a free radical, the unpaired electron that remains in the molecule is not left with harmful effects; rather, it distributes or "delocalizes" throughout the compound's aromatic ring. This process stabilizes the molecule and prevents it from becoming a new source of oxidation.

Due to these actions, thymol and carvacrol safeguard essential elements of our cells. They lower the deterioration of fats (lipid peroxidation) in cellular membranes, safeguard proteins from harm, and assist in maintaining DNA against detrimental changes induced by oxidative stress. This protection is vital for sustaining cell health, particularly when confronted with stress from environmental influences or illness.

In addition to directly neutralizing free radicals, these compounds may also enhance the body's antioxidant defenses. According to research, thymol and carvacrol may have an impact on important antioxidant enzymes, such as glutathione peroxidase, catalase, and superoxide dismutase (SOD). These enzymes play a significant role in maintaining the balance between

harmful oxidants and protective antioxidants within the body, so boosting their activity helps cells defend against damage more effectively.

1.9 Mechanism of action of eucalyptus oil

1.9.1 Its bioactive, antioxidant, and antibacterial qualities are a result of multiple modes of action and are represented as follows:

1. **Antimicrobial activity:** Eucalyptol, also known as 1,8-cineole, is the main component of eucalyptus oil. It has various functions, including notable antibacterial properties. Bacterial cell lysis and death result from this compound's disruption of the cell membranes of bacteria. By this, bacteria cannot gather or replicate again as their cell wall is inhibited. According to several studies, eucalyptus oil works well against a variety of bacteria, including both Gram-positive and Gram-negative, such as Escherichia coli and Staphylococcus aureus. In addition, the mechanism by which it exerts these effects involves several critical steps:

2. **Disruption of Bacterial Cell Membranes:** Eucalyptol has a hydrophobic nature that allows it to embed itself into the lipid bilayer of bacterial cell membranes. Essential ions, nucleotides, and other critical intracellular materials leak out of the membranes as a result of this integration's increased fluidity and permeability. Due to this, when cells undergo lysis, the bacteria are destroyed.

3. **Inhibition of Cell Wall Synthesis:** In addition to impairing the membrane's integrity, eucalyptol can hinder the production of essential bacterial cell wall components, hence affecting the structural stability of the bacterium.

4. **Interference with Enzymatic Systems:** The disruption of the membrane can impact membrane-bound enzymes that are crucial for bacterial respiration and metabolic processes, further inhibiting the growth and replication of bacteria.

Empirical research provides substantial support for this form of operation. Harkat-Madouri et al., (2015) said that eucalyptus oil works very well against germs like Staphylococcus aureus and Escherichia coli.). Through the destruction of these pathogens' cell membranes, which greatly increases their permeability and ultimately results in cell death, the oil reaches this amazing accomplishment.

1.9.2 Antioxidant activity: Shah et al., (2023) reported that many phytochemicals found in eucalyptus oil have antioxidant properties. In order to fight free radicals, which can lead to oxidative stress and cell damage, this action is essential to occur. In tests like DPPH and FRAP, the oil's strong antioxidant ability has been demonstrated.

A lot of the antioxidant advantages of eucalyptus oil come from the fact that it has a lot of phytochemicals, especially phenolic compounds like eucalyptol. These remarkable substances possess the power to neutralize harmful ROS, showcasing the oil's impressive ability to protect and enhance cellular health.

Here are some key mechanisms:

1. **Free Radical Scavenging:** Phenolic compounds in eucalyptus oil donate hydrogen atoms or electrons to neutralize free radicals, such as DPPH (2,2-diphenyl-1-picrylhydrazyl). This process helps prevent oxidative damage to lipids, proteins, and nucleic acids.

2. **Metal Ion Chelation:** Some components of eucalyptus oil can chelate transition metal ions (e.g., Fe^{2+}, Cu^{2+}), which catalyze the formation of ROS through Fenton reactions.

3. **Enhancement of Endogenous Antioxidant Systems:** Research suggests that eucalyptus essential oil may enhance the activity of intracellular antioxidant enzymes (e.g., superoxide dismutase and catalase), further protecting cells against oxidative stress.

Eucalyptus oil exhibits notable radical scavenging and lowering capabilities in tests like DPPH and FRAP, demonstrating its potential as an antioxidant.

1.10 Optimizing Safety and Synergy of Essential Oils in Pharmaceutical Formulations

Williams, (2020) said that in pharmaceutical formulations, it is necessary to dilute essential oils before incorporating them into the formula for topical use due to their potent nature and their potential for skin irritation or allergic reactions when used in concentrated forms. Diluting certain essential oils preserves their beneficial properties while minimizing potential hazards. To fully realize their captivating potential, essential oils should be elegantly blended with a carrier oil or a suitable solvent, such as methanol or ethanol, ensuring a harmonious fusion that enhances their aromatic properties. The concentration of the diluted essential oil in the formula typically ranges from 1% to 5%, depending on its intended use. Additionally, essential oils, such as eucalyptus and thyme, possess numerous therapeutic properties. However, if they are used undiluted, some people may get skin discomfort. Therefore, it is recommended to do a patch test before widespread use. Additionally, the regulatory guidelines emphasized the importance of dilution and proper usage to ensure safety and efficacy.

ÖzüiÇli et al., (2023) approved that because of their distinct characteristics and modes of operation, they have the amazing capacity to improve the end product's quality and efficacy. Furthermore, eucalyptus oil has antibacterial properties against resistant types of bacteria, including Methicillin-resistant Staphylococcus aureus, which makes it more effective in drug formulations intended to combat infections. This also increases the effectiveness of antibiotic creams against bacterial resistance or other infections. Essential oils of thyme and eucalyptus include chemicals that are effective against a range of diseases, including viruses and bacteria. Because they can damage microbial cell membranes and cause cell death, they are a useful component of formulations used to treat infections. In addition, based on synergistic effects with other oils and according to studies, thyme essential oil can boost the antibacterial activity of other essential oils, such as eucalyptus. When administered combined, these oils have stronger biocidal effects than when applied separately, suggesting that they may work in concert to improve therapeutic outcomes. At the end, these oils exhibit considerable antioxidant properties, which can protect pharmaceutical formulations from oxidative deterioration. This characteristic not only maintains the stability of active ingredients but also improves the overall therapeutic efficacy of the product.

1.11 Comprehensive Testing of Essential Oil Formulations

The microbiological limit test and minimum inhibitory concentration are two assays for essential oils. Following preparation and during stability studies, a microbial limit test will be performed on the final product to assess its quality. Additionally, the DPPH free radical scavenging activity assay will be developed to demonstrate the antioxidant activity of the essential oils and to confirm the antioxidant activity of the finished product. Assay test for the active pharmaceutical ingredient for the final product will also be made during the stability study. Additional physical tests concerning texture, color, PH, odor, etc., will be examined. The ability of a preservative system to shield pharmaceutical items against microbial contamination over the course of their shelf life is assessed by preservative efficacy testing (PET), often referred to as antimicrobial effectiveness testing. This testing involves intentionally inoculating the product with specific microorganisms, typically including Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Candida albicans, and Aspergillus Niger. After that, periodic microbiological enumeration is carried out for a predetermined period of time, often 28 days. The test is performed according to pharmacopeial standards, such as USP 51, Ph. Eur. 5.1.3, and JP. Each organism's log decrease serves as the basis for acceptance criteria. Because contamination is more likely to occur after repeated exposure to the environment, PET is essential for topical and multi-dose formulations. This test will be performed on one sample from each of two cream formulas, both incorporating a 1.0% w/v concentration of carefully selected thyme and eucalyptus essential oils. Our objective is to assess the efficacy, safety, and antimicrobial activity of these remarkable oils within the finished product. Furthermore, this study will showcase the potential of essential oils to serve as a protective shield against microbial contamination, ensuring the integrity of pharmaceutical products throughout their shelf life. (El-Kased & El-Kersh, 2022)

In addition, the concentrations of essential oils used in the minimum inhibitory concentration test were selected at 0.5% v/v, 1.0% v/v, 2.0% v/v, 3.0% v/v, and 4.0% v/v. For the DPPH assay test, the same concentrations were applied, but expressed as w/v% in comparison to the ascorbic acid reference standard. Based on the results of these two tests, various mixtures of the two oils as well as individual oils will be incorporated into pharmaceutical preparations as antioxidants and natural preservatives. The choice for the concentrations of essential oils from 0.5 to 4.0 because it represents a scientifically accepted and pharmaceutically relevant interval in which essential oils consistently demonstrate measurable antimicrobial and antioxidant activity while remaining suitable for topical pharmaceutical formulations. Concentrations below 0.5% often show weak or inconsistent inhibition, whereas concentrations above 4% may offer no significant additional efficacy due to activity saturation and may increase the risk of irritation, instability, or unacceptable sensory properties. Previous studies have shown that thyme and eucalyptus essential oils typically exhibit effective minimum inhibitory concentrations and strong DPPH radical scavenging activity within this range, making it appropriate for identifying the lowest effective concentration that balances efficacy, safety, and formulation feasibility. Soković et al., (2010).

1.12 Objectives: The key goals of this study are outlined below:

1. To assess the powerful antioxidant and antibacterial activity of both eucalyptus and thyme essential oils.

2. To incorporate different concentrations of both essential oils into pharmaceutical preparations (containing fusidic acid as active ingredient, methyl paraben as chemical preservative, mafenide acetate as active ingredient, and also methyl paraben as chemical preservative, and finally vitamin D3 crystalline as active ingredient, and butylhydroxytoluene as a chemical antioxidant by replacing the chemical preservative and antioxidants with these oils).

3. To study the impact of essential oils on active pharmaceutical ingredients in the final product, enhancing efficacy and overall quality.

4. To conduct stability studies on final products for six months in stability ovens, maintained at storage conditions of 40 ℃ and 75% relative humidity.

5. To explore how essential oils significantly influence key physical properties, including pH, color, and odor, in the final product.

6. To determine the minimum inhibitory concentration of both thyme and eucalyptus essential oils.

7. To determine the antimicrobial activity of thyme and eucalyptus essential oils on clinical bacterial and fungal isolates.

The Antimicrobial Effectiveness test or efficacy test will be done on the following standard references:

1. *S. aureus*
2. *P. aeruginosa*
3. *E. coli*
4. *C. albicans*
5. *A. niger*

Chapter Two:

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2. Literature review

Essential oils are natural, volatile compounds produced by aromatic plants and have been valued for centuries, especially since the Middle Ages, when Arabs first used steam or hydro-distillation methods to extract them. One of the most studied and important essential oils is thyme oil, which comes from the plant *Thymus vulgaris*. Thyme oil is widely used because of its strong biological activities. It is among the top ten essential oils globally, known for its powerful antiseptic, antioxidant, antimicrobial, and antifungal effects. The key active ingredient in thyme oil is thymol, a compound proven to be very effective against many harmful fungi that attack plants. Thymol is not only important in agriculture but is also extensively used in dental care and as a base for producing menthol. The antimicrobial power of thyme oil comes from the mixture of several chemical compounds, including *p*-cymene, *γ*-terpinene, ledol, and aromadendrene, with thymol and *p*-cymene being the most abundant. These substances work together, often enhancing each other’s effects, which increases the overall effectiveness of the oil. Because of these properties, thyme oil is widely applied in food preservation to prevent spoilage, in medical products for its healing benefits, and in agriculture as a natural pesticide. The impressive body of research focusing on its chemical makeup and biological activities confirms thyme oil's potential as a natural and safe alternative to synthetic antimicrobial and antioxidant agents. This highlights its growing importance in various industries that seek eco-friendly and health-conscious solutions. (Abozid, 2013)

Natural substances that have biological and pharmacological effects are becoming more popular as safer and more effective options compared to traditional anti-inflammatory medicines. Common anti-inflammatory drugs are often used, but they can lead to unwanted side effects like stomach issues, liver harm, and a higher chance of heart-related problems. Because of this, doctors and scientists are always looking for natural treatments that work in a similar way to medicines but have less chance of causing harm. Among these promising natural agents, Eucalyptus globulus,

also called blue gum, is special because of its long history of being used for medicine and its well-known health benefits. Eucalyptus globulus makes an essential oil that has a lot of strong, active ingredients, which help it offer many different health benefits.

Natural substances with biological and pharmacological activities are gaining increasing attention as safer and effective alternatives to traditional anti-inflammatory drugs. Conventional anti-inflammatory medications, although widely used, often cause adverse side effects such as gastrointestinal irritation, liver damage, and increased risk of cardiovascular problems. Because of this, researchers and healthcare professionals are continuously searching for natural remedies that provide similar therapeutic benefits but with fewer risks. Among these promising natural agents, Eucalyptus globulus, commonly known as blue gum, stands out due to its long history of medicinal use and well-documented pharmacological properties.

Eucalyptus globulus produces an essential oil that is rich in highly bioactive compounds, which contribute to its diverse health benefits. The major components of this volatile oil include eucalyptol (also called 1,8-cineole), α-pinene, δ-limonene, α-terpineol, globulol, α-terpineol acetate, and alloaromadendrene. Among these, eucalyptol is the most abundant and pharmacologically significant, known especially for its potent antimicrobial, anti-inflammatory, and mucolytic effects. Eucalyptol has been extensively studied and shown to exhibit strong antibacterial effects against a variety of harmful pathogens, including Escherichia coli, Proteus species, and Staphylococcus aureus. This makes the oil particularly useful in treating respiratory infections and skin wounds, where bacterial contamination is a concern.

In addition to its antimicrobial activity, the essential oil of Eucalyptus globulus has been traditionally used to relieve symptoms of inflammatory conditions such as rheumatism and neuralgia. When applied topically, the oil penetrates the skin and provides pain-relieving and anti-inflammatory effects, reducing swelling and discomfort in affected joints and muscles. This is attributed largely to the oil's capacity to modulate inflammatory pathways and inhibit pro-inflammatory cytokines, as demonstrated in several pharmacological studies. Moreover, eucalyptol facilitates respiratory health by loosening mucus in the airways, making it easier to breathe for patients with chronic bronchitis or asthma.

Recent scientific reviews have also highlighted the antioxidant properties of eucalyptus oil, which help protect cells from oxidative stress, a key contributor to chronic inflammatory diseases. This dual function, combining both anti-inflammatory and antioxidant effects, enhances the therapeutic potential of Eucalyptus globulus in modern medicine. Furthermore, the oil's natural antiseptic properties support wound healing and skin care, leading to its use in various dermatological and cosmetic products. (Belkhodja, 2021)

The need for safer and more effective methods for human use rather than synthetic preservatives and antioxidants, and their potential toxicity, has motivated researchers to action. Among these

substitutes, essential oils of eucalyptus (Eucalyptus globulus) and thyme (Thymus vulgaris) have shown promise as options. These oils exhibit strong antibacterial and antioxidant properties due to their high content of bioactive constituents.

They are therefore very helpful in the pharmaceutical industry as natural preservatives, stabilizers, and antioxidants. Thyme essential oil is known to contain significant levels of two potent antibacterial compounds, thymol and carvacrol. Moreover, thyme oil works well against many harmful bacteria that are important in the pharmaceutical field and can cause serious, infectious diseases. It also helps fight some strong types of Escherichia coli and Staphylococcus aureus. (Cimino, 2021)

Oussalah et al., (2007) said that Thymol and carvacrol boldly invade bacterial cell membranes, finally leading to their destruction and rendering them powerless. Because Gram-positive bacteria lack the outer layer that protects them, these substances are more potent against them. This outer membrane acts as a barrier, making Gram-negative bacteria more resistant to various antibiotics and chemical preservatives. The combination of thymol and carvacrol improves antibacterial effects, reduces bacterial growth, and may lower the risk of antibiotic resistance.

Sharifi-Rad et al., (2023) approved that Eucalyptus oil helps thyme oil work better against bacteria, mostly because it has a lot of 1,8-cineole, which is also called eucalyptol. This compound fights bacteria and germs that affect the lungs and skin. Besides that, the phenolic part of eucalyptus oil also helps make it very good at fighting against harmful substances in the body. This remarkable property effectively neutralizes harmful free radicals, including DPPH (2,2-diphenyl-1-picrylhydrazyl) and hydroxyl radicals, offering a powerful defense against oxidative stress. Antioxidant properties are important in medicine because they help keep drugs stable and make them last longer by stopping damage from oxidation.

Al-Rimawi et al., (2024) reported that in practical pharmaceutical applications, both thyme and eucalyptus essential oils have demonstrated their effectiveness as natural preservatives in various drug formulations; however, thyme oil is used more frequently than eucalyptus. Thyme oil, in particular, has been successfully incorporated into products such as dexamethasone sodium phosphate syrup and vitamin D3 oral drops. These applications demonstrate thyme oil's ability to protect active pharmaceutical ingredients (APIs) by maintaining their chemical stability without negatively affecting critical quality parameters like pH or other physicochemical properties during extended stability testing. Such stability is crucial for ensuring the safety, efficacy, and shelf-life of pharmaceutical products, making thyme oil an attractive preservative option.

At low concentrations, thyme oil exhibits strong inhibitory effects on a wide range of bacteria and fungi. This antimicrobial activity confirms its potential to replace synthetic preservatives, which are often associated with safety concerns. Common synthetic preservatives such as potassium sorbate and methylparaben, although effective, have raised issues related to allergic reactions and toxicity with long-term use. Thus, the demand for natural, safer alternatives has intensified in pharmaceutical formulation research. Thyme oil's key bioactive compound, thymol, is recognized

in the literature for its robust antibacterial and antifungal properties. It effectively targets both gram-positive and gram-negative bacteria, which underscores its broad-spectrum preservative ability.

Similarly, compounds like oleuropein from olive leaves exhibit significant antimicrobial effects and are gaining attention for their potential use in pharmaceutical products. Oleuropein's inclusion not only supports antimicrobial preservation but also helps maintain formulation stability, ensuring that the product's physicochemical characteristics remain uncompromised. Research demonstrates that when used in optimal concentrations, natural extracts such as thyme oil and oleuropein do not merely preserve the product but can also work synergistically to enhance the overall antimicrobial efficacy. This synergy can result in improved protection against microbial contamination, extending shelf-life, and improving patient safety.

The increasing scientific evidence supports the trend toward replacing synthetic preservatives with natural extracts, which are considered more sustainable and less likely to cause adverse health reactions. Natural preservatives align well with current regulatory and consumer demands for [clean-label] pharmaceutical and cosmetic products, ultimately promoting public health and environmental sustainability.

However, despite these promising results, further comprehensive studies are necessary to evaluate the full range of effectiveness and safety of these natural compounds across different pharmaceutical formulations. This includes long-term toxicity studies, assessment of potential allergenic effects, and interactions with various drug molecules. Expanding this research will help optimize concentrations and combinations of natural extracts to maximize their preservative potential. At the end, thyme and eucalyptus oils, along with other natural bioactive extracts, offer a compelling alternative to traditional synthetic preservatives. Their integration into pharmaceutical formulations has the potential to improve product stability and therapeutic efficacy while reducing the health risks associated with commonly used synthetic chemicals. Continued research and development in this field are essential to fully realize their benefits and support the movement toward safer, more natural pharmaceutical products.

Calo et al., (2015) added that eucalyptus and thyme oils hold remarkable promise when paired with natural ingredients or antibiotics. This combination can create synergistic effects that improve both antimicrobial and antioxidant activity. Antibiotic resistance is a significant health concern of our time, and such synergy may enable lower dosages of synthetic antibiotics, reducing side effects.

Sharifi-Rad et al., (2023) gave an example that using thyme and eucalyptus essential oils together with fusidic acid in an antibiotic cream applied to the skin showed great promise for improving the treatment of stubborn bacterial infections. These natural oils can help the antibiotic work more effectively by weakening the bacteria's protective defenses, such as biofilms, which often make infections difficult to eradicate. This combined approach may enhance the overall antimicrobial activity, reducing treatment time and possibly minimizing the risk of antibiotic resistance

developing. Such synergistic effects between essential oils and conventional antibiotics offer a valuable strategy in pharmaceutical formulations aimed at combating resistant microbes.

Bilia et al., (2014) said that although preliminary studies highlight the beneficial effects of these oils, thorough clinical validation is necessary before they can fully replace synthetic preservatives in medicine. Such validation must include comprehensive safety assessments to detect any potential toxic or allergic reactions, as well as well-designed clinical trials to confirm their effectiveness and optimal dosages in real patient populations. Regulatory approval processes will also require robust data demonstrating that these natural compounds do not interfere negatively with active pharmaceutical ingredients or the overall stability of the final product.

Hamad et al. (2024) conducted a comprehensive study on the leaves of Eucalyptus globulus, which contain a diverse range of plant chemicals that suggest potential medicinal uses. Using a Soxhlet extractor with 60% ethanol, extracts from eucalyptus leaves were found to strongly block the creation of advanced glycation end-products (AGEs). At a concentration of 120 ppm, the inhibition was around 29.9%, and at 300 ppm, it was about 44.4%.

AGEs are bad molecules that form when sugars react with proteins or fats without the help of enzymes. These reactions lead to oxidative stress, inflammation, and damage to tissues, which can play a role in long-term diseases like diabetes. Eucalyptus extracts may stop AGEs from forming because they have a lot of phenolic compounds. These compounds can catch harmful substances like reactive carbonyl groups and free radicals that take part in glycation. This helps stop the build-up of AGEs. This antioxidant ability was also shown through the extracts' strong ability to remove harmful free radicals, which helps protect cells from damage caused by reactive oxygen species (ROS) and lowers the damage to fats in cell membranes.

The polarity of the solvent greatly affected the kinds and quantities of active compounds that were extracted. For example, the 60% ethanol extracts and soaked samples had the highest total phenolic content, whereas ethyl acetate extracts showed higher levels of flavonoids and alkaloids, including important phytochemicals like gallic acid, rutin, syringic acid, and kaempferol. These compounds work together in a way that strengthens their effects: phenolic acids and flavonoids can bind to metal ions that speed up harmful chemical reactions, stop enzymes that create harmful substances, and affect the body's signals that control inflammation. Kaempferol and rutin work to reduce inflammation by blocking certain proteins and enzymes that cause inflammation, such as cytokines and enzymes like cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Antibacterial tests found that extracts from eucalyptus leaves work better against Gram-positive bacteria, like Staphylococcus aureus, than Gram-negative bacteria such as Escherichia coli and Pseudomonas aeruginosa.

This difference comes from the way their cell walls are built: Gram-positive bacteria have a thicker layer of peptidoglycan but don’t have an outer membrane like Gram-negative bacteria, which makes them easier for phenolic compounds and essential oils to penetrate and damage their membranes. The antimicrobial effect works in several ways. It can make the bacterial cell leak its

contents, mess up the cell's charge balance, stop certain enzymes from working, and prevent the bacteria from making their DNA and RNA. All of these actions together cause the bacteria to die. Together, these chemical processes like fighting sugar damage by neutralizing harmful chemicals, protecting cells from oxidation, and killing bacteria by affecting their membranes and how they work show that eucalyptus leaf extracts have a wide range of possible medicinal uses. Their many useful health effects make them good natural preservatives and active ingredients in pharmaceutical formulations.

When combined with thyme oil, which works in a similar way to fight off germs and protect against damage through chemicals like thymol and *p*-cymene, eucalyptus-based products offer a strong foundation for creating mixtures that work better together. These combinations might make preservatives work better and improve treatment results, providing a safer and more natural choice compared to man-made chemicals in medicine.

Retnowati et al., (2025) said that there is a study that looks at how effective eucalyptus oil from Melaleuca leucadendra is against fungi. This plant is commonly used in Indonesia for its strong ability to fight different types of microbes. Earlier studies show that eucalyptus oil has many active substances, including a type of compound called 1,8-cineole, which is also known as eucalyptol. This compound is usually the main part of the oil. A study looking at different types of Eucalyptus found that 1,8-cineole can make up over 70% of the oil in medicines made from Eucalyptus. This monoterpene oxide affects microbial cell membranes and walls by making them more porous and breaking down their protective layers, which helps in fighting microbes.

In this study, the researchers aimed to check how well eucalyptus oil works against the fungus Candida albicans, which causes several infections like oral thrush and vaginal yeast infections, especially in people with weak immune systems. The study used a design where only a final test was done after treatment. Fungal cultures were given oil at five different levels of strength: 20%, 40%, 60%, 80%, and 90%. The effect was checked using a method called well diffusion to see how big the areas without growth were. This way of doing things lets you compare how well different concentrations of antifungal work directly. The results show that eucalyptus oil created noticeable areas of inhibition around Candida albicans, and importantly, the size of these areas grew larger as the concentration of the oil increased.

For example, the area where bacteria couldn't grow increased from 26.36 mm at 20% concentration to 61.36 mm at 90% concentration, which was more effective than the antifungal medicine nystatin at similar concentrations. The statistical analysis showed that the inhibitory effect was strong and important ($p < 0.05$), which means the antifungal activity is reliable and effective. These results show that higher oil levels are linked to a greater ability to fight fungi. This effect is probably because the sample had a lot of 1,8-cineole in it. When considering all the results together, it suggests that eucalyptus oil, which contains a higher concentration of active ingredients such as 1,8-cineole, may serve as a helpful alternative or adjunctive treatment for infections caused by Candida albicans. The study also shows that the chemicals in eucalyptus oil can change a lot based on the environment and the type of soil, which can affect how strong its ability to fight

microbes is. So, it's important to carefully set standards for the oil's makeup in any use or future improvements. Overall, this study contributes to the growing body of work that shows plant-based extracts are safe, effective, and natural options for fighting infections, especially as drug resistance becomes more common.

Chapter Three:

Methods and materials

This study utilized several materials, including the following:

3.1 Microbial limit test was done according to Beit Jala pharmaceutical company, (2022), SOP for Microbiological Examination of Nonsterile Pharmaceutical Products.

Materials needed for microbial limit test are shown in Table 3.1A

Table 3.1A

Tryptic soy agar
Tryptic soy broth
Sabouaraud dextrose agar
Sabouaraud dextrose broth
Sterile glassware
Laminar Flow Cabinet
Autoclave
Glass test tubes
Sterile pipette
Incubator

Both the essential oils and the final pharmaceutical product are subject to this test. It is employed to assess the microbiological composition of pharmaceutical goods that are not sterile. Additionally, this testing verifies that pharmaceutical products meet established safety and quality requirements by quantifying and detecting particular microorganisms that may be present or generated throughout the process in raw materials, intermediates, and finished products.

Preparation for mold & yeast media **(Sabouaraud dextrose broth)**

30.0 grams of the powder were dissolved in one liter of purified water. The mixture was then heated in the microwave for 5 minutes. Afterward, it was placed in the autoclave at 121 °C for 15 minutes. Finally, the pH was adjusted to 5.6 ± 0.2 by the dropwise addition of sterile dilute hydrochloric acid or sterile dilute sodium hydroxide, as required for laboratory use.

A total of 30.0 grams of the powder consists of the following substances:

5.0 g of Peptic Digest of Animal Tissue.

5.0 g of Pancreatic Digest of Casein.

20.0 g of Dextrose.

Preparation for mold & yeast media **(Sabouaraud dextrose agar)**

65.0 grams of the powder were suspended in one liter of purified water and mixed thoroughly. The mixture was then heated with frequent agitation and boiled for 1 minute to ensure complete dissolution of the powder. Afterwards, it was autoclaved at 121 °C for 15 minutes. The final pH for laboratory use was adjusted to 5.6 ± 0.2, as measured using a calibrated pH meter and adjusted, if necessary, by the dropwise addition of sterile dilute hydrochloric acid or sterile dilute sodium hydroxide. Following autoclaving, the media were distributed onto sterile plates under a laminar flow cabinet and allowed to dry and solidify on the plates for subsequent use in the micro test.

65.0 grams of the powder consist of the following substances:

5.0 g of Peptic Digest of Animal Tissue.

5.0 g of Pancreatic Digest of Casein.

40.0 g of Dextrose

15.0 g of Agar.

Preparation for bacterial media **(tryptic soy broth)**

30.0 grams of the powder were suspended in one liter of purified water. The mixture was gently warmed in the microwave until the powder was fully dissolved. It was then autoclaved at 121 °C for 15 minutes. The final pH for laboratory use was adjusted to 7.3 ± 0.2 by carefully adding small amounts of sterile dilute hydrochloric acid or sterile dilute sodium hydroxide while continuously checking with a calibrated pH meter.

A total of 30.0 grams of the powder consists of the following substances

17.0 g of Pancreatic Digest of Casein.

3.0 g of Papaic Digest of Soybean.

2.5 g of Dextrose.

5.0 g of Sodium Chloride.

2.5 g of Dipotassium Phosphate.

Preparation for bacterial media **(tryptic soy agar)**

40.0 grams of the powder were suspended in one liter of purified water and mixed thoroughly. The mixture was heated with continuous agitation and boiled for 1 minute to ensure the powder was completely dissolved. It was then autoclaved at 121 °C for 15 minutes. The final pH for laboratory use was 7.3 ± 0.2 by carefully adjusting with sterile dilute hydrochloric acid or sterile dilute sodium hydroxide as needed and verified with a calibrated pH meter. After autoclaving, the media were poured onto sterile plates under a laminar flow cabinet and allowed to dry and solidify on the plates for use in micro tests.

40.0 grams of the powder consists of the following materials

15.0 g of Pancreatic Digest of casein.

5.0 g of Papaic Digest of Soybean

5.0 g of Sodium Chloride

15.0 g of Agar.

Procedure for microbial limit test for essential oils and final pharmaceutical product:

90.0 ml of the broth media was measured and transferred into a sterile glass bottle. The procedure was conducted within a laminar flow cabinet to maintain the sterility of the media. A specified quantity of oil or the finished product was added to the glass bottle and mixed thoroughly. 1.0 ml was withdrawn from the dilution and applied onto the prepared agar media. The glass bottle was placed in an incubator at 35°C for 3 days to promote bacterial growth. The agar plate was incubated for 7 days at 23°C to facilitate the growth of yeast and mold. (Myemba et al., 2022)

3.2 Minimum inhibitory concentration test was done according to (Gheorghita et al., 2022). Five concentrations of both eucalyptus and thyme essential oils were tested. The concentrations were 0.5% v/v, 1.0% v/v, 2.0% v/v, 3.0% v/v, and 4.0% v/v. Four types of bacteria were used: Staphylococcus aureus, Staphylococcus epidermidis, E. coli, and Pseudomonas aeruginosa. Broth microdilution method was utilized for the test.

Chemicals, biomaterials, and equipment's needed for MIC test are listed in Table 3.2A

Table 3.2A

Essential oils: eucalyptus and thyme
Tryptic soy broth (TSB) for bacterial culture
0.9 % saline to keep the bacteria suspended
Bacterial Strains: *E. coli*, *S. aureus*, *S. epidermidis*, *P. aeruginosa*
Microtubes: For serial dilutions
Pipettes and tips
Vortex mixer
UV Spectrophotometer
Incubator
Test tubes
UV Cuvette
96-Well Microplate
Rack
Laminar flow cabinet (LFC)

Procedure:

3.2.1 Preparation of bacterial suspension

Inoculum preparation:

1. A bacterial suspension for each strain was prepared by suspending colonies in 0.9% saline until the turbidity reached approximately 1×10^8 CFU/mL.

2. The cell concentration of each suspension was verified by measuring the turbidity with a UV spectrophotometer at 650 nm, using 0.9% saline as a blank.

3. For *S. aureus*, an absorbance between 0.3 and 0.45 corresponded to roughly $1–3 \times 10^8$ CFU/mL.

4. For *P. aeruginosa*, *S. epidermidis*, and *E. coli,* an absorbance between 0.2 and 0.3 corresponded to roughly $1–3 \times 10^8$ CFU/mL.

5. To prepare inoculum for testing, 10.0 mL of 0.9% saline was placed in each of four separate test tubes, and 1.0 mL of the respective bacterial suspension was added to each tube.

6. The absorbance of each bacterial suspension was measured individually to confirm cell density, with 0.9% saline used as the blank.

3.2.2 Serial dilutions of essential oils:

1. Stock solutions of thyme and eucalyptus essential oils were prepared in TSB media.

2. 10.0 ml of tryptic soy broth was used for each dilution for the five concentrations.

3. For the 0.5% v/v oil solution, 0.05 mL of oil was added to 9.95 mL of TSB.

4. For the 1.0% v/v oil solution, 0.1 mL of oil was added to 9.9 mL of TSB.

5. For the 2.0% v/v oil solution, 0.2 mL of oil was added to 9.8 mL of TSB.

6. For the 3.0% v/v oil solution, 0.3 mL of oil was added to 9.7 mL of TSB.

7. For the 4.0% v/v oil solution, 0.4 mL of oil was added to 9.6 mL of TSB.

8. Each mixture was mixed well using a vortex mixer.

3.2.3 Microdilution assay:

1. A volume of 0.2 ml (200 µL) of the prepared bacterial suspension was transferred to a test tube.

2. The diluted essential oil with tryptic soy broth (TSB) was added to the bacteria and mixed well using a vortex mixer. The final concentration of bacteria was 1×10^6 CFU.

3. The bacteria with essential oils and TSB were incubated in an incubator for 3 days at 35 °C.

4. A negative control containing only TSB and essential oils was also incubated for 3 days at 35 °C.

5. A positive control containing bacteria suspension and TSB was incubated for 3 days at 35 °C.

3.2.4 Determination of MIC:

1. Each well was checked visually for turbidity after incubation.
2. The MIC was determined as the lowest concentration of essential oil that stopped visible growth, showing no turbidity compared to the positive and negative control wells.

3.3 DPPH free radical scavenging activity assay: was done according to (DNAW, 2023). DPPH stands for 2,2-Diphenyl-1-picrylhydrazyl-hydrate. In this test, the essential oil was mixed with DPPH, a stable purple-colored free radical. If the oil had antioxidant properties, the DPPH was reduced, and the purple color faded. The more the color faded, the higher the antioxidant activity was. This color change was measured to determine the strength of the antioxidant. This test was conducted at five concentrations of both eucalyptus and thyme essential oils: 0.5% w/v, 1.0% w/v, 2.0% w/v, 3.0% w/v, and 4.0% w/v.

Chemicals and materials needed for the DPPH test are listed in Table 3.3A

Table 3.3A

2,2-Diphenyl-1-picrylhydrazyl-hydrate (DPPH)
Absolute methanol
L- ascorbic acid
Distilled water
UV spectrophotometer
UV cuvette
Test tubes
Rack
Micropipette
Volumetric flasks

Procedure:

1. The essential oil concentrations ranging from 0.5% to 4.0% w/v were prepared in absolute methanol.
2. The standard concentrations of ascorbic acid, ranging from 0.5 % to 4.0% w/v in methanol were prepared.
3. A 0.0634 M DPPH solution was made by dissolving 25.0 milligrams of DPPH in 1000 milliliters of absolute methanol.
4. Separately, 0.1 ml of the standard and essential oil were mixed with 4.0 ml of the 0.0634 M DPPH solution.
5. The mixture was stirred well and then kept in the dark for 30 minutes.
6. The absorbance of the samples and the standard was measured using a UV spectrophotometer set at 515 nm.
7. Absolute methanol was used as the blank.

DPPH % inhibition = $(Absorbance\ control\ - Absorbance\ sample) \div (\ Absorbance\ sample) \times 100\%$

Note: The absorbance of the control refers to the DPPH solution without any analyte, such as essential oils.

3.4 Preservative efficacy test (PET): (was done according to Beit Jala Pharmaceutical Company. (2024). SOP for Antimicrobial Effectiveness Testing)

Procedure:

3.4.1 Preparation of bacterial suspension

Inoculum preparation:

The culture conditions for preparing the inoculum are outlined in Table 3.4.1A

Table 3.4.1A

Organism	Suitable medium	Incubation Temperature	Inoculum Incubation Time	Microbial Recovery Incubation Time
E. coli	SCD, SCDA	32.5 ± 2.5 ºC	18-24 hours	3-5 days
P. aeruginosa	SCD, SCDA	32.5 ± 2.5 ºC	18-24 hours	3-5 days
S. aureus	SCD, SCDA	32.5 ± 2.5 ºC	18-24 hours	3-5 days
C. albicans	SAB, SABS	22.5 ± 2.5 ºC	44-52 hours	3-5 days
A. niger	SAB, SABS	22.5 ± 2.5 ºC	6-10 days	3-7 days

Note:

SCD: This stands for Soybean-Casein Digest Medium, also known as Tryptic Soy Broth **(TSB)**. It is a general-purpose medium used for the growth of both aerobic and anaerobic bacteria, including those that are Gram-positive and those that are Gram-negative.

SCDA: This stands for Soybean-Casein Digest Agar, commonly referred to as Tryptic Soy Agar **(TSA)**. It serves as a general-purpose agar for the enumeration and isolation of bacteria from various samples.

SAB: This refers to Sabouraud Dextrose Broth, which is specifically designed for the selective growth of fungi, including yeasts and molds.

SABS: This stands for Sabouraud Dextrose Agar, which is used for the selective isolation of fungi (especially molds and yeasts) from mixed samples.

1. A bacterial suspension was prepared for each strain by carefully suspending isolated colonies in a sterile 0.9% saline solution. The procedure continued until the turbidity of the suspension reached approximately 1×10^8 CFU/mL, indicating a concentrated bacterial population.

2. To ensure accuracy, the concentration of each suspension was verified by measuring the turbidity using a UV spectrophotometer set at a wavelength of 650 nm. A 0.9% saline solution was used as a blank for calibration, allowing for precise readings of the bacterial density in the samples

3. For *S. aureus*, an absorbance of 0.3-0.45 was used (about $1\text{-}3 \times 10^8$ CFU).

4. For *P. aeruginosa* and *E. coli*, an absorbance of 0.2-0.3 was used (about $1\text{-}3 \times 10^8$ CFU).

5. For *C. albicans* and *A. niger*, an absorbance of ≤ 1.0 was used (about $1\text{-}3 \times 10^8$ CFU).

6. To prepare the inoculum for testing, 10.0 mL of 0.9% saline was placed in each of five separate test tubes, followed by the addition of 1.0 mL of the respective bacterial suspension to each tube.

7. The absorbance of each bacterial suspension was then measured individually to confirm cell density, using 0.9% saline as the blank.

3.4.2 Preparation of the media for general microorganisms (letheen broth and letheen agar)

1. Preparation of letheen broth: The solution was prepared by suspending 25.7 grams of the powder in 1 liter of purified water and mixing it thoroughly. The mixture was heated with frequent agitation and brought to a boil for 1 minute to ensure the powder was completely dissolved. It was then autoclaved at 121 ℃ for 15 minutes. The final pH was adjusted to 7.3 ± 0.2 to provide optimal conditions for bacterial growth, using sterile dilute hydrochloric acid or sterile dilute sodium hydroxide as needed, and confirmed with a calibrated pH meter.

A total of 25.7 grams of the powder consists of the following materials:

10.0 g of Proteose Peptone No.3.

5.0 g of Beef Extract.

0.7 g of Lecithin.

5.0 g of Polysorbate 80.

5.0 g of Sodium Chloride.

2. Preparation of letheen agar: The solution was prepared by suspending 32.0 grams of the powder in 1 liter of purified water and mixing it thoroughly. The mixture was heated with frequent agitation and brought to a boil for 1 minute to ensure the powder was completely dissolved. It was then autoclaved at 121 ºC for 15 minutes. The final pH was adjusted to 7.3 ± 0.2 to provide optimal conditions for bacterial growth, using sterile dilute hydrochloric acid or sterile dilute sodium hydroxide as needed, and confirmed with a calibrated pH meter.

A total of 32.0 grams of the powder comprises the following materials:

3.0 g of Beef Extract.

5.0 g of Pancreatic Digest of Casein.

1.0 g of Dextrose.

15.0 g of Agar

7.0 g of Polysorbate 80.

1.0 g of Lecithin.

3.4.3 Inoculation of Bacteria, Yeast, and Mold with the Product and Serial Dilution Using Letheen Broth

1. 0.2 ml of each prepared bacterial, yeast, and mold suspension was added to 20.0 g of the product in a sterile glass bottle with mixing gently.

2. From the mixture, 1.0 mL was taken and added to 9.0 mL of Letheen broth to make a 10^{-1} dilution.

3. Then, 1.0 mL from the 10^{-1} dilution was added to 9.0 mL of Letheen broth to make a 10^{-2} dilution.

4. Next, 1.0 mL from the 10^{-2} dilution was added to 9.0 mL of Letheen broth to make a 10^{-3} dilution.

5. After that, 1.0 mL from the 10^{-3} dilution was added to 9.0 mL of Letheen broth to make a 10^{-4} dilution.

6. Finally, 1.0 mL from the 10^{-4} dilution was added to 9.0 mL of Letheen broth to make a 10^{-5} dilution.

7. As a control, only the prepared bacteria, yeast, and mold suspensions were diluted using Letheen broth, without adding the product.

8. For the control group, 1.0 mL of each prepared microorganism (bacteria, yeast, and mold) was added to 9.0 ml of Letheen broth, and the same serial dilution steps were done from 10^{-1} to 10^{-5}.

3.4.4 Incubation of the Product with Microorganisms in Agar

1. After serial dilution of both the control and the product + microorganisms using Letheen broth, 1.0 ml from each dilution was added to 14.0 mL of melted Letheen agar in suitable plates under a laminar flow cabinet.

2. The plates were left for 15 minutes to dry and solidify.

3. Plates containing bacteria were incubated in the incubator for 3 days at 35 °C.

4. Plates containing yeast and mold were incubated in the incubator for 7 days at 23 °C.

5. The test was carried out over one month at four-time intervals:

First week (t0),

Second week (t7),

Third week (t14), Fifth week (t28).

3.5 The formulation for each pharmaceutical product is outlined below, followed by a description of the preparation procedure:

3.5.1 Anti-bacterial cream: was done according to Beit Jala Pharmaceutical Company. (2022). SOP for the formula, preparing and analyzing antibacterial cream according to USP method.

The formula for the Anti-bacterial cream is shown Table 3.5.1 (A)

Table 3.5.1 (A)

Chemical name	Amount	Function
Fusidic acid **(Active pharmaceutical ingredient)**	0.1 kg	Anti-bacterial agent
Excipients		
Cetosteryl alcohol lanette O, kolliwax CSA 50)	0.35 kg	Emollient
Macrogol cetosteryl ether (BRIJ CS 12 -SS-(RB))	0.075 kg	Emulsifying agent
Macrogol cetosteryl ether (BRIJ CS 25 -PA-(RB))	0.075 kg	Emulsifying agent
Light mineral oil (crystal oil Protol P)	0.6 kg	Vehicle
Propylene glycol	0.4 kg	Solvent
Methyl Parahydroxybenzoate, (Nipagin M, Methyl paraben)	0.005 kg	Antimicrobial preservative
Purified water	3.395 kg	Solvent

Total amount of cream is **5.0 kg**

Note: The essential oils will be used in the formula instead of Methyl Parahydroxybenzoate, (Nipagin M, Methyl paraben).

Preparation procedure:

1. Lanette (BRIJ CS 12 -SS-(RB)) and (BRIJ CS 25 -PA-(RB)) were transferred into the primary mixing vessel and heated to approximately 80°C to form the oily phase.

2. The entire quantity of purified water was poured into a separate container and heated to about 60°C.

3. Crystal oil was moved to another container and heated to approximately 60°C.

4. Fusidic acid was combined with the crystal oil and stirred until fully dissolved, which took about 5 to 10 minutes.

5. The aqueous phase was gradually added to the oily phase and mixed thoroughly until an emulsion was formed.

6. The cooling system was activated while mixing continued, maintaining the temperature at 40°C.

7. Upon reaching around 40°C, propylene glycol was incorporated and mixed thoroughly.

8. After the propylene glycol dissolved, the fusidic acid mixture with crystal oil was added to the blend and mixed well for an additional 10 minutes.

9. The system was cooled completely, and the final cream was transferred into appropriate tubes for storage and use.

After preparing the cream, thyme and eucalyptus essential oils were added as follows:

1. The total cream weighed 5.0 kg and was divided into 5 equal parts, each weighing 1.0 kg.

2. In the first part, 30.0 g of eucalyptus oil was mixed into the cream for about 10 minutes, making a 3% w/w eucalyptus oil concentration.

3. In the second part, 15.0 g of eucalyptus oil and 15.0 g of thyme oil were added and mixed well for about 10 minutes, making 1.5% w/w of each oil in this cream portion.

4. In the third part, 10.0 g of eucalyptus oil and 10.0 g of thyme oil were stirred continuously for about 10 minutes, resulting in 1.0% w/w of each oil in this portion.

5. In the fourth part, 5.0 g of eucalyptus oil and 5.0 g of thyme oil were stirred continuously for about 10 minutes, giving 0.5% w/w of each oil in this portion.

6. The last part was used as a negative control, so no oils were added to it.

3.5.1 (B) Assay for the active pharmaceutical ingredient:

After the finished product was obtained, it was tested using High-Performance Liquid Chromatography (HPLC) to determine the percentage of the active pharmaceutical ingredient, Fusidic acid. Following this analysis, the product was placed in an oven for stability studies, which were conducted over a period of six months at storage conditions of 40 ℃ and 75% relative humidity.

Initially, the product was tested at time zero (t_0). After one month, it underwent another HPLC test, referred to as t_{30} days. A third test was conducted after three months, known as t_{90} days, and finally, a last test was performed at the end of the six months, called t_{180} days

The analysis was conducted using the following method:

The reagents used in the analysis method included fusidic acid (reference standard), phosphoric acid, acetonitrile HPLC grade, and Methanol HPLC grade.

The HPLC conditions were:

The mobile phase was composed of three reagents in the following percentages: A) 10% methanol (HPLC grade), B) 35% phosphoric acid (0.05 M), and C) 55% acetonitrile (HPLC grade).

The diluent used is the same prepared mobile phase.

The wavelength was 235 nm

The column used was Lichrosphere RP-18, 125 × 4 mm, 5 µm, the flow rate was 1.0 mL/min, and the injection volume was 20.0 µL.

Standard preparation: The standard solution was prepared by accurately weighing 600.0 mg of fusidic acid and transferring it into a 200 mL volumetric flask. The material was dissolved in the prepared mobile phase, and the solution was sonicated for 15 minutes to ensure complete dissolution. Subsequently, a 10.0 mL aliquot of this stock solution was quantitatively transferred into a 100 mL volumetric flask and diluted to volume with the mobile phase. The resulting working standard solution had a final concentration of 30.0 mg of fusidic acid per 100 mL and was mixed thoroughly prior to HPLC analysis.

Sample preparation: The sample solution was prepared by accurately weighing 1.50 g of the cream formulation and transferring it into a 100 mL volumetric flask. The flask was filled to volume with the prepared mobile phase, and the mixture was stirred and gently warmed for 15 minutes to ensure complete dissolution of the cream matrix. The solution was then allowed to cool to room temperature and filtered through Whatman filter paper to remove any insoluble excipients. The resulting filtrate corresponded to a fusidic acid concentration of 30.0 mg per 100 mL and was used directly for HPLC analysis.

The assay was calculated by using the following law.:

% Assay result $= (Response\ area\ of\ sample \div Response\ ares\ of\ standard) \times (Conc\ of\ standard\ \div Conc\ of\ sample) \times 100\%$

(Response area of sample / area of standard) × (conc of standard / conc of sample) × 100 %

Limit of fusidic acid is **95.0 – 105.0 %**

After preparing the product, the pH test will be checked at time zero (t0) and during stability studies, as described below:

5.0 g of the cream was dissolved in 50 mL of purified water and heated with stirring until a homogeneous mixture was achieved. After cooling, the pH was measured at 20-22 ºC by using a suitable pH meter.

Limit: 4.3-6.0

3.5.2 Anti-infective cream: was done according to Beit Jala Pharmaceutical Company. (2017). SOP for the formula, preparing and analyzing anti-infective cream according to USP method.

The formula for the Anti-infective cream is described in Table 3.5.2 (A)

Table 3.5.2 (A)

Chemical name	Amount	Function
Mafenide acetate (**Active pharmaceutical ingredient)**	0.3372 kg	Anti-infective agent
Excipients		
Cetosteryl alcohol lanette O, kolliwax CSA 50)	0.24 kg	Emulsifying agent
Macrogol cetosteryl ether (Emulgen B2 Koliphor CS 20)	0.03 kg	Emulsifying agent
White soft paraffin oil	0.36 kg	Emollient
Propylene glycol	0.24 kg	Water-miscible cosolvent
Methyl Parahydroxybenzoate, (Nipagin M, Methyl paraben)	0.003	Antimicrobial preservative
Purified water	1.7898 kg	Solvent

Total amount of cream is **3.0 kg**

Note: The essential oils will be used in the formula instead of Methyl Parahydroxybenzoate, (Nipagin M, Methyl paraben).

Preparation procedure:

1. Lanette O, white petroleum, and Koliphor CS 20 were transferred into the primary mixing vessel and heated to approximately 80°C to form the oily phase.

2. The entire quantity of purified water was poured into the melting container and heated to a temperature between 80 and 85°C to prepare the aqueous phase.

3. Mafenide acetate was added to the aqueous phase and stirred for five minutes until fully dissolved.

4. The aqueous phase containing the active pharmaceutical ingredient was combined with the oily phase and mixed thoroughly until an emulsion was formed.

5. Propylene glycol was added to the mixture and mixing continued for approximately 5 to 10 minutes.

6. The cooling system was activated while mixing was maintained at around 40°C.

7. After cooling, mixing was continued for an additional five minutes before the cream was transferred into appropriate tubes.

After preparing the cream, thyme and eucalyptus essential oils were added as follows:

1. The total cream weighed 3.0 kg and was divided into 5 parts: four parts each weighing 0.5 kg, and one part weighing 1.0 kg.

2. For the first part, 15.0 g of eucalyptus oil was added to 0.5 kg of cream and mixed continuously for about 10 minutes, resulting in a 3.0% w/w concentration of eucalyptus oil in the cream.

3. For the second part, 7.5 g of eucalyptus oil and 7.5 g of thyme oil were added to 0.5 kg of cream and stirred continuously for 10 minutes, giving a 1.5% w/w concentration of each oil in the cream.

4. For the third part, 5.0 g of eucalyptus oil and 5.0 g of thyme oil were added to 0.5 kg of cream and mixed continuously for 10 minutes, resulting in a 1.0% w/w concentration of each oil.

5. For the fourth part, 2.5 g of eucalyptus oil and 2.5 g of thyme oil were added to 1.0 kg of cream and stirred continuously for 10 minutes, giving a 0.5% w/w concentration of each oil in the cream.

6. The last part, weighing 0.5 kg, was used as a negative control, so no oils were added to it.

3.5.2 (B) Assay for the active pharmaceutical ingredient:

After the final product was made, it was tested with High-Performance Liquid Chromatography (HPLC) to find out how much of the active ingredient, Mafenide acetate, it contained. Then, the product was put in an oven to check its stability over six months for stability studies at storage conditions at temperature of 40 ºC and 75% relative humidity.

The first test was done right at the start (t_0). After one month, another HPLC test was done (t_{30} days). A third test was carried out after three months (t_{90} days), and the last test was done at the end of six months (t_{180} days).

The analysis was performed using the following method:

The reagents used in the analysis method composed of mafenide acetate (reference standard) sodium acetate, octane sulfonic acid sodium salt, methanol HPLC grade, and 20% ethanol as diluent

The HPLC conditions were:

The mobile phase was composed of 2 percent each of two reagents: A, methanol (HPLC grade), and B, a solution of 0.1 g sodium acetate and 0.1 g octane sulfonic acid sodium salt per 500 mL of purified water. The final composition was 15% A and 85% B.

The wavelength was 230 nm

The column used was Lichrosphere RP- select B, 125 × 4 mm, 5 µm, the flow rate was 1.0 ml/min, and the injection volume was 20.0 µL.

Standard preparation: The standard solution was prepared by accurately weighing 22.4 mg of mafenide acetate and transferring it into a 200 mL volumetric flask. The compound was dissolved in 20% (v/v) ethanol, and the solution was sonicated for 15 minutes to ensure complete dissolution. The flask was then brought to volume with the same solvent and mixed thoroughly, yielding a final standard concentration of 11.20 mg of mafenide acetate per 100 mL.

Sample preparation: The sample solution was prepared by accurately weighing 2.0 g of the cream formulation and transferring it into a 200 mL volumetric flask. The flask was filled to volume with 20% (v/v) ethanol, and the mixture was warmed and stirred for 15 minutes to ensure complete dissolution of the cream matrix. After cooling to room temperature, a 10.0 mL aliquot of the solution was quantitatively transferred into a 100 mL volumetric flask and diluted to volume with 20% ethanol. The solution was subsequently filtered through Whatman filter paper to remove insoluble excipients. The resulting filtrate corresponded to a mafenide acetate concentration of 11.20 mg per 100 mL and was used for HPLC analysis.

The assay was calculated by using the following law.

% Assay result $= (Response\ area\ of\ sample \div Response\ ares\ of\ standard) \times (Conc\ of\ standard\ \div Conc\ of\ sample) \times 100\%$

Limit of mafenide acetate acid is **90.0 – 110.0 %**

The pH will be checked immediately after the product is prepared (at time zero) and again during stability studies, as described below:

5.0 g of the cream was dissolved in 50 mL of purified water and was heated with stirring until a uniform mixture was formed. After the mixture was cooled, the pH was measured at 20–22 ºC using a suitable pH meter, with the electrode placed in the solution until a stable reading was obtained.

Limit: 6.0-7.5

3.5.3 Vitamin D3 oral drop: was done according to Beit Jala Pharmaceutical Company. (2016). SOP for the formula, preparing and analyzing vitamin D3 oral drops according to USP method.

The formula of vitamin D3 oral drop is shown in Table 3.5.3(A)

Table 3.5.3(A)

Chemical name	Amount	Function
Vitamin D3 crystals **(Active pharmaceutical ingredient)**	0.87125 g	Antihypocalcemic, anti-hypoparathyroid agents, and essential vitamins
Excipients		
Tween 80	0.0625 kg	Solubilizer, surface active agent
Glycerin	1.625 kg	Solvent and vehicle
Citric acid anhydrous	2.5 g	Buffering and acidifying agent
Butylhydroxytoluene	0.625 g	Antioxidant
Orange oil flavor	0.833 g	Flavoring agent
Purified water	q.s to 2.5 liter	Solvent and vehicle

Total amount of the product is **4.19 liters**

Note: The essential oils will be used in the formula instead of Butylhydroxytoluene.

Preparation procedure:

1. The fluorescent light was turned off, and a sodium lamp was used instead, as vitamin D3 crystals are sensitive to fluorescent light.

2. First, the mixing tank was filled with Tween 80. Next, vitamin D3 crystals were added to the tank and stirred for about 15 minutes until fully dissolved.

3. Then, glycerin was poured into the mixture using a mixing head, and the mixture was stirred for an additional 15 minutes.

4. After that, the mixture was transferred to the mixing tank, and orange flavoring was added.

5. Finally, a solution of citric acid was dissolved in filtered water and added to the tank.

6. Enough clean water was then added to reach the final desired volume.

After preparing the vitamin D3 oral drops, thyme and eucalyptus oils were added as follows:

1. The product was divided into 7 parts to add different concentrations of essential oils.
2. In the first part, 6.0 g of eucalyptus oil was added to 0.3 liters of the product to make a 2.0% w/v oil concentration.
3. In the second part, 1.5 g of eucalyptus essential oil was added to 0.3 liters of the product to make a 0.5% w/v oil concentration.
4. In the third part, 6.0 g of thyme oil was added to 0.3 liters of the product to make a 2.0% w/v oil concentration.
5. In the fourth part, 1.5 g of thyme oil was added to 0.3 liters of the product to make a 0.5% w/v oil concentration.
6. In the fifth part, a mixture of 0.75 g of both oils was added to 0.3 liters of the product to make a final 0.5% w/v concentration for both oils (0.25% w/v of each oil).
7. In the sixth part, a mixture of 1.5 g of both oils was added to 0.3 liters of the product to make a final 1.0% w/v concentration for both oils (0.50% w/v of each oil).
8. The last part was used as a control, so no essential oils were added to it.

3.5.3 (B) Assay for the active pharmaceutical ingredient:

Once the final product was made, it was checked using High-Performance Liquid Chromatography (HPLC) to measure how much of the active ingredient, vitamin D3 crystalline, it had. After that, the product was placed in an oven to test how stable it would be over a period of six months for stability studies at storage conditions at temperature of 40 ºC and 75% relative humidity.

The first test was done right at the beginning (t_0). Another HPLC test was done after one month (t_{30} days). A third test was done after three months (t_{90} days), and the final test was done after six months (t_{180} days).

The analysis was done using the following method:

The reagents used in the analysis method consisted of vitamin D3 crystalline (reference standard), acetonitrile HPLC grade, methanol HPLC grade, and 70% ethanol as diluent.

The HPLC conditions were:

The mobile phase was composed of two reagents in the following percentages:

A (25% methanol): B (75% acetonitrile).

The wavelength was 268 nm

The column used was Lichrosphere RP18e, 150-4.6 4 mm, 5 µm(L1), the flow rate was 1.2 mL/min, and the injection volume was 20.0 µL.

Standard solution preparation: The standard solution was prepared by accurately weighing 30.0 mg of crystalline vitamin D_3 and transferring it into a 100 mL volumetric flask. The flask was filled to volume with 70% (v/v) ethanol as the diluent, and the solution was stirred for 10 minutes to ensure complete dissolution of the crystalline material. Subsequently, a 10.0 mL aliquot of this solution was quantitatively transferred into a second 100 mL volumetric flask and diluted to volume with 70% ethanol, followed by stirring for an additional 10 minutes. The resulting working standard solution had a final concentration of 3.00 mg of vitamin D_3 per 100 mL and was used for HPLC analysis.

Sample solution preparation: The sample solution was prepared by accurately transferring 10.0 mL of the vitamin D_3 oral drops formulation into a 100 mL volumetric flask. The flask was filled to volume with 70% (v/v) ethanol and stirred for 10 minutes to ensure complete dissolution and homogeneity of the formulation. The resulting solution corresponded to a vitamin D_3 concentration of 3.00 mg per 100 mL and was directly subjected to HPLC analysis.

The assay was calculated by using the following law.

% Assay result $= (Response\ area\ of\ sample \div Response\ ares\ of\ standard) \times (Conc\ of\ standard\ \div Conc\ of\ sample) \times 100\%$

Limit of vitamin D3 crystalline acid is 80.0-120.0 %

The pH will be measured immediately after product preparation (at time zero) and again during stability studies, as described below:

25 mL of the prepared product was poured into a 50 mL beaker, and the pH was measured at 20–22 ℃ using a suitable pH meter. The electrode was placed in the solution until a stable reading was obtained.

Limit: 2.0-4.0

Chapter Four:

Results and Discussion:

In this section, the results will be presented in an orderly manner, starting with the essential oils and continuing to the products one by one, including all tests conducted during the study.

4.1 Microbial limit test:

Overview: Microbial limit testing (MLT) is a critical quality control procedure used to assess the microbial purity and safety of pharmaceutical products, especially those derived from natural sources such as essential oils. In this study, microbial limit tests were performed on various formulations, including thyme and eucalyptus essential oils, antibacterial and anti-infective creams, and Vitamin D_3 oral drops. The tests included both direct inoculation in enrichment broth and enumeration of total aerobic microbial count and total combined yeast and mold count.

The results of the microbial limit test for the finished products and essential oils are presented below:

4.1.1 Microbial Limit Test for Essential Oils

a. Direct Transfer (Broth Media) Test: The results of the direct inoculation of thyme and eucalyptus essential oils into Tryptic Soy Broth (TSB) and Sabouraud Dextrose Broth (SDB) showed no visible turbidity in any of the media. The broth remained clear throughout the incubation period, indicating the absence of bacterial or fungal contamination in the essential oil samples, as shown in **Table 4.1.1 (A).**

Table 4.1.1 (A)

Medium	Result of thyme oil	Result of eucalyptus oil
Tryptic soy broth	Clear	Clear
Sabouraud dextrose broth	Clear	Clear

The absence of turbidity in TSB and SDB for both thyme and eucalyptus essential oils indicates no microbial growth. This suggests that both oils are free from bacterial, yeast, and mold contamination.

According to Hammer et al., (2006), this result reflects the natural antimicrobial properties of the essential oils. Compounds like thymol in thyme and 1,8-cineole in eucalyptus have been shown to possess strong antimicrobial activity by disrupting microbial membranes. Thus, these essential oils meet microbiological quality standards for use in pharmaceutical preparations and show no microbial burden when directly incubated in nutrient-rich broth media.

b. Total Count Test

Quantitative microbial assessments on Tryptic Soy Agar (TSA) and Sabouraud Dextrose Agar (SDA) confirmed the results of the broth media tests. Both oils yielded colony-forming units (CFU) below 10 CFU/mL for bacteria and fungi, which is well within the USP pharmacopeial limits (<200 CFU/mL for bacteria and <20 CFU/mL for fungi) according to accepted limit range for non-sterile pharmaceutical products tested in BJP. These results validate the microbial safety of both essential oils for use in topical and oral formulations. This result confirms that thyme and eucalyptus oils are microbiologically acceptable for non-sterile pharmaceutical use. It also supports their potential use as natural antimicrobial preservatives, as both exhibit strong inhibitory effects against a wide range of microorganisms.

Table 4.1.1 (B): Microbial limit test, Total count test for thyme and eucalyptus essential oils

Medium	Limits on agar CFU/ml	Result of thyme oil	Result of eucalyptus oil
Tryptic soy agar	Bacteria $< 200\ (10^2)$	< 10 CFU	< 10 CFU
Sabouraud dextrose agar	Yeast and mold $< 20\ (10^1)$	< 10 CFU	< 10 CFU

4.2 Microbial Testing of Antibacterial Cream Formulations

a. Broth Media Test

All antibacterial cream samples, including those containing 0.5–3.0% w/v concentrations of eucalyptus and thyme oils, exhibited no microbial growth in either TSB or SDB. Even the negative control, which lacked any preservatives or essential oils, remained clear. This finding suggests a low initial microbial load in the formulation ingredients or effective aseptic handling during

manufacturing. This finding supports the established antimicrobial activity of thymol and eucalyptol, which disrupt microbial membranes and inhibit growth. Also, this clarity may be attributed to good manufacturing practices (GMP) in pharmaceutical preparations in the pharmaceutical guidelines, a low initial microbial load, or the characteristics of the cream base that do not support microbial growth.

Microbial limit test – Direct transfer (broth media) test for Anti-bacterial cream is shown in Table 4.2.1

Medium	Result of sample 1 (3.0% w/w conc of eucalyptus oil)	Result of sample 2 (1.5% w/w mix conc of each oil)	Result of sample 3 (1.0% w/w mix conc of each oil)	Result of sample 4 (0.5% w/w mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
Tryptic soy broth	Clear	Clear	Clear	Clear	Clear
Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear

b. Total Count Test

Based on Damjanović-Vasilić, (2011), the CFU/mL results for all antibacterial cream samples were consistently below 10 CFU/mL for both bacterial and fungal counts, including the preservative-free control. These results indicate that the antimicrobial activity of eucalyptus and thyme oils at all tested concentrations was sufficient to maintain microbial safety, making them microbiologically acceptable for non-sterile topical pharmaceutical use, and in compliance with pharmacopeial guidelines. The low microbial counts observed across all samples suggest that the essential oils, particularly at higher concentrations, play a role in maintaining microbial quality by suppressing microbial growth over time, particularly at higher concentrations. This sustained antimicrobial effect reduces the risk of contamination, supports product stability, and aligns with pharmacopeial requirements for effective preservation during storage and use This is likely due to their antimicrobial components, such as thymol and 1,8-cineole, which are well-studied for their bactericidal and fungicidal properties. Interestingly, the negative control also exhibited minimal microbial load, indicating either high production hygiene or the presence of excipients in the cream base that may naturally inhibit microbial growth. The total count test results for the cream are illustrated in **Table 4.2.2**

Table 4.2.2

Medium	Limits on agar CFU/ml	Result of sample 1 (3.0% conc of eucalyptus oil)	Result of sample 2 (1.5% mix conc of each oil)	Result of sample 3 (1.0% mix conc of each oil)	Result of sample 4 (0.5% mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
Tryptic soy agar	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
Sabouraud dextrose agar	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU

4.3 Microbial Testing of Anti-infective Cream Formulations

All tested anti-infective cream samples, including those with low concentrations of essential oils (0.5% w/w) and even the unpreserved controls, showed no turbidity in broth media and microbial counts below detectable limits (<10 CFU/mL), confirming their microbiological safety. The absence of bacterial and fungal growth in both tryptic soy and Sabouraud dextrose broths across all formulations, whether preserved with eucalyptus oil, a thyme-eucalyptus blend, or unpreserved, highlights the strong antimicrobial properties of essential oil compounds, such as thymol and eucalyptol, as well as the high hygiene standards maintained during preparation. Interestingly, the negative control also remained contamination-free, suggesting that the base cream may resist microbial growth due to its low water activity or the presence of mildly antimicrobial excipients. These findings align with good manufacturing practices and demonstrate that all formulations are well within acceptable microbial limits for non-sterile products, which allows up to 200 CFU/mL for bacteria and 20 CFU/mL for fungi, confirming the creams are clean, stable, and safe for use. Ratajczak et al., (2015). The direct transfer media results are shown in **Table 4.3.1**, and the total count results are shown in **Table 4.3.2**.

Table 4.3.1

Medium	Result of sample 1 (3.0% w/w conc of eucalyptus oil)	Result of sample 2 (1.5% w/w mix conc of each oil)	Result of sample 3 (1.0% w/w mix conc of each oil)	Result of sample 4 (0.5% w/w mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
Tryptic soy broth	Clear	Clear	Clear	Clear	Clear
Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear

Table 4.3.2

Medium	Limits on agar CFU/ml	Result of sample 1 (3.0% w/w conc of eucalyptus oil)	Result of sample 2 (1.5% w/w mix conc of each oil)	Result of sample 3 (1.0% w/w mix conc of each oil)	Result of sample 4 (0.5% w/w mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
Tryptic soy agar	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
Sabouraud dextrose agar	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU

4.4 Microbial Limit Test for Vitamin D Oral Drops

a. Direct Transfer Test

All Vitamin D_3 oral drop samples, including those containing various concentrations of eucalyptus oil, thyme oil, and their combinations, showed no microbial growth in either tryptic soy broth or Sabouraud dextrose broth, with all broths remaining clear. Even the negative control sample, which contained no preservatives or antioxidants, showed no signs of contamination, indicating that the formulation was prepared under excellent hygienic conditions with minimal initial microbial load. The absence of bacteria, yeast, and mold across all samples suggests strong microbiological integrity of the oil-based formulation, likely supported by the natural antimicrobial properties of thymol and eucalyptol in the essential oils, which can disrupt microbial cell membranes and inhibit growth. These results reflect high production standards and confirm

that all samples were clean and microbiologically safe at the time of testing. ŽT Rakuša et al., (2021).

The broth media results are shown in **Table 4.4.1**.

Table 4.4.1

Medium	Result of sample 1 (0.5% w/v conc of eucalyptus oil)	Result of sample 2 (2.0 % w/v cone of eucalyptus oil)	Result of sample 3 (0.5% w/v conc of thyme oil)	Result of sample 4 (2.0% w/v conc of thyme oil)	Result of sample 5 (0.5% w/v mix of the two oils, 0.25% w/v of each one)	Result of sample (1.0% w/v mix of the two oils, 0.5% w/v of each one)	Negative control, (without any chemical or natural antioxidant)
Tryptic soy broth	Clear	Clear	Clear	Clear	Clear	Clear	Clear
Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear	Clear	Clear

b. Total Count Test

All samples, including the ones with essential oils (eucalyptus, thyme, or a mix of both) and the negative control without any antioxidants, showed very low microbial counts:

Tryptic Soy Agar (for bacteria): Each sample had fewer than 10 colony-forming units (CFU/ml), which is much lower than the allowed limit of 200 CFU/ml.

Sabouraud Dextrose Agar (for yeast and mold): Each sample also had less than 10 CFU/ml, which is well below the limit of 20 CFU/ml.

This means all the samples passed the microbial limit test and were clean and safe from bacterial, yeast, and mold contamination, even the sample without any antioxidant. The findings suggest that the physicochemical properties of the formulation, such as low water activity, lipophilicity, and the potential inherent antimicrobial effects of the carrier oils, may help suppress microbial growth even without added preservatives. The total count test results are shown in **Table 4.4.2**.

Table 4.4.2

Medium	**Limits on agar CFU/ml**	**Result of sample 1 (0.5% w/v conc of eucalyptus oil)**	**Result of sample 2 (2.0 % w/v cone of eucalyptus oil)**	**Result of sample 3 (0.5% w/v conc of thyme oil)**	**Result of sample 4 (2.0% w/v conc of thyme oil)**	**Result of sample 5 (0.5% w/v mix of the two oils, 0.25% w/v of each one)**	**Result of sample (1.0% w/v mix of the two oils, 0.5% w/v of each one)**	**Negative control, (without any chemical or natural antioxidant)**
Tryptic soy broth	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
Sabouraud dextrose broth	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU

4.5 Microbial limit test for all samples during stability study: The tables below present the microbial limit test results for all product samples during the stability study, ensuring their quality, purity, and safety.

4.5.1 For antibacterial cream:

Microbial limit test – Direct transfer (broth media) test results for Anti-bacterial cream during stability study are shown in Table 4.5.1 (A)

Time duration	Medium	Result of sample 1 (3.0% w/w conc of eucalyptus oil)	Result of sample 2 (1.5% w/w mix conc of each oil)	Result of sample 3 (1.0% w/w mix conc of each oil)	Result of sample 4 (0.5% w/w mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
T_{zero}	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear
T_{zero}	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear
$T_{30\ days}$	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear
$T_{30\ days}$	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear
$T_{90\ days}$	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear
$T_{90\ days}$	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear
$T_{180\ days}$	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear
$T_{180\ days}$	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear

Table 4.5.1(B): Microbial limit test – Total count test for Anti-bacterial cream during stability study.

Time duration	Medium	Limits on agar CFU/ml	Result of sample 1 (3.0% w/w conc of eucalyptus oil)	Result of sample 2 (1.5% w/w mix conc of each oil)	Result of sample 3 (1.0% w/w mix conc of each oil)	Result of sample 4 (0.5% w/w mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
T_{zero}	Tryptic soy agar	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
T_{zero}	Sabouraud dextrose agar	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{30\ days}$	Tryptic soy agar	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{30\ days}$	Sabouraud dextrose agar	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{90\ days}$	Tryptic soy agar	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{90\ days}$	Sabouraud dextrose agar	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{180\ days}$	Tryptic soy agar	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{180\ days}$	Sabouraud dextrose agar	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU

4.5.2 For anti-infective cream:

Table 4.5.2 (A): Microbial limit test – Direct transfer (broth media) test for Anti-infective cream during stability study.

Time duration	Medium	Result of sample 1 (3.0% w/w conc of eucalyptus oil)	Result of sample 2 (1.5% w/w mix conc of each oil)	Result of sample 3 (1.0% w/w mix conc of each oil)	Result of sample 4 (0.5% w/w mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
T_{zero}	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear
T_{zero}	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear
$T_{30\ days}$	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear
$T_{30\ days}$	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear
$T_{90\ days}$	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear
$T_{90\ days}$	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear
$T_{180\ days}$	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear
$T_{180\ days}$	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear

Table 4.5.2 (B): Microbial limit test – Total count test for Anti-infective cream during stability study.

Time duration	Medium	Limits on agar CFU/ml	Result of sample 1 (3.0% w/w conc of eucalyptus oil)	Result of sample 2 (1.5% w/w mix conc of each oil)	Result of sample 3 (1.0% w/w mix conc of each oil)	Result of sample 4 (0.5% w/w mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
T_{zero}	Tryptic soy agar	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
T_{zero}	Sabouraud dextrose agar	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{30\ days}$	Tryptic soy agar	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{30\ days}$	Sabouraud dextrose agar	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{90\ days}$	Tryptic soy agar	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{90\ days}$	Sabouraud dextrose agar	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{180\ days}$	Tryptic soy agar	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{180\ days}$	Sabouraud dextrose agar	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU

4.5.3 For vitamin D3 oral drops:

Table 4.5.3 (A): Microbial limit test – Direct transfer (broth media) test for Vitamin D3 oral drops during stability study.

Time duration	Medium	Result of sample 1 (0.5% w/v conc of eucalyptus oil)	Result of sample 2 (2.0 % w/v cone of eucalyptus oil)	Result of sample 3 (0.5% w/v conc of thyme oil)	Result of sample 4 (2.0% w/v conc of thyme oil)	Result of sample 5 (0.5% w/v mix of the two oils, 0.25% w/v of each one)	Result of sample (1.0% w/v mix of the two oils, 0.5% w/v of each one)	Negative control, (without any chemical or natural antioxidant)
T_{zero}	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear	Clear	Clear
T_{zero}	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear	Clear	Clear
$T_{30\ days}$	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear	Clear	Clear
$T_{30\ days}$	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear	Clear	Clear
$T_{90\ days}$	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear	Clear	Clear
$T_{90\ days}$	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear	Clear	Clear
$T_{180\ days}$	Tryptic soy broth	Clear	Clear	Clear	Clear	Clear	Clear	Clear
$T_{180\ days}$	Sabouraud dextrose broth	Clear	Clear	Clear	Clear	Clear	Clear	Clear

Table 4.5.3 (B): Microbial limit test – Total count test for Vitamin D3 oral drops during stability study.

Time duration	Medium	Limits on agar CFU/ ml	Result of sample 1 (0.5% w/v conc of eucalyptus oil)	Result of sample 2 (2.0 % w/v cone of eucalyptus oil)	Result of sample 3 (0.5 % w/v conc of thyme oil)	Result of sample 4 (2.0 % w/v conc of thyme oil)	Result of sample 5 (0.5 % w/v mix of the two oils, 0.25 % w/v of each one)	Result of sample (1.0 % w/v mix of the two oils, 0.5% w/v of each one)	Negative control, (without any chemical or natural antioxidant)
T_{zero}	Tryptic soy broth	Bacteria < 200 (10^2)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
T_{zero}	Sabouraud dextrose broth	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{30\ days}$	Tryptic soy broth	Bacteria < 200	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{30\ days}$	Sabouraud dextrose broth	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{90\ days}$	Tryptic soy broth	Bacteria < 200	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{90\ days}$	Sabouraud dextrose broth	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{180\ days}$	Tryptic soy broth	Bacteria < 200	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU
$T_{180\ days}$	Sabouraud dextrose broth	Yeast and mold <20 (10^1)	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU	< 10 CFU

4.5.4 Comparative Analysis and Implications: Across all tested formulations, essential oils, creams, and oral drops, the microbial load during the stability study remained significantly below the USP pharmacopeial limits for non-sterile pharmaceutical products, which are typically:

<200 CFU/mL for total aerobic microbial count (TAMC).

<20 CFU/mL for total yeast and mold count (TYMC).

As specified by the United States Pharmacopeia (USP) and European Pharmacopoeia (Ph. Eur.). These findings collectively confirm the microbiological safety and preservative effectiveness of eucalyptus and thyme essential oils in both topical and oral pharmaceutical applications. Notably, the antimicrobial performance was consistent even at the lowest tested concentration of 0.5% w/w, supporting the rationale for their use in clean-label or preservative-free natural formulations. Moreover, the absence of contamination in negative control samples across all product types suggests that the formulation environment and process met good manufacturing practice (GMP) standards, and essential oil components likely possess residual antimicrobial activity that persists through formulation and packaging.

The results of the microbial limit tests demonstrated that both eucalyptus and thyme essential oils possess effective antimicrobial properties suitable for use as natural preservatives in pharmaceutical formulations. All tested samples, including those with minimal oil concentrations and those without any added preservatives, complied with pharmacopeial microbial safety criteria. These outcomes highlight the potential of essential oils not only as active pharmaceutical ingredients (APIs) with therapeutic benefits but also as functional excipients contributing to the microbial stability of finished products. This dual function supports the development of natural, preservative-free products with enhanced consumer appeal and compliance with clean-label standards.

4.6 Minimum inhibitory concentration test:

The MIC assay was conducted to determine the lowest concentration of thyme and eucalyptus essential oils required to inhibit visible growth of four bacterial strains: *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa*. The broth turbidity method was employed, with visual changes in turbidity and odor used as indicators of microbial growth.

The absorbance of bacterial suspensions was measured at 650 nm using a UV–visible spectrophotometer. This wavelength, in the red region of the visible spectrum, is commonly used to estimate bacterial growth based on optical density. The Absorbance of bacterial suspension results are presented in Table **4.6.1**.

Table 4.6.1

Sample (Bacteria)	ABS (650)	Acceptable limit range
Blank	—	
S. aureus	0.282	0.3-0.45
S. epidermidis	0.252	0.2-0.3
E. coli	0.196	0.2-0.3
P. aeruginosa	0.202	0.2-0.3

The results of the minimum inhibitory concentration (MIC) test conducted at 650 nm revealed absorbance values that indicate effective bacterial inhibition. As expected, the blank sample displayed no absorbance. *S. aureus* showed an absorbance of 0.282, which is slightly below its typical range of 0.3 to 0.45, suggesting a minor reduction in bacterial population compared to the standard range of 1 to 3 × 10^8 CFU. *S. epidermidis* recorded an absorbance of 0.252, while *E. coli* and *P. aeruginosa* had values of 0.196 and 0.202, respectively, which are within or just below the expected range of 0.2 to 0.3 for their species. These results indicate that the essential oils tested were effective in inhibiting bacterial growth, reaching levels close to or within the standard MIC ranges for high bacterial loads (10^8 CFU). This demonstrates promising antimicrobial activity in vitro under the test conditions. Such quantitative data underscore promising in vitro potency for anti-infective formulations, aligning with pharmacopoeial benchmarks (MIC ≤0.5–2% v/v oils) and supporting development of stable, broad-spectrum topical/oral products under GMP conditions. Balouiri et al., (2016).

The results for the minimum inhibitory concentration for eucalyptus oil are shown in Table 4.6.1 (A).

Microorganisms	Concentration	0.5%v/v	1.0%v/v	2.0%v/v	3.0%v/v	4.0%v/v
S. aureus		Negative	Negative	Negative	Negative	Negative
S. epidermidis		Negative	Negative	Negative	Negative	Negative
E. coli		Positive	Positive	Negative	Negative	Negative
P. aeruginosa		Positive	Positive	Positive	Negative	Negative
Negative control (Product + media)		Negative	Negative	Negative	Negative	Negative

Table 4.6.1 (B): The results for positive control

Microorganisms	Positive control (Media + bacteria)
S. aureus	Positive
S. epidermidis	Positive
E. coli	Positive
P. aeruginosa	Positive

Note:

Negative: It means there is no visible growth of microorganisms, turbidity, or change in color of the solution, resulting in a clear solution without any unpleasant odor

Positive: It indicates visible growth of microorganisms, turbidity, and a change in the solution's color, accompanied by a bad odor.

The minimum inhibitory concentration (MIC) test for eucalyptus oil demonstrated strong antibacterial activity, especially at higher concentrations. Both *S. aureus* and *S. epidermidis* were completely inhibited across all tested concentrations (0.5%–4.0% v/v), indicating high susceptibility of Gram-positive bacteria. For Gram-negative bacteria, *E. coli* showed growth at 0.5% and 1.0% v/v but was fully inhibited at 2.0% v/v and above, whereas *P. aeruginosa* required 3.0%–4.0% v/v for complete suppression, highlighting a concentration-dependent effect. These results show that even minimal amounts of eucalyptus oil (0.05 mL for 0.5% v/v to 0.4 mL for 4.0% v/v) are sufficient to inhibit microbial growth.

The broth microdilution technique used in this study is a widely accepted method for determining MIC by monitoring changes in turbidity, the presence or absence of visible growth, and other indicators such as odor (Wiegand, Hilpert, & Hancock, 2008). Negative controls remained free of contamination, while positive controls confirmed bacterial viability, validating the reliability of the test. Overall, eucalyptus oil exhibited broad-spectrum antibacterial activity, with particularly strong effects against Gram-positive bacteria and concentration-dependent inhibition of Gram-negative strains, confirming its effectiveness as a natural antimicrobial agent.

Table 4.6.1 (C): The minimum inhibitory concentration (MIC) of eucalyptus oil required to prevent visible microbial growth is summarized below

Microorganism	MIC Value (Eucalyptus Oil)
S. aureus	≤ 0.5% v/v
S. epidermidis	≤ 0.5% v/v
E. coli	2.0% v/v
P. aeruginosa	3.0% v/v

Table 4.6.2: The results for the minimum inhibitory concentration of thyme oil are detailed below

Microorganisms	Concentration	0.5%v/v	1.0%v/v	2.0%v/v	3.0%v/v	4.0%v/v
S. aureus		Positive	Negative	Negative	Negative	Negative
S. epidermidis		Negative	Negative	Negative	Negative	Negative
E. coli		Negative	Negative	Negative	Negative	Negative
P. aeruginosa		Positive	Positive	Positive	Positive	Positive
Negative control (Product + media)		Negative	Negative	Negative	Negative	Negative

Table 4.6.2 (A): The results for positive control

Microorganisms	Positive control (Media + bacteria)
S. aureus	Positive
S. epidermidis	Positive
E. coli	Positive
P. aeruginosa	Positive

Note:

Negative Result: This means there is no visible growth of microorganisms, no turbidity, and no change in the color of the solution. As a result, the solution remains clear and has no unpleasant odor.

Positive Result: This indicates visible growth of microorganisms, turbidity, and a change in the color of the solution, along with a foul odor.

The minimum inhibitory concentration (MIC) results for thyme essential oil indicated that Staphylococcus aureus was inhibited starting at a concentration of 1.0%. Complete inhibition of *S. epidermidis* and *E. coli* occurred at all tested concentrations (0.5% to 4.0%), demonstrating strong antibacterial activity even at the lowest concentration. However, *P. aeruginosa* showed resistance at all concentrations tested, with positive microbial growth observed from 0.5% to 4.0%. The negative control (product + media) remained clear, confirming its sterility, while the positive control (product + bacteria) showed microbial growth for all tested organisms, validating the test system. These results suggest that thyme oil is particularly effective against Gram-positive cocci and *E. coli* but not effective against *P. aeruginosa*, even at higher concentrations.

Table 4.6.2 (B): The minimum inhibitory concentration (MIC) of thyme oil, which effectively inhibits visible microbial growth, is clearly summarized below

Microorganism	MIC Value (Eucalyptus Oil)
S. aureus	1.0% v/v
S. epidermidis	≤ 0.5% v/v
E. coli	≤ 0.5% v/v
P. aeruginosa	> 4.0% v/v (Resistant)

Thyme oil demonstrated significant antimicrobial activity, particularly effective against *S. aureus*, *S. epidermidis*, and *E. coli*, inhibiting these bacteria at low concentrations. In contrast, eucalyptus oil exhibited strong effectiveness against *S. aureus* and *S. epidermidis* but needed higher concentrations to impact *E. coli* and *P. aeruginosa*. Additionally, thyme oil showed no effect on *P. aeruginosa*, underscoring the necessity of strain-specific testing when developing antimicrobial products.

4.7 DPPH (2,2-Diphenyl-1-Picryl-Hydrazyl-Hydrate) free radical Scavenging activity assay:

The antioxidant activity of the essential oils was evaluated using the DPPH (2,2-Diphenyl-1-Picrylhydrazyl) assay. The reduction in DPPH absorbance at 515 nm was used to calculate the percentage of free radical scavenging activity.
Standard used: L- Ascorbic acid free acid

Table 4.7A: The results For DPPH test for essential oils and L ascorbic acid standard are shown below

Standard, sample, and control	Absorbance	% DPPH inhibition
Control	0.658	
Standards		
0.5 % w/v ascorbic acid	0.599	8.96%
1.0 % w/v ascorbic acid	0.518	21.30%
2.0% w/v ascorbic acid	0.461	29.93%
3.0% w/v ascorbic acid	0.413	37.23%
4.0% w/v ascorbic acid	0.402	38.90%
Samples		
0.5 % w/v eucalyptus oil	0.622	5.47%
1.0 % w/v eucalyptus oil	0.605	8.0%
2.0 % w/v eucalyptus oil	0.495	24.77%
3.0 % w/v eucalyptus oil	0.402	38.90%
4.0 % w/v eucalyptus oil	0.394	40.12%
0.5 % w/v thyme oil	0.510	22.50%
1.0 % w/v thyme oil	0.424	35.56%
2.0 % w/v thyme oil	0.329	50.0%
3.0 % w/v thyme oil	0.266	59.57%
4.0 % w/v thyme oil	0.248	62.31%

The percentage of DPPH inhibition for the sample and standard is calculated using the following equations:

%DPPH inhibition for sample $= (Absorbance\ control\ - Absorbance\ sample) \div (\ Absorbance\ sample) \times 100\%$

%DPPH inhibition for standard $= (Absorbance\ control\ - Absorbance\ standard) \div (\ Absorbance\ standard) \times 100\%$

4.7.1 Standard (Ascorbic Acid) Curve: As expected, ascorbic acid exhibited dose-dependent scavenging activity. At 4.0 % w/v, it reached 38.90% inhibition.

Graph 4.7.1A: The graph of Ascorbic Acid Curve

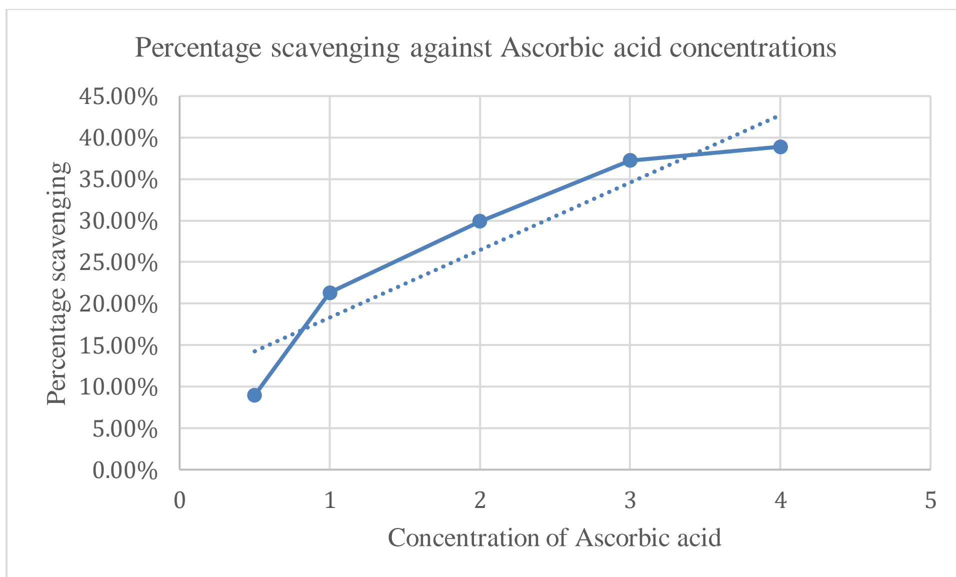


Table 4.7.2: Antioxidant Activity of Eucalyptus Oil: Eucalyptus oil showed moderate antioxidant activity. At 4.0%, the maximum scavenging was 40.12%, comparable to 4.0% w/v of ascorbic acid. These results indicate a concentration-dependent antioxidant capacity, although lower than that of thyme oil.

4.7.2A: The graph of Eucalyptus oil curve

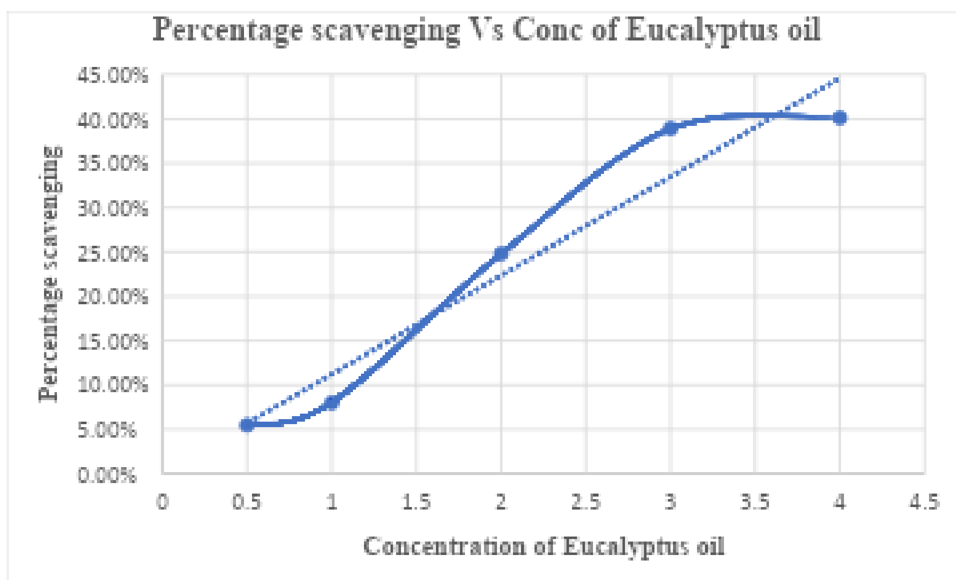
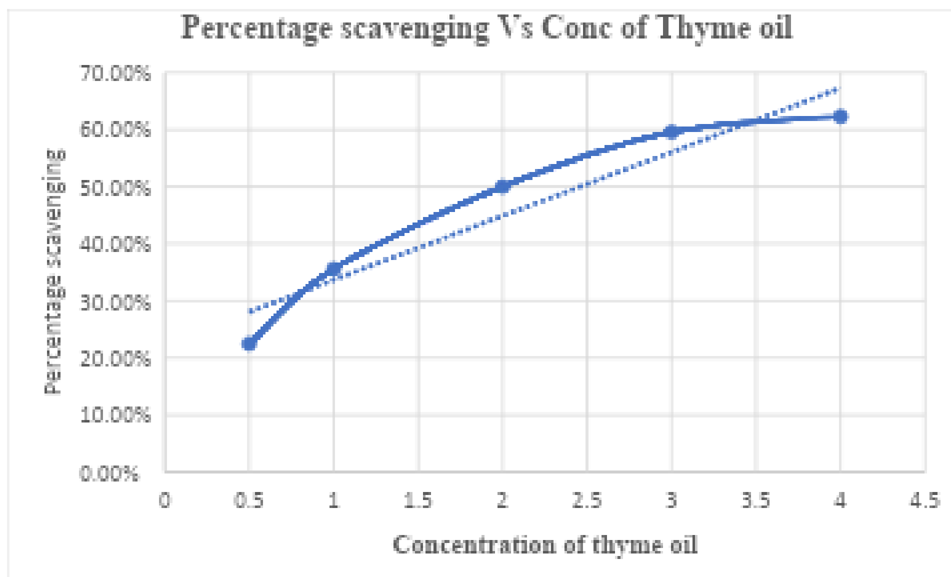


Table 4.7.3A: Antioxidant Activity of Thyme Oil: Thyme oil exhibited significantly higher free radical scavenging activity compared to eucalyptus oil. Thyme oil's activity surpassed that of ascorbic acid at comparable concentrations, suggesting a rich phenolic content contributing to its antioxidant power.

4.7.3B: The graph of Thyme oil Curve



The results indicate that eucalyptus and thyme essential oils have significant antimicrobial and antioxidant activities, though their effectiveness varies depending on concentration and species.

In the antioxidant evaluation, both essential oils exhibited dose-dependent free radical scavenging activity in the DPPH assay, confirming their ability to neutralize stable free radicals. Thyme essential oil showed significantly stronger antioxidant activity, which is well supported by its high content of phenolic compounds such as thymol and carvacrol, known for their hydrogen-donating capacity and strong redox properties. These compounds enhance oxidative stability in formulations and contribute to protection against free radical–induced degradation.(Brand-Williams et al., 1995)

Together, these findings support the potential application of eucalyptus and thyme essential oils as multifunctional active ingredients in pharmaceutical and cosmeceutical formulations, providing both antimicrobial preservation and antioxidant protection. Their natural origin offers an attractive alternative to synthetic preservatives and antioxidants; however, further in vivo investigations and formulation-based studies are necessary to confirm long-term efficacy, physicochemical stability, and safety under real-use conditions before final product development (Reichling et al., 2009).

4.8 Preservative efficacy test:

Preservative efficacy testing (PET), also known as antimicrobial effectiveness testing, evaluates the ability of a preservative system to inhibit microbial growth in pharmaceutical products. The main goal of this testing is to ensure product safety during manufacturing, storage, and use.

In this study, we assessed the antimicrobial preservation of antibacterial and anti-infective creams that contained 1.0% w/w combined essential oils (thyme and eucalyptus oils). We conducted challenge tests against five microbial strains: *S. aureus*, *P. aeruginosa*, *E. coli*, *C. albicans*, and *A. niger.*

The evaluation took place over a 28-day observation period, with assessments at 0, 7, 14, and 28 days. We used serial dilutions from 10^{-1} to 10^{-6} to count the surviving microbial populations. This dilution range ensures countable colony numbers, minimizes plating errors, and allows reliable determination of microbial reduction over time, as recommended by pharmacopeial and microbiological standard methods for antimicrobial effectiveness testing. (USP, 2024). According to pharmacopeial standards (USP/Ph. Europe.), an acceptable preservative system must demonstrate:

For Bacteria: NLT 2 log reduction from the initial count at 14 days and no increase from the 14-day count at 28 days

For Fungi: No increase from the initial calculated count at 14 and 28 days.

4.8.1 Absorbance Validation of Microbial Suspensions: Absorbance measurements were used to validate the presence and turbidity of bacterial, yeast, and mold suspensions by measuring optical density using a UV–visible spectrophotometer at 650 nm. This wavelength lies in the red region of the visible spectrum and is commonly applied to estimate microbial growth because light scattering at this range correlates with cell concentration in turbid suspensions. This approach provides a rapid and reliable confirmation of microbial inoculum density prior to experimental use, ensuring consistency and validity of microbiological assays. (CLSI, 2018)

The results are presented in the **Table 4.8.1 (1)**

Table 4.8.1 (1): Absorbance of bacterial suspension

Sample (Bacteria)	ABS (650)	Acceptable limit range
Blank	—	
S. aureus	0.416	0.3-0.45
E. coli	0.235	0.2-0.3
P. aeruginosa	0.283	0.2-0.3
C. albicans	1.016	≤ 1.0
A. niger	0.940	≤ 1.0

The absorbance values obtained for *S. aureus*, *E. coli*, and *P. aeruginosa* fall within their specified acceptable ranges, indicating that the bacterial suspensions were standardized appropriately and

contained sufficient cell densities for challenge testing. Similarly, the absorbance values for *C. albicans* and *A. niger* were at or below the acceptable upper limit, confirming adequate yeast and mold loads without exceeding recommended turbidity levels. These results demonstrate that all microbial suspensions were viable, dense, and suitable for use in a preservative efficacy test, ensuring that any observed reduction in microbial counts can be attributed to the antimicrobial activity of thyme and eucalyptus essential oils rather than insufficient initial inoculum.

Using standardized and sufficiently high microbial loads is critical in preservative efficacy testing, as recommended by pharmacopeial guidelines, to rigorously evaluate the ability of natural preservatives to inhibit or reduce microbial growth over time. The confirmed absorbance values support the reliability and validity of subsequent challenge test results, particularly when assessing essential oils as alternatives to synthetic preservatives in pharmaceutical formulations. Adequate initial microbial concentrations ensure meaningful comparison across sampling intervals and provide robust evidence of preservative performance against bacteria, yeast, and mold commonly associated with product contamination. (European Pharmacopoeia, 2023)

4.8.1(A) Preservative Efficacy Test for Antibacterial Cream

The antibacterial cream containing 1.0% w/w combined thyme and eucalyptus oils was challenged with the test organisms. Microbial counts were recorded over four time points.

Bacterial Challenge Results:

S. aureus and *S. epidermidis:* No growth detected at any time point.

P. aeruginosa: Initial recovery at dilutions 10^{-3} and 10^{-4} on day 0, but fully eradicated by day 7.

E. coli: Initially recovered at higher dilutions (>1000 CFU/mL), but eliminated by day 7.

Fungal Challenge Results:

C. albicans: Initial recovery at dilutions 10^{-1} and 10^{-2}, but no growth observed from day 7 onward.

A. Niger: No detectable growth at any time point.

The antibacterial cream met all pharmacopeial PET criteria, showing that all bacteria showed ≥2-log reduction by day 14 and no regrowth at day 28, and fungal organisms showed no increase from initial counts. This confirms the adequacy of the essential oil blend (thyme and eucalyptus at 1.0% w/w) as an effective natural preservative system.

The report on the efficacy testing of preservatives used for the 1.0% w/w of antibacterial cream is shown in **Table 4.8.1 (B)**

Table 4.8.1 (B)

Product	Testing interval	Preservatives used	Preservatives concentration	Dilution
Anti-bacterial cream	T0	1. Thyme oil. 2. Eucalyptus oil.	1.0 % w/w 1.0 % w/w	As is

Microorganism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	**6538**	**0**	0	0	0	0	0	
		7	0	0	0	0	0	
		14	0	0	0	0	0	
		28	0	0	0	0	0	
P. aeruginosa	**9027**	**0**	0	73	32	0	0	
		7	0	0	0	0	0	
		14	0	0	0	0	0	
		28	0	0	0	0	0	
E. coli	**8739**	**0**		>1000	>1000	74	0	
		7	0	0	0	0	0	
		14	0	0	0	0	0	
		28	0	0	0	0	0	
C. albicans	**10231**	**0**	200	11	0	0	0	
		7	0	0	0	0	0	
		14	0	0	0	0	0	
		28	0	0	0	0	0	
A. niger	**16404**	**0**	0	0	0	0	0	
		7	0	0	0	0	0	
		14	0	0	0	0	0	
		28	0	0	0	0	0	

The control dilution for the 1.0% w/w Antibacterial cream is shown in **Table 4.8.1 (C)**

Control Dilution						Day
10^{-1}	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	
			440	56		**0**
			400	68		**7**
			>>1000	>1000		**14**
				>1000	460	**28**
			44	5		**0**
			>1000	90		**7**
			>1000	350		**14**
				702	68	**28**
			20	2		**0**
			543	50		**7**
			>>1000	>>1000		**14**
				>>1000	>1000	**28**
		220	39	3		**0**
		323	25	6		**7**
			>1000	>1000		**14**
			>1000	200		**28**
		240	20	3		**0**
		>1000	120	10		**7**
		>1000	200	18		**14**
		>1000	400	58		**28**

Control dilution was done as the same of the sample, and the control is diluted microorganisms with the media to make comparison for the efficacy of essential oils to kill microorganisms.

4.8.2 Preservative Efficacy Test for Anti-Infective Cream

A similar test protocol was applied to the anti-infective cream formulation using the same concentration of preservatives.

Bacterial Challenge Results

S. aureus: No growth detected at any point.

P. aeruginosa: Moderate growth initially (10^{-3} and 10^{-4} dilutions) on day 0; eradicated by day 7.

E. coli: High initial contamination (>1000 CFU/mL), cleared completely by day 7.

Fungal Challenge Results

C. albicans: Initial moderate growth (10^{-1} to 10^{-4}), no growth detected from day 7 onward.

A. niger: No growth throughout the testing period.

The anti-infective cream also complied with pharmacopeial PET requirements. Both bacterial and fungal populations were eliminated or controlled within the prescribed timeframe, affirming the preservative efficiency of the essential oil system.

Control Comparison

The control samples lacking preservatives showed persistent or increased microbial contamination over the 28-day study period, with bacterial counts remaining above 1000 CFU/mL and no measurable reduction in viable microorganisms, alongside pronounced fungal growth. These results confirm the inability of unpreserved formulations to control microbial proliferation and clearly contrast with the preserved samples, thereby demonstrating the essential role of thyme and eucalyptus essential oils in effectively inhibiting bacterial and fungal growth and maintaining microbiological stability, as required by pharmacopeial standards for antimicrobial effectiveness testing. (United States Pharmacopeial Convention, 2023; European Pharmacopoeia Commission, 2023).

The report on the efficacy testing of preservatives used for the 1.0% w/w of anti-infective cream is shown in **Table 4.8.2 (A)**

Table 4.8.2 (A)

Product	Testing interval	Preservatives used	Preservatives concentration	Dilution
Anti-infective cream	T0	1. Thyme oil. 2. Eucalyptus oil.	1.0% w/w 1.0% w/w	As is

Microorganism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	**6538**	**0**	0	0	0	0	0	
		7	0	0	0	0	0	
		14	0	0	0	0	0	
		28	0	0	0	0	0	
P. aeruginosa	**9027**	**0**	0	73	32	5	1	
		7	0	0	0	0	0	
		14	0	0	0	0	0	
		28	0	0	0	0	0	
E. coli	**8739**	**0**	>1000	>1000	>1000	75	10	
		7	0	0	0	0	0	
		14	0	0	0	0	0	
		28	0	0	0	0	0	
C. albicans	**10231**	**0**	100	11	39	3	2	
		7	0	0	0	0	0	
		14	0	0	0	0	0	
		28	0	0	0	0	0	
A. niger	**16404**	**0**	0	0	0	0	0	
		7	0	0	0	0	0	
		14	0	0	0	0	0	
		28	0	0	0	0	0	

Table 4.8.2 (B):

Control Dilution						Day
10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
			440	56		**0**
			400	68		**7**
			>>1000	>1000		**14**
				>1000	460	**28**
			44	5		**0**
			>1000	90		**7**
			>1000	350		**14**
				702	68	**28**
			20	2		**0**
			543	50		**7**
			>>1000	>>1000		**14**
				>>1000	>1000	**28**
		220	39	3		**0**
		323	25	6		**7**
			>1000	>1000		**14**
			>1000	200		**28**
		240	20	3		**0**
		>1000	120	10		**7**
		>1000	200	18		**14**
		>1000	400	58		**28**

The results of the preservative efficacy tests strongly indicate that a 1.0% w/w combination of thyme and eucalyptus essential oils is effective in both antibacterial and anti-infective cream formulations. These essential oils successfully inhibited the growth of both bacteria and fungi, fulfilling pharmacopeial preservation standards. This finding emphasizes the potential of these natural oils as enchanting, eco-friendly alternatives to synthetic preservatives in topical pharmaceutical and cosmetic products, paving the way for a more sustainable future.

Table 4.8.3: The following report provides a comprehensive analysis of a negative control sample of antibacterial cream without any chemical or natural preservatives

Microorganism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	**6538**	**0**	0	0	0	460	60	
		7	0	0	0	490	80	
		14	0	0	0	<< 1000	<1000	
		28	0	0	0	0	<1000	490
P. aeruginosa	**9027**	**0**	0	0	0	465	15	
		7	0	0	0	<1000	100	
		14	0	0	0	<1000	366	
		28	0	0	0	0	712	77
E. coli	**8739**	**0**	0	0	0	32	12	
		7	0	0	0	550	60	
		14	0	0	0	>>1000	>>1000	
		28	0	0	0	0	>>1000	>1000
C. albicans	**10231**	**0**	0	0	250	50	11	
		7	0	0	330	83	19	
		14	0	0	0	>1000	>1000	
		28	0	0	0	>1000	>1000	
A. niger	**16404**	**0**	0	0	250	30	7	
		7	0	0	>1000	135	18	
		14	0	0	>1000	215	32	
		28	0	0	>1000	420	64	

Table 4.8.4: The following report provides a comprehensive analysis of a negative control sample of anti-infective cream without any chemical or natural preservatives

Microorganism 10^6 CFU/ml	ATCC No.	Day	Test Dilution					
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
S. aureus	**6538**	**0**	0	0	0	470	70	
		7	0	0	0	500	85	
		14	0	0	0	<< 1000	<1000	
		28	0	0	0	0	<1000	505
P. aeruginosa	**9027**	**0**	0	0	0	480	20	
		7	0	0	0	<1000	110	
		14	0	0	0	<1000	385	
		28	0	0	0	0	723	88
E. coli	**8739**	**0**	0	0	0	25	15	
		7	0	0	0	465	55	
		14	0	0	0	>>1000	>>1000	
		28	0	0	0	0	>>1000	>1000
C. albicans	**10231**	**0**	0	0	260	54	10	
		7	0	0	345	94	25	
		14	0	0	0	>1000	>1000	
		28	0	0	0	>1000	>1000	
A. niger	**16404**	**0**	0	0	190	28	9	
		7	0	0	>1000	118	27	
		14	0	0	>1000	225	56	
		28	0	0	>1000	400	78	

The preservative effectiveness test (PET) was done for both the antibacterial cream with fusidic acid and the anti-infective cream with mafenide acetate. The test used samples that didn't have any preservatives, either chemical or natural. The test was done to find out how well the basic mixtures naturally protect against microbes before adding natural preservatives like thyme and eucalyptus essential oils. In both creams, when bacteria and fungi like *S. aureus*, *P. aeruginosa*, *E. coli*, *C. albicans*, and *A. niger* were added at the start, they all grew a lot during the 28 days of observation. The bacterial results showed that neither formulation met the needed ≥2 log reduction in 14 days, and neither kept a steady count over 28 days, as required by the pharmacopoeial standards (USP <51>, 2023).

For example, *E. coli* and *C. albicans*, the number of colonies was higher than 1000 CFU/mL by day 14, which suggests that the microbes were growing actively instead of being kept under control. Similarly, the results for fungal challenges with *C. albicans* and *A. niger* showed a big rise in the number of live fungi at days 14 and 28, showing that neither of the cream bases was able to stop the growth of these fungi. This result shows that the natural ability of fusidic acid and mafenide acetate to fight microbes on their own wasn't enough to keep the products safe, because these medicines work to treat infections, not as general preservatives that stop all types of bacteria and fungi. Without preservatives, microbes could live and grow, which shows how important it is to use good antimicrobial stabilizers to keep the product safe when it's stored and used. These results show that neither of the cream formulas can protect itself from spoiling, and they provide an important starting point for checking how well natural preservatives work in future tests. The growth of microbes in the control samples shows that adding thyme and eucalyptus essential oils to the products, as was done, helps make them more resistant to contamination and better at killing microbes. This improves the quality of the product and ensures it meets the required standards set by pharmacopoeial guidelines.

4.9 Assay results for the active pharmaceutical ingredient and pH levels in the finished products:

4.9.1 Anti-bacterial cream: the assay results are shown in **Table 4.9.1 (A)**

Table 4.9.1 (A)

Time period	Result of sample 1 (3.0% w/w conc of eucalyptus oil)	Result of sample 2 (1.5% w/w mix conc of each oil)	Result of sample 3 (1.0% w/w mix conc of each oil)	Result of sample 4 (0.5% w/w mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
T_{Zero}	96.8%	96.6%	101.0%	96.9%	97.3%
$T_{30\ days}$	96.5%	96.6%	100.4%	96.0%	97.6%
$T_{90\ days}$	96.3%	96.6%	100.2%	96.1%	97.4%
$T_{180\ days}$	96.0%	96.3%	99.8%	95.8%	97.2%

The pH results for the are shown in **Table 4.9.1 (B)**

Time period	Result of sample 1 (3.0% w/w conc of eucalyptus oil)	Result of sample 2 (1.5% w/w mix conc of each oil)	Result of sample 3 (1.0% w/w mix conc of each oil)	Result of sample 4 (0.5% w/w mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
T_{Zero}	4.53	4.51	4.50	4.53	4.52
$T_{30\ days}$	4.51	4.50	4.50	4.54	4.52
$T_{90\ days}$	4.55	4.49	4.46	4.42	4.48
$T_{180\ days}$	4.52	4.44	4.44	4.40	4.45

All samples, including those with varying concentrations of essential oils and the negative control, maintained fusidic acid content close to initial values (96.8–101.0%) over 180 days. Most stable sample: Sample 3 (1.0% mixed oils) had the highest initial value (101.0%) and showed very little degradation (99.8% at day 180). The pH remained consistent in the acidic range (4.40–4.55) throughout the study. A slight drop was noted over time, especially in lower oil concentrations (Sample 4). The pH is suitable for skin application and supports drug stability and compatibility.

4.9.2 Anti-infective cream: the assay results are described in **table 4.9.2 (A)**

Table 4.9.2 (A)

Time period	Result of sample 1 (3.0% w/w conc of eucalyptus oil)	Result of sample 2 (1.5% w/w mix conc of each oil)	Result of sample 3 (1.0% w/w mix conc of each oil)	Result of sample 4 (0.5% w/w mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
T_{Zero}	95.1%	99.3%	98.9%	102.3%	103.0%
$T_{30\ days}$	95.0%	96.1%	98.6%	99.8%	101.8%
$T_{90\ days}$	94.9%	95.6%	97.8%	99.1%	100.8%
$T_{180\ days}$	93.4%	95.4%	97.2%	98.4%	98.5%

The pH results are described in Table **4.9.2 (B)**

Time period	Result of sample 1 (3.0% w/w conc of eucalyptus oil)	Result of sample 2 (1.5% w/w mix conc of each oil)	Result of sample 3 (1.0% w/w mix conc of each oil)	Result of sample 4 (0.5% w/w mix conc of each oil)	Result of sample 5 (Negative control, product without any chemical or natural preservative)
T_{Zero}	6.62	6.59	6.62	6.61	6.60
$T_{30\ days}$	6.50	6.60	6.61	6.62	6.60
$T_{90\ days}$	6.61	6.59	6.60	6.59	6.58
$T_{180\ days}$	6.58	6.54	6.57	6.52	6.50

Greater variability than the anti-bacterial cream, especially in the control sample: 103% at T0 dropping to 98.5% at T180. Sample 1 (3.0% eucalyptus oil) showed more degradation (95.1% → 93.4%), which could imply some instability at higher oil concentrations. Sample 2 (1.5% mix) had a more stable profile (99.3% → 95.4%), suggesting moderate oil blends offer better protection for the API. The pH started around 6.60 and slightly decreased to ~6.50, with minimal variation between samples.

As a conclusion, Mafenide acetate shows acceptable stability, especially in high oil concentrations. Essential oils could play a protective role, but the base formulation needs optimization.

4.9.3 vitamin D3 oral drops: the assay results are illustrated in **Table 4.9.3 (A)**

Table 4.9.3 (A)

Time period	Result of sample 1 (0.5% w/v conc of eucalyptus oil)	Result of sample 2 (2.0 % w/v cone of eucalyptus oil)	Result of sample 3 (0.5% w/v conc of thyme oil)	Result of sample 4 (2.0% w/v conc of thyme oil)	Result of sample 5 (0.5% w/v mix of the two oils, 0.25% w/v of each one)	Result of sample 6 (1.0% w/v mix of the two oils, 0.5% w/v of each one)	Result of sample 7 negative control, (without any chemical or natural antioxidant)
T_{Zero}	98.0%	98.9%	97.7%	99.2%	99.3%	101.1%	99.6%
$T_{30\ days}$	97.8%	99.2%	98.4%	100.1%	100.2%	100.0%	99.3%
$T_{90\ days}$	97.8%	98.0%	97.6%	97.5%	97.4%	97.3%	97.8%
$T_{180\ days}$	96.7%	96.8%	96.7%	96.8%	96.7%	96.6%	97.3%

The pH results are illustrated in **Table 4.9.3 (B)**

Time period	Result of sample 1 (0.5% w/v conc of eucalyptus oil)	Result of sample 2 (2.0 % w/v cone of eucalyptus oil)	Result of sample 3 (0.5% w/v conc of thyme oil)	Result of sample 4 (2.0% w/v conc of thyme oil)	Result of sample 5 (0.5% w/v mix of the two oils, 0.25% w/v of each one)	Result of sample 6 (1.0% w/v mix of the two oils, 0.5% w/v of each one)	Result of sample 7 negative control, (without any chemical or natural antioxidant)
T_{Zero}	3.03	2.99	3.04	3.01	3.04	3.05	3.01
$T_{30\ days}$	2.96	2.95	3.05	2.99	2.97	3.02	3.02
$T_{90\ days}$	2.91	2.92	3.00	2.91	2.96	3.00	2.99
$T_{180\ days}$	2.90	2.90	2.98	2.92	2.93	2.97	2.96

Initial high concentrations (up to 101.1%) dropped slightly to ~96.6–97.3% by day 180. Negative control remained fairly stable (99.6% → 97.3%), but the oil blends maintained better stability overall. Most effective sample: Sample 2 (2.0% eucalyptus oil) and sample 4 (2.0% thyme oil) showed minimal degradation. The pH started acidic (2.99–3.05), which is typical for oil-based vitamin D3. Gradual decrease to ~2.90–2.97 at T180. The acidic environment is acceptable and protective for vitamin D3, though sustained acidity may need consideration for long-term mucosal tolerance.

As a conclusion, Vitamin D3 remained chemically and physically stable across all formulations. The use of essential oils slightly improved retention and possibly acted as antioxidants.

Chapter Five:

5.1 Conclusion

This specific study has examined the therapeutic applications of eucalyptus and thyme essential oils as well as their possible application as natural antioxidants and preservatives. Both oils have effectively stabilized certain medicinal compounds in topical creams and vitamin D3 oral drops, and they have demonstrated significant efficacy against a broad range of bacteria. Microbial limit tests found that all samples, including those containing essential oils and the negative control, maintained excellent microbiological safety, with countable colonies of less than 10 CFU/mL of bacteria, yeast, and mold, and clear broth media without turbidity. Particularly, eucalyptus oil had potent antibacterial properties that grew stronger with concentration. When compared to the positive strains, the negative Gram bacteria showed more resistance. Gram-positive bacteria and *E. Coli* were strongly inhibited by thyme oil; however, *P. aeruginosa* was barely affected. Tests of DPPH radical scavenging activity showed that eucalyptus oil had modest radical scavenging activity, although thyme oil was more effective than the control in scavenging free radicals.

In tests of preservation efficacy, a 1.0% w/w thyme and eucalyptus oil combination in anti-bacterial and anti-infective creams inhibited microbiological development for 28 days. *S. aureus* and *A. niger* were completely inhibited at every time point by day 7, and *P. aeruginosa*, *E. coli*, and *C. albicans* were no longer detectable. This achievement satisfied the stability and microbial reduction pharmacopeial standards. In contrast, control formulations lacking preservatives continued to show microbial growth, demonstrating the essential oil blend's efficacy as a natural preservative. According to the laboratory results, all formulations for the active medicinal ingredients exhibited outstanding chemical stability. In the anti-bacterial cream and anti-infective cream, Fusidic acid and Mafenide acetate, respectively, maintained concentrations close to their initial value in 180 days, with only slight changes at higher essential oil concentrations. In the

same way, the Vitamin D3 oral drops were also stable chemically, while the essential oils added were able to provide additional antioxidant protection and improved retention.

The pH measurements for all formulations stayed within the appropriate ranges for their intended applications, further confirming both formulation stability and suitability for skin or oral use. In summary, the study's findings suggest that thyme and eucalyptus essential oils may both operate as multipurpose natural preservatives and antioxidants, demonstrating their potential as safer alternatives to pharmaceutical products' artificial preservatives and antioxidants. Incorporating natural substances into cream and oral drop formulations enhances their efficacy and quality while ensuring chemical and microbiological stability, as well as microbiological safety. The results would thus create readily sustainable and high-grade pharmaceutical formulations, and essential oils do show ample promise in formulation, as the pharmaceutical products aim for consumer welfare and ready availability.

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الاستخدام الفعّال لزيوت الزعتر والكينا العطرية كمُركّبات حافظة ومضادات أكسدة طبيعية في المستحضرات الصيدلانية

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الملخص:

تزداد أهمية الزيوت العطرية المستخلصة من Thyme و Eucalyptus نظرًا لفوائدها العلاجية القوية، وخصائصها المضادة للبكتيريا والأكسدة، وقدرتها على العمل كمُركّبات حافظة طبيعية. تهدف هذه الدراسة إلى تقييم إمكانية استخدام هذه الزيوت كبدائل طبيعية للمواد الحافظة ومضادات الأكسدة الصناعية في كريمات الجلد ومكملات فيتامين D3، مما يُسهم في جعل المستحضرات أكثر أمانًا واستقرارًا وفعالية. تمت إضافة الزيوت بتراكيز مختلفة إلى كريمات مضادة للبكتيريا وأخرى مضادة للعدوى، إضافة إلى قطرات فموية من فيتامينD3.

تضمنت الدراسة اختبار الفعالية الميكروبية للزيوت ضد أنواع متعددة من الكائنات الحية الدقيقة، شملت البكتيريا موجبة الغرام مثل *S. aureus* و *S. epidermidis*، والبكتيريا سالبة الغرام مثل E. coli و P. aeruginosaإضافة إلى الخميرة C. albicans والعفن As. Niger كما تم تقييم النشاط المضاد للأكسدة باستخدام اختبار DPPH free radical scavenging assay.

درست التجربة تأثير الزيوت العطرية على الثبات الكيميائي لثلاث مواد فعالة هي fusidic acid، mafenide acetate، crystalline vitamin D3على مدى 180 يومًا، مع متابعة تغيرات قيمة ال pH لتقييم التوافق بين المكونات في التركيبة. أظهرت الاختبارات الميكروبية أن جميع العينات كانت خالية تقريبًا من التلوث الجرثومي، إذ بلغ عدد الكائنات الحية الدقيقة أقل من 10 CFU/mL، ولم يُلاحظ أي عكارة في الأوساط الغذائية.

أظهر زيت Eucalyptus نشاطًا قويًا مضادًا للبكتيريا يعتمد على التركيز؛ حيث ثبّط نمو *S. aureus* و *S. epidermidis*عند جميع التراكيز (0.5–4.0%v/v) ، بينما تم تثبيط E. coli عند تركيز 2.0%v/v فأكثر، و *P. aeruginosa* عند تركيز ≥3.0%v/v. أما زيت Thyme فقد أظهر فعالية عالية ضد *S.*

*epidermidis*و *E. coli* عند جميع التراكيز، وبدأت فعاليته ضد *S. aureus* من تركيز 1.0% فأعلى، في حين بقيت *P. aeruginosa* مقاومة.

في اختبار DPPH، أظهر زيت Thyme قدرة أعلى على إزالة الجذور الحرة، بنسبة تثبيط بلغت 62.3% عند تركيز 4.0%، متفوقًا على ascorbic acid المستخدم كمرجع بتركيز 100 µg/mL والذي بلغت نسبة تثبيطه 38.9%. أما زيت Eucalyptus فبلغت فعاليته 40.1% عند التركيز ذاته.

أظهرت تجربة الكريم المحتوي على 1.0%w/v من زيوت Thyme و Eucalyptus قدرة فعالة على تثبيط نمو S. aureus, *S. epidermidis*, *As. Niger* في جميع الفترات الزمنية. أما *P. aeruginosa* و *E. coli*, *C. albicans* فقد اختفت تمامًا بعد اليوم السابع، وبحلول اليوم 28 لم تُسجل أي عودة للنمو، بما يتوافق مع معايير دستور الأدوية. كما أظهر الكريم المضاد للعدوى فعالية في الحفاظ على خلو العينات من الملوثات البكتيرية والفطرية لمدة 28 يومًا.

أظهرت العينات الضابطة الخالية من المواد الحافظة زيادة في نمو البكتيريا والفطريات، مما يؤكد فعالية خليط الزيوت العطرية كمُركّبات حافظة طبيعية. أظهرت تحاليل المواد الفعالة ثباتًا كيميائيًا مرتفعًا؛ إذ بقيت نسبة fusidic acidفي الكريم المضاد للبكتيريا بين 96.0% و101.1% من الكمية الابتدائية، في حين انخفضت بنسبة طفيفة من 101.1% إلى 99.8% بعد 180 يومًا. تراوحت نسبة mafenide acetate في الكريم المضاد للعدوى بين 93.4% و103%، وكان تركيز 1.5%w/w من الزيت الأكثر ثباتًا، بينما شهد تركيز 3.0%w/w تحللًا أكبر نسبيًا. أما قطرات فيتامين D3 الفموية فحافظت على قوة تتراوح بين 96.6% و101.1%، خصوصًا عند احتوائها على 2.0% من زيت .Eucalyptus أوThyme

كما بقيت قيم الـ pHضمن الحدود المثالية للثبات والاستخدام؛ إذ تراوحت بين 4.40–4.55 للكريم المضاد للبكتيريا، و6.50–6.62 للكريم المضاد للعدوى، و2.90–3.05 لقطرات فيتامينD3.

تُظهر النتائج أن زيوت Thyme و Eucalyptus تعمل بفعالية كمُركّبات حافظة طبيعية ذات نشاط قوي مضاد للبكتيريا، مع قدرة عالية على مقاومة الأكسدة، مما يُسهم في حماية المنتجات من التلوث الميكروبي،

والحفاظ على ثباتها الكيميائي وفعاليتها العلاجية. وتشير الدراسة إلى أن استخدام هذه الزيوت يُعد خيارًا بيئيًا آمنًا ومستدامًا يمكن أن يحل محل المواد الحافظة ومضادات الأكسدة الصناعية في المستحضرات الصيدلانية.

الكلمات المفتاحية: الثيمول (Thymol), اختبار التركيز المثبط MIC , اختبار الحدود الميكروبية MLT , اختبار DPPH,

(Cineole – 1,8) 1.8- السينول , زيت الزعتر , زيت الكينيا , المواد الحافظة , مضادات الأكسدة.

Appendix

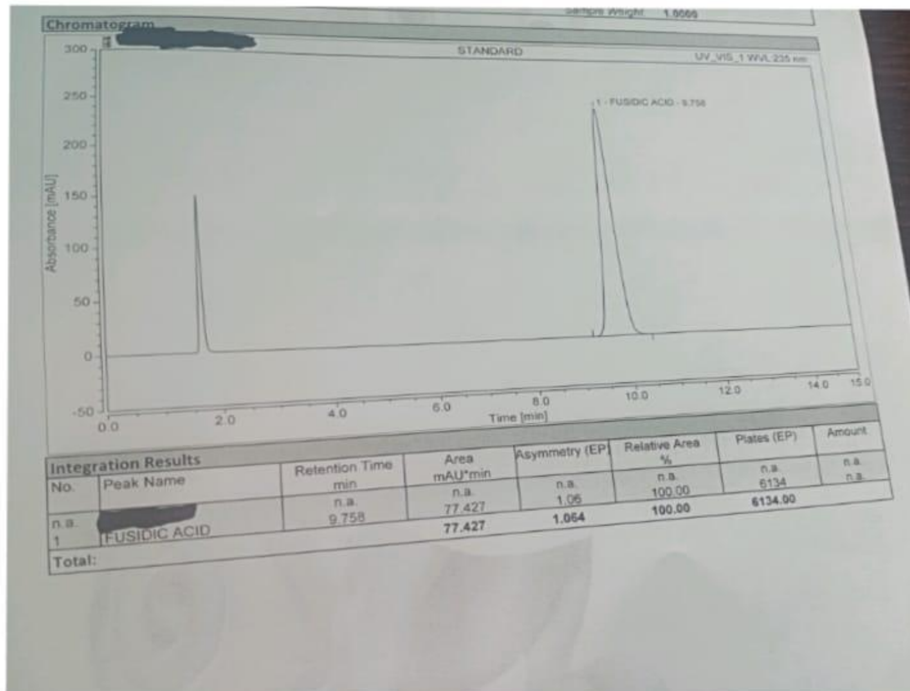
Chromatographic analysis using HPLC was performed on all test samples across the three pharmaceutical formulations to assess the identity and purity of the active pharmaceutical ingredients (APIs). Duplicate injections of standards and each sample ensured method reproducibility. Retention times for all sample peaks were consistent with the standards (within ±0.05 min), confirming the chemical identity of each API. No extraneous peaks indicative of degradation or interaction with preservatives were observed, demonstrating chemical stability across the 6-month period. The chromatographic data reinforced the findings of the quantitative assay, affirming that both natural preservatives and formulation matrices effectively maintained the integrity of the APIs under study.

The graphs below display the chromatograms from analyzing the active pharmaceutical ingredient (Fusidic Acid) in the antibacterial cream, including both standard and sample analyses.

1.Standard (two injections):

Integration Results

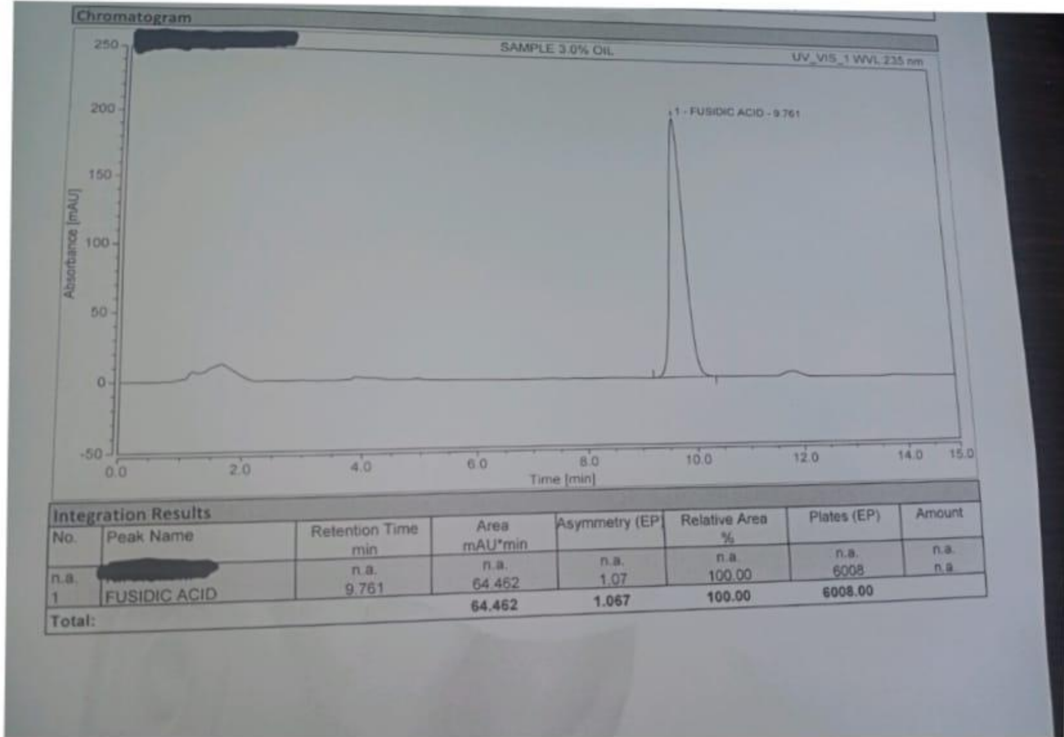
No.	Peak Name	Retention Time min	Area mAU*min	Asymmetry (EP)	Relative Area %	Plates (EP)	Amount
n.a.		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	FUSIDIC ACID	9.760	77.352	1.07	100.00	6117	n.a.
Total:			77.352	1.068	100.00	6117.00	



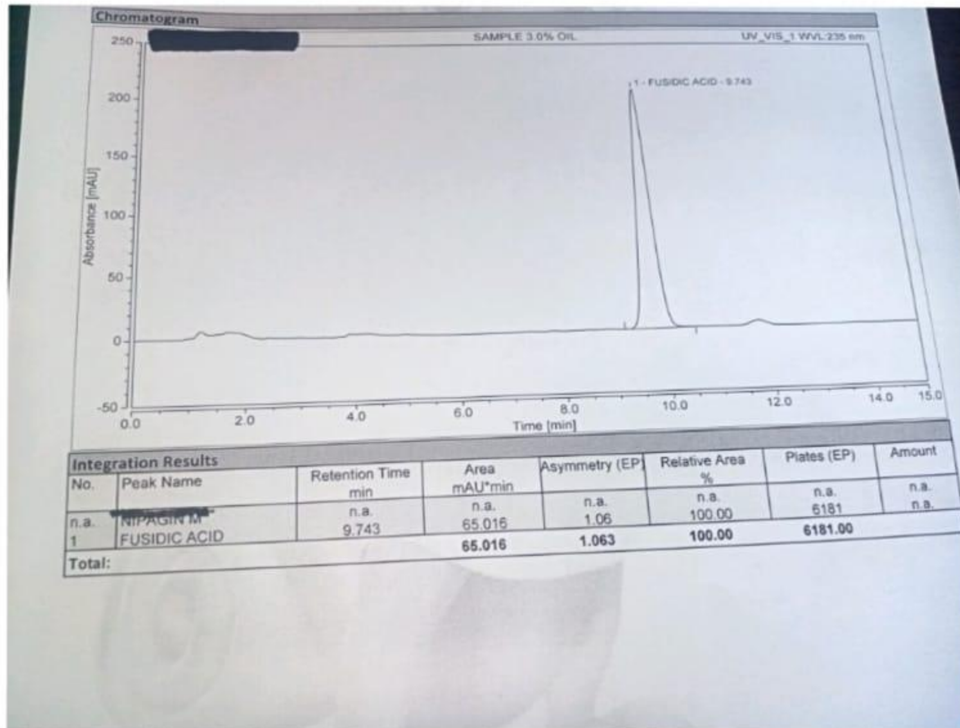
Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Asymmetry (EP)	Relative Area %	Plates (EP)	Amount
n.a.		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	FUSIDIC ACID	9.758	77.427	1.06	100.00	6134	n.a.
Total:			77.427	1.064	100.00	6134.00	

2. Sample 1 (3.0% w/v conc of eucalyptus oil) 2 injections:



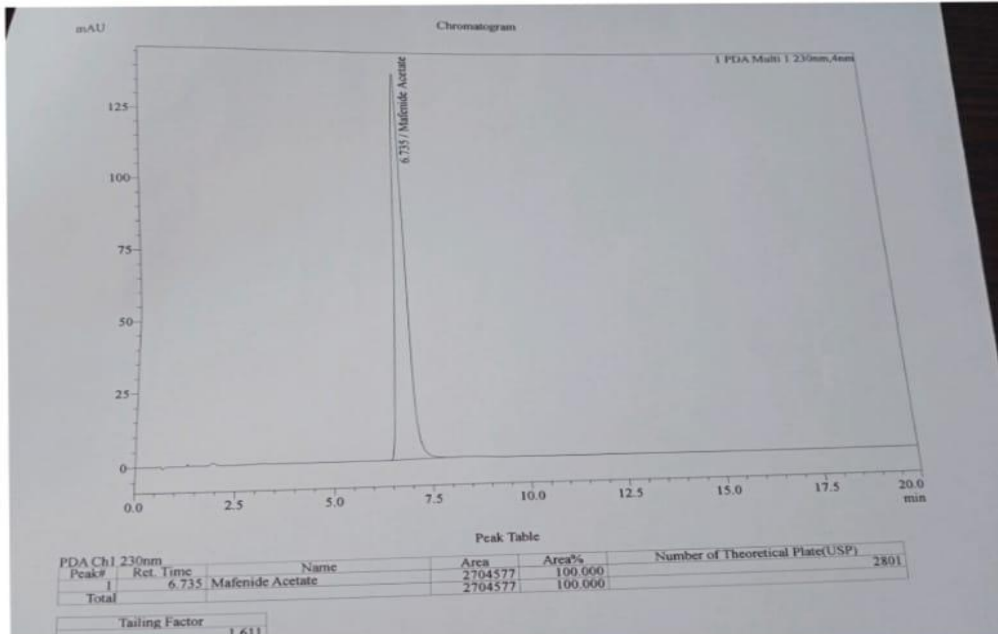
No.	Peak Name	Retention Time min	Area mAU*min	Asymmetry (EP)	Relative Area %	Plates (EP)	Amount
n.a.	[illegible]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	FUSIDIC ACID	9.761	64.462	1.07	100.00	6008	n.a.
Total:			64.462	1.067	100.00	6008.00	

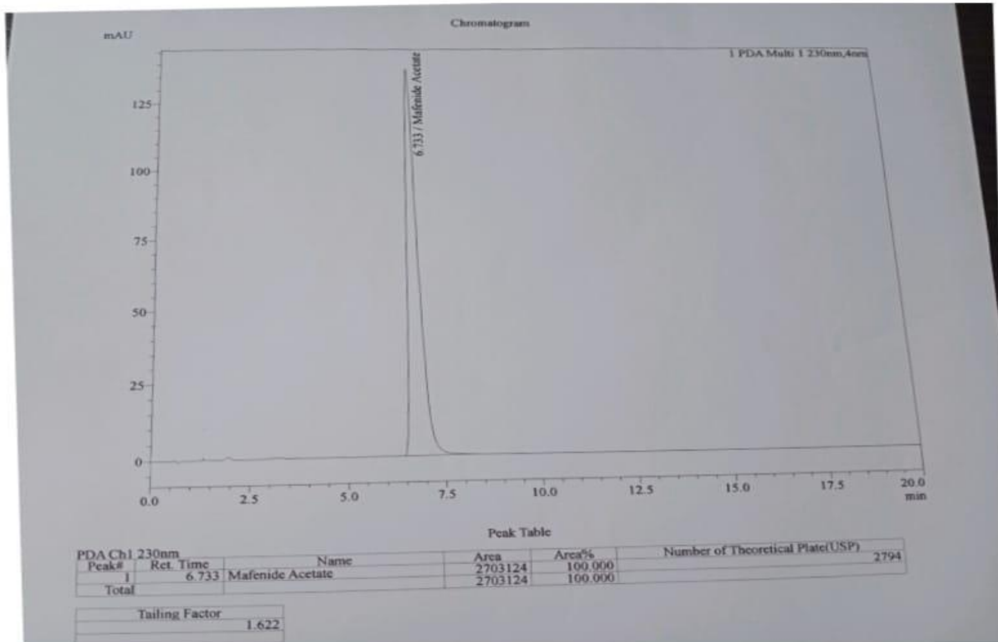


No.	Peak Name	Retention Time min	Area mAU*min	Asymmetry (EP)	Relative Area %	Plates (EP)	Amount
n.a.	NIPAGIN M	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
1	FUSIDIC ACID	9.743	65.016	1.06	100.00	6181	n.a.
Total:			65.016	1.063	100.00	6181.00	

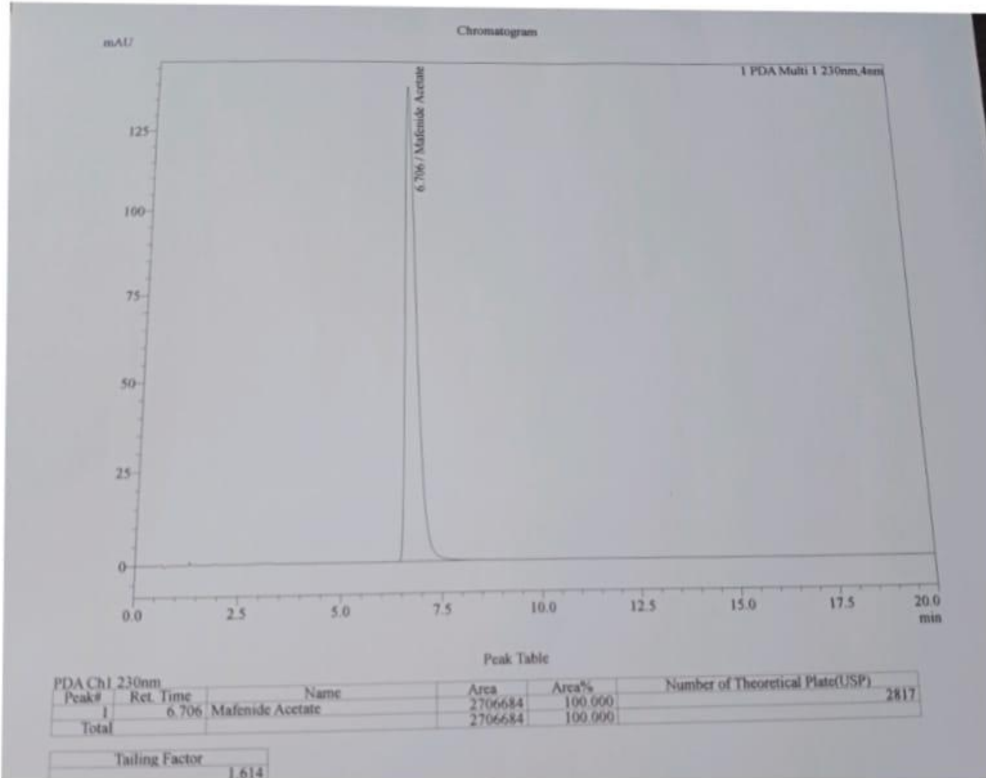
The graphs below present the chromatograms from the analysis of the active pharmaceutical ingredient (Mafenide Acetate) in the anti-infective cream, including both standard and sample analyses:

1. Standard (two injections):





2. Sample 1 (3.0% w/v conc of eucalyptus oil) 2 injections:

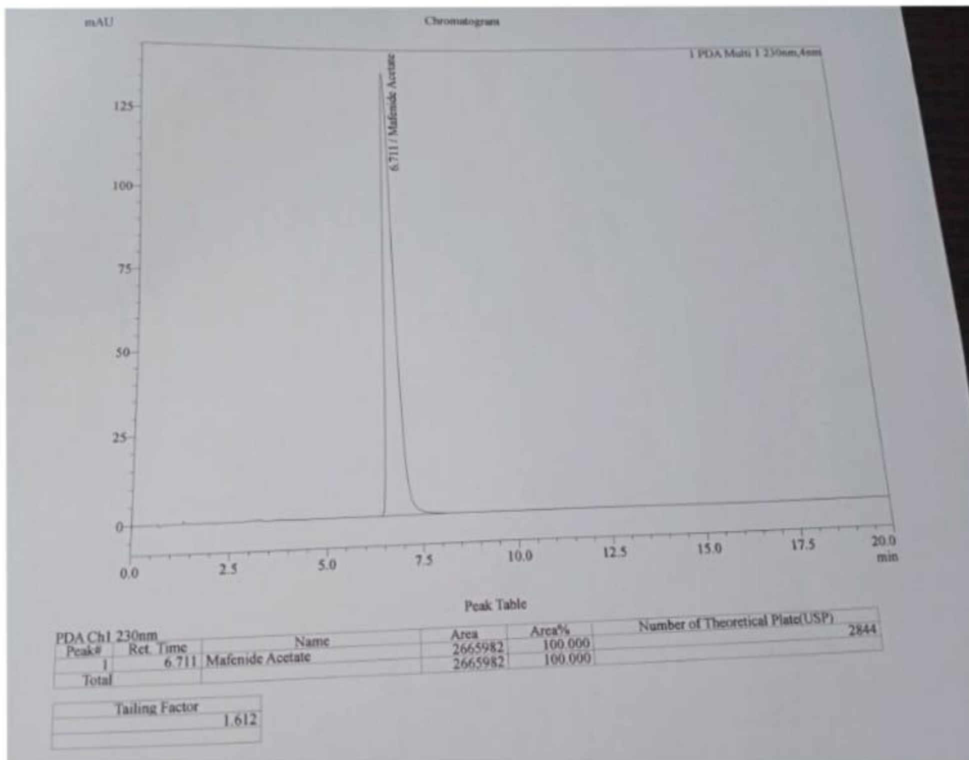


Peak Table

PDA Ch1 230nm

Peak#	Ret. Time	Name	Area	Area%	Number of Theoretical Plate(USP)
1	6.706	Mafenide Acetate	2706684	100.000	2817
Total			2706684	100.000	

Tailing Factor
1.614



Peak Table

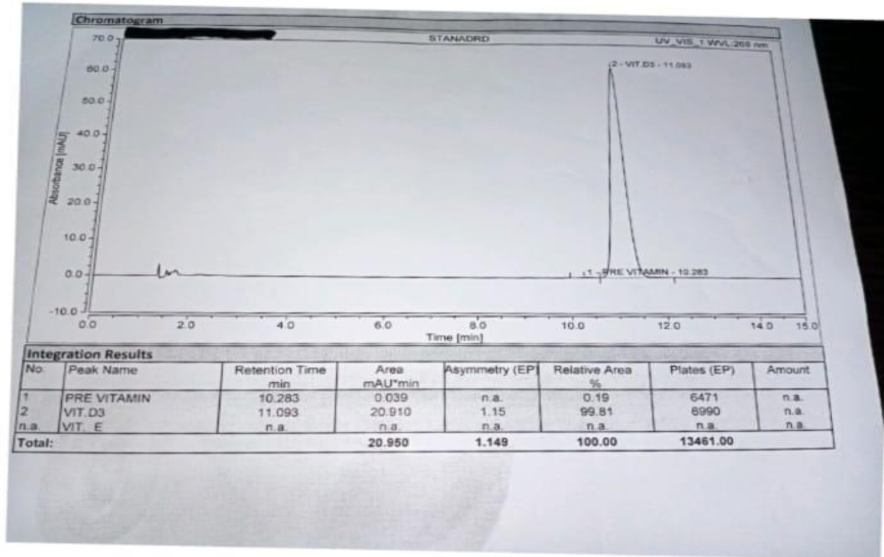
PDA Ch1 230nm

Peak#	Ret. Time	Name	Area	Area%	Number of Theoretical Plate(USP)
1	6.711	Mafenide Acetate	2665982	100.000	2844
Total			2665982	100.000	

Tailing Factor
1.612

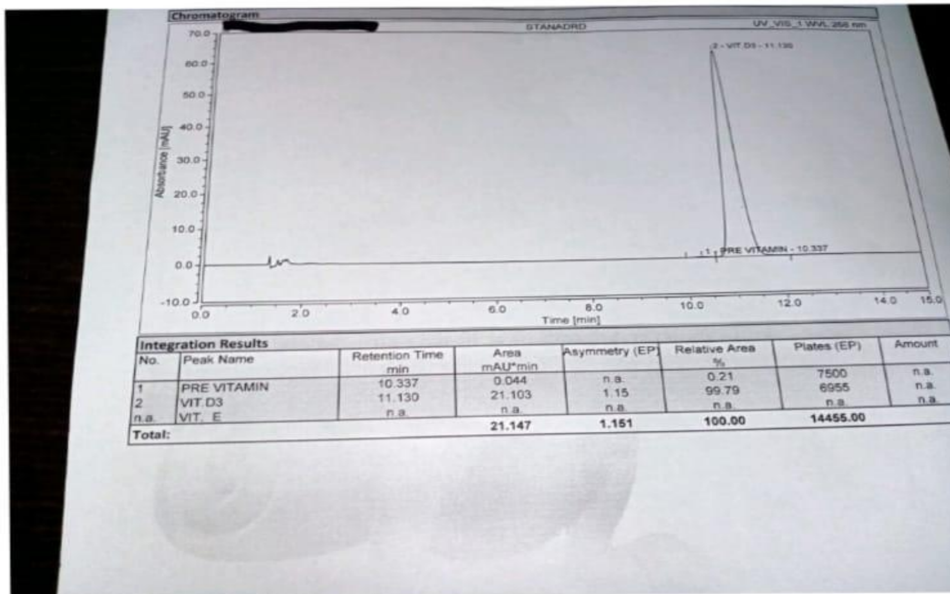
The graphs below display the chromatograms from the analysis of the active pharmaceutical ingredient (Vitamin D3 crystalline) in the vitamin D3 oral drops product, which includes analyses of both the standard and the sample.

1. Standard (2 injections):



Integration Results

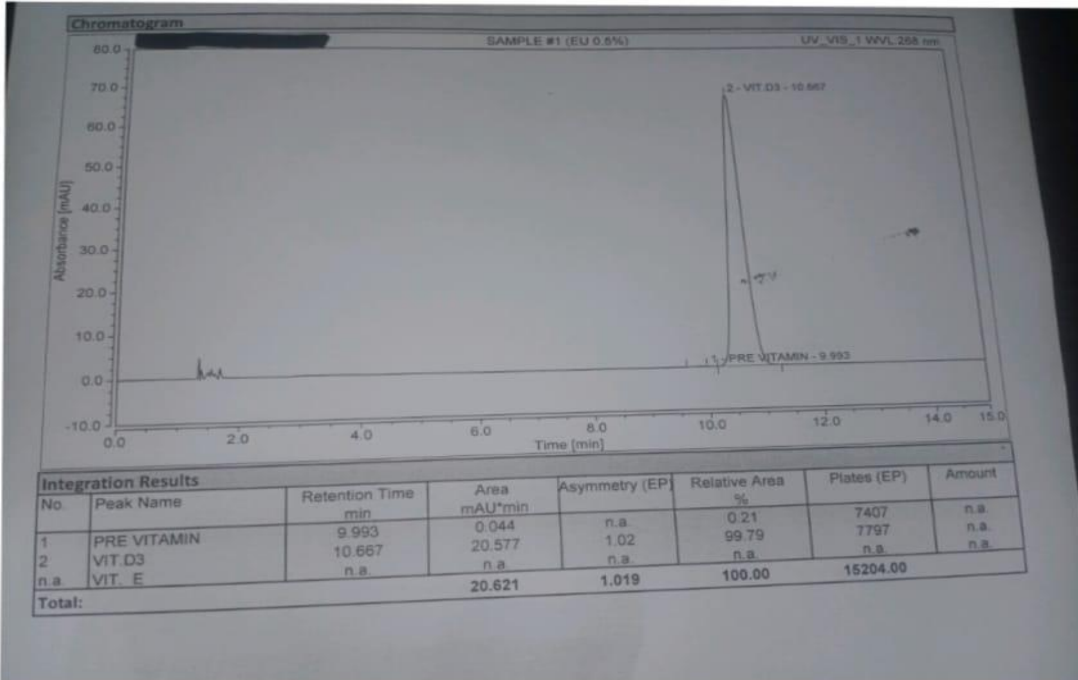
No.	Peak Name	Retention Time min	Area mAU*min	Asymmetry (EP)	Relative Area %	Plates (EP)	Amount
1	PRE VITAMIN	10.283	0.039	n.a.	0.19	6471	n.a.
2	VIT.D3	11.093	20.910	1.15	99.81	6990	n.a.
n.a.	VIT. E	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			20.950	1.149	100.00	13461.00	



Integration Results

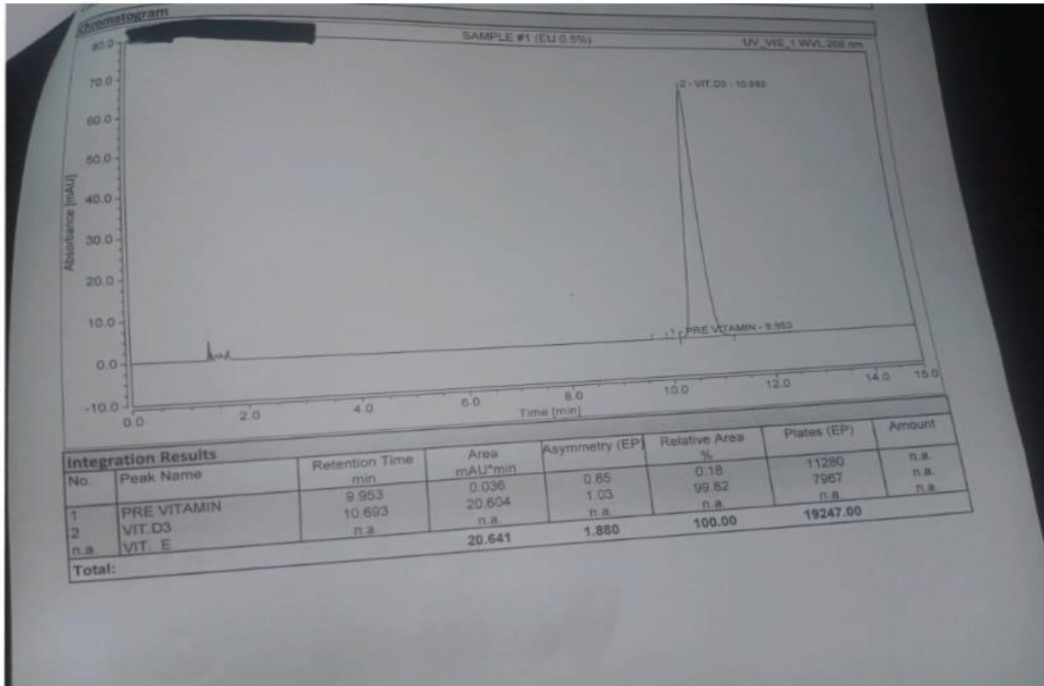
No.	Peak Name	Retention Time min	Area mAU*min	Asymmetry (EP)	Relative Area %	Plates (EP)	Amount
1	PRE VITAMIN	10.337	0.044	n.a.	0.21	7500	n.a.
2	VIT.D3	11.130	21.103	1.15	99.79	6955	n.a.
n.a.	VIT. E	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			21.147	1.151	100.00	14455.00	

2. sample 1 (0.5% w/v conc of eucalyptus oil) 2 injections:



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Asymmetry (EP)	Relative Area %	Plates (EP)	Amount
1	PRE VITAMIN	9.993	0.044	n.a.	0.21	7407	n.a.
2	VIT.D3	10.667	20.577	1.02	99.79	7797	n.a.
n.a.	VIT. E	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			20.621	1.019	100.00	15204.00	



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Asymmetry (EP)	Relative Area %	Plates (EP)	Amount
1	PRE VITAMIN	9.953	0.036	0.85	0.18	11280	n.a.
2	VIT.D3	10.693	20.604	1.03	99.82	7967	n.a.
n.a.	VIT. E	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Total:			20.641	1.880	100.00	19247.00	