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Application of bioactive compounds in food bio-preservation and infection protection

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***

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***

الملخص

يمثل تلوث المحاصيل الحمضية بالفطريات مشكلة كبيرة في الجزائر. الهدف الرئيسي من هذه الدراسة هو الحد من نمو الفطريات في ثمار البرتقال باستخدام المستحلبات النانوية المستخلصة من نباتات *Origanum majorana*، *Citrus limon*، *Cymbopogon citratus* و *Foeniculum vulgare*. تم استخراج الزيوت الأساسية من هذه النباتات بطريقة التقطير المائي، كما تم إنتاج مستحلباتها النانوية باستخدام الموجات فوق الصوتية. تم تحليل التركيب الكيميائي باستخدام جهاز الكروماتوغرافية الغازية والمطيافية الكتلية، ثم تم تقييم الخصائص الفيزيائية والكيميائية للمستحلبات النانوية. تم تقييم الفعالية المضادة للميكروبات مخبريا. اضافة الى ذلك، تم تقييم الإمكانات المضادة للفطريات للبردقوش والشمر حاسوبيا. أظهر تحليل الكروماتوغرافية الغازية والمطيافية الكتلية أن الاستحلاب النانوي يزيد من تراكيز التربينين-4-أول (terpinen-4-ol) و سيس بيتا تربينيول (cis-β-terpineol) في البردقوش، وكذلك الإستراجول (estragole) والأنيثول (anethole) في الشمر، بالإضافة الى النيرال (neral) والجرانيال (geranial) في كل من زيت الليمون وزيت عشبة الليمون، مع انخفاض مستويات الفا تربينين (α-Terpinene)، الفينكون (fenchone)، الليمونين (limonene)، قاماتربينين (γ-terpinene)، وبيتا ميرسين (β-myrcene) .أظهرت صور المجهر الا لكتروني النافذ للمستحلبات النانوية مورفولوجية كروية بأبعاد نانوية متنوعة. أظهر التحليل الجزيئي والتحليل التطوري العرقي للفطريات المعزولة من ثمار البرتقال تطابقًا بنسبة 100% مع *P. expansum* و *P. digitatum*. أظهرت الزيوت الأساسية فعالية أكبر مقارنةً مع مستحلباتها النانوية، حيث تراوحت قيم الحد الأدنى من التركيز المثبط (MIC) بين 0.12% و2% حجم/ حجم ضد البكتيريا ومن 0.03% إلى1 % حجم/ حجم ضد الفطريات. علاوة على ذلك، تم تثبيط الغالبية العظمى من الفطريات المختبرة تمامًا بواسطة عشبة الليمون، من ناحية أخرى، أظهرت *F.vulgare* أضعف نشاطية مضادة للميكروبات. تمت ملاحظة قيم قوية للطاقة الحرة للتفاعل في التفاعلات بين الزيوت الأساسية أو المستحلبات النانوية وإنزيمات الفطريات، مع درجات ربط ملحوظة (-6.6 إلى-7.0 كيلوكالوري/مول) للمكونات العطرية. تطبيق المستحلبات النانوية للبردقوش، الليمون وعشبة الليمون كغلاف على ثمار البرتقال منع بشكل كبير نمو فطريات *P. expansum* و *P. digitatum* مقارنة بالعينات غير المغلفة. استخدام المستحلبات النانوية يقلل من التغيرات السلبية في معايير جودة ثمار البرتقال طوال فترة التخزين، بما في ذلك الحموضة القابلة للمعايرة (TA)، الرقم الهيدروجيني(pH) ، المواد الصلبة الذائبة الكلية (TSS)، تركيز حمض الأسكوربيك، فقدان الوزن والصلابة. لم تؤثر المستحلبات النانوية ل*O.majorana* و*C. limon* على الخصائص الحسية للفواكه المغلفة مقارنةً بالمستحلب النانوي ل*C. citratus*.بشكل عام، أظهرت المستحلبات النانوية التي تم اختبارها فعاليتها العالية في إدارة مسببات الأمراض التي تؤثر على ثمار البرتقال.

الكلمات المفتاحية: *Origanum majorana*، *Cymbopogon citratus*، *Citrus limon*، *Penicillium digitatum*، *Penicillium expansum*، الزيوت الاساسية، المستحلبات النانوية، برتقال تومسون.

Abstract

Fungal contamination of citrus crops represents a significant issue in Algeria. The primary aim of this study is to limit fungal growth on orange fruits using the nanoemulsions obtained from plants of *Origanum majorana, Citrus limon, Cymbopogon citratus and Foeniculum vulgare.* The essential oils from these plants were extracted using hydrodistillation method, and their nanoemulsions were produced using ultrasonication method. The chemical composition was analyzed using a GC-MS system, and the physicochemical properties of the nanoemulsions were assessed. The antimicrobial efficacy was evaluated *in vitro*. Furthermore, the antifungal potential of marjoram and fennel was assessed *in silico*. The GC-MS analysis demonstrated that the nanoemulsification increases the concentrations of terpinen-4-ol and cis-β-terpineol in marjoram, as well as estragole and anethole in fennel, in addition to neral and geranial in both lemon and lemongrass oils, while reducing the levels of α-terpinene, fenchone, limonene, γ-terpinene, and β-myrcene. The nanoparticles exhibited a narrow particle size distribution, indicating high stability and monodispersity of the nanoemulsions. The TEM imaging of the nanoemulsions showed a spherical morphology with diverse nanometer-scale dimensions. The molecular and the phylogenetic analysis of the isolated fungi from orange fruits showed 100% homology to *P. expansum* and *P. digitatum*. the essential oils exhibited greater potency compared to their nanoemulsions, with MIC values spanning from 2% to 0.12% against bacteria and from 1% to 0.03% against fungus. Furthermore, the majority of the tested fungi were completely inhibited by lemongrass. On the other hand, *F. vulgare* showed the weakest antimicrobial activity. Strong binding-free energy values were observed in the interactions between essential oil or nanoemulsions and fungal enzymes, with notable docking scores (−6.6 to −7.0 kcal/mol) for aromatic components. The application of nanoemulsified marjoram, lemon and lemongrass as a coating on orange fruits significantly inhibit the growth of *P. expansum* and *P. digitatum* molds compared to uncoated samples. The use of nanoemulsions minimize negative changes in quality parameters of orange fruits throughout storage, encompassing titratable acidity (TA), pH, total soluble solids (TSS), ascorbic acid concentration, weight loss and firmness. The nanoemulsified *O. majorana* and *C. limon* did not influence the sensory attributes of the coated fruits as compared to the *C. citratus* nanoemulsion. Overall, the tested nanoemulsions demonstrated their high efficacy in managing pathogens affecting orange fruits.

Key words: *Origanum majorana*, *Foeniculum vulgare*, *Cymbopogon citratus*, *Citrus limon*, *Penicillium digitatum*, *Penicillium expansum*, nanoemulsion, Thomson orange.

Résumé

La contamination fongique des cultures d'agrumes représente un problème important en Algérie. L'objectif principal de cette étude est de limiter la croissance fongique sur les fruits d'orange en utilisant les nanoemulsions obtenues à partir de plantes d'*Origanum majorana*, *Citrus limon, Cymbopogon citratus* et *Foeniculum vulgare*. Les huiles essentielles de ces plantes ont été extraites par hydrodistillation et leurs nanoémulsions ont été obtenues par ultrasonication. La composition chimique a été analysée à l'aide d'un appareil GC-MS, puis les propriétés physico-chimiques des nanoémulsions ont été évaluées. L'efficacité antimicrobienne a été évaluée *in vitro*. De plus, le potentiel antifongique de la marjolaine et du fenouil a été évalué *in silico*. L'analyse GC-MS a démontré que la nanoémulsification augmente les concentrations de terpinen-4-ol et de cis-β-terpinéol dans la marjolaine, ainsi que d'estragole et d'anéthole dans le fenouil, en plus de néral et de géranial dans les huiles de citron et de citronnelle, tout en réduisant les niveaux de α-terpinène, de fenchone, de limonène, de γ-terpinène et de β-myrcène. Les nanoparticules ont montré une distribution étroite des tailles de particules, indiquant une grande stabilité et une monodispersité des nanoémulsions. L'imagerie MET des nanoémulsions a montré une morphologie sphérique avec des dimensions variées à l'échelle nanométrique. L'analyse moléculaire et phylogenetique des moisissures isolées des fruits d'orange a montré une homologie de 100 % avec *P. expansum* et *P. digitatum*. Les huiles essentielles ont montré une plus grande efficacité par rapport à leurs nanoémulsions, avec des valeurs de CMI allant de 2 % à 0,12 % contre les bactéries et de 1 % à 0,03 % contre les moisissures. De plus, la majorité des moisissures testées ont été complètement inhibés par la citronelle. D'autre part, *F. vulgare* a montré la plus faible activité antimicrobienne. Des valeurs élevées d'énergie libre de liaison ont été observées dans les interactions entre les huiles essentielles ou les nanoémulsions et les enzymes fongiques, avec des scores de docking notables (−6,6 à −7,0 kcal/mol) pour les composants aromatiques. L'application de nanoémulsions de la marjolaine, le citron et la citronelle comme revêtement sur les fruits d'orange inhibe significativement la croissance des moisissures *P. expansum* et *P. digitatum* par rapport aux échantillons non revêtus. L'utilisation de nanoémulsions minimise les changements négatifs dans les paramètres de qualité des fruits d'orange tout au long du stockage, englobant l'acidité titrable (AT), le pH, les solides solubles totaux (SST), la concentration en acide ascorbique, la perte de poids et la fermeté. Les nanoémulsions d'*O.majorana* et de *C. limon* n'ont pas influencé les attributs sensoriels des fruits enrobés par rapport à la nanoémulsion de *C. citratus*. Dans l'ensemble, les nanoémulsions testées ont démontré leur haute efficacité dans le control des pathogènes affectant les fruits d'orange.

Mots-clés : *Origanum majorana, Foeniculum vulgare, Cymbopogon citratus, Citrus limon, Penicillium digitatum, Penicillium expansum,* nanoémulsion, orange Thomson.

List of Abbreviations

- ***A. flavus*:** *Aspergillus flavus*
- ***A. parasiticus* :** *Aspergillus parasiticus*
- **amu:** atomic mass unit
- **bp:** base pair
- **Ben A:** beta-tubuline.
- ***C. citratus* DC :** *Cymbopogon citratus (*De Candolle)
- ***C.limon* (L) :** *Citrus limon* (Linnaeus)
- **CCEO :** *Cymbopogon citratus* essential oil
- ***Citrus sinensis* L. :** *Citrus sinensis* Linnaeus
- **CLEO:** *Citrus limon* essential oil
- **CMC:** Carboxy Methyl Cellulose
- **DLS:** Dynamic Light Scatering
- **DON:** Deoxynivalenol
- **EF1:** elongation factor 1
- ***F. vulgare*:** *Foeniculum vulgare*
- **FAO:** Food and Agriculture Organization
- **FVEO:** *Foeniculum vulgare* essential oil.
- **GC-MSD:** Gas Chromatography-Mass selective Detector
- **HD:** Hydrodistillation
- **HP-5 MS:** Phenyl, Methylpolysiloxane, Mass spectrometry
- **ITS:** Internal Transcribed Spacer
- **LGEO:** Lemongrass essential oil
- **N:** Newton
- **NE:** Nanoemulsion.
- **NECC:** Nanoemulsified *Cymbopogon citratus*
- **NECL:** Nanoemulsified *Citrus limon*
- **NEFV:** Nanoemulsified *Foeniculum vulgare*.
- **NEOM:** Nanoemulsified *Origanum majorana*.
- **NFT75-006:** Frensh standard for the preparation of calibration gas mixture
- **NIST:** National Institute of Standards and Technology
- **OMEO:** *Origanum majorana* essential oil.
- **OTA:** Ochratoxin A

- ***P. expansum*:** *Penicillium expansum*
- **TEF1:** Transcription Elongation Factor 1
- **TEM:** Transmission Electron Microscopy
- **ZEA:** Zearalenone

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Introduction

Introduction

Citrus fruits are among the most economically significant fruit crops worldwide. According to AgriAlgerie (2024), the total production of citrus crops in Algeria is approximately 18 million quintals during the 2024/2025 season, with Thomson orange being the main variety. Regarding the foremost production of orange fruits, Blida state was the first in rank (Boudedja et al., 2024). Thomson orange (*Citrus sinensis* (L.) is rich in vitamin C, sugar, organic acids, which create a suitable environment for pathogens (Karmakar & De, 2019). Citrus fruits in Algeria face considerable post-harvest losses, primarily due to fungal infections by *Penicillium digitatum* and *Penicillium italicum*, commonly referred to green and blue molds respectively, these pathogens are responsible for the deterioration of the fruit quality which significantly affect the fruit industry and lead to enormous economic losses and a considerable health negative effects (Rafiee & Ramezanian, 2022). Usually, chemical fungicides such as imazalil, sulfite, sodium bicarbonate, thiabendazole, fludioxonil, pyrimethanil, and azoxystrobin are used as preservative agents against deteriorating fungi in fruits (Zhang et al., 2009). However, the overuse of the aforementioned synthetic fungicides for the preservation of fruits leads to the development of fungal resistance; moreover, several diseases and health side effects were found related to the consumption of crops treated with these chemicals (Papoutsis et al., 2019). In addition, a research investigation conducted by Mebdoua et al. (2017) on Algerian fruits revealed that 57.5% of samples had at least one pesticide residue, underscoring the prevalent application of chemical treatments in agricultural production. Despite the efficacy of these chemicals, their use raises concerns about environmental consequences and potential health problems ; for example, sulfites, commonly used as additives in fruits, can induce allergic reactions, asthma, and possibly cancer (Farid et al., 2023). This circumstance requires urgently new alternatives to replace chemical preserving agents. Aromatic plants with their high content in bioactive compounds represent an attracting option (Maghenzani et al., 2018). The Essential oils are secondary metabolites of aromatic plants, biodegradable, non-toxic substances, and environmentally sustainable, with a wide range of biological activities (Cháfer et al., 2012).

Terpenes and phenolics are the main bioactive compounds in essential oils, known for their remarkable antimicrobial activity (Ricardo-Rodrigues et al., 2024). Moreover, essential oils consist of chemicals that may exhibit synergistic or antagonistic effects, consequently, it is challenging microorganisms to develop resistance to their actions (Abimbola & Bolatito, 2024).

Various studies have demonstrated the antimicrobial activities of essential oils and their compounds. *Citrus limon* essential oil possesses a significant antibacterial and antifungal properties against various tested strains (Anastasopoulou et al. 2024; Kačániová et al. 2024). Research by Najmi et al. (2023) indicated that cinnamon and clove essential oils exhibited effective antifungal activity, *T. vulgaris* showed very strong activity against *S. aureus* in the study by Aouf et al. (2022). Aminifard & Bayat (2017) evaluated the activity of black caraway and anise essential oils against green mold in blood orange fruit.

Many studies have proven the potent antimicrobial activity of *Origanum majorana*, the EO of this plant is rich in monoterpens such (sabinene hydrate and terpinen-4-ol) which are responsible for its potent antimicrobial activity (Sellami et al., 2009). Wild fennel essential oil has been documentad as strong inhibitor against *Aspergillus* and *C. albicans* (Belabdelli et al., 2020). *Citrus limon* and *Cymbopogon citratus* essential oils exhibit significant antifungal action, especially lemongrass. Numerous studies, for instance, demonstrated that lemongrass volatile oil exhibits considerable antimicrobial, and anti-aflatoxinogenic activities (Boudechicha et al., 2023). Kgang et al. (2022) established the *in vitro* combination of lemon and lemongrass oils negative impact on the growth *P. expansum*.

Nonetheless, the application of essential oils is limited due to their sensibility to light, oxygen, high temperature and low solubility in aqueous solvents, furthermore, their potent flavor and aroma may induce unfavorable organoleptic alterations in food (Reis et al., 2022).

Recent innovative technologies are being used to overcome the drawbacks associated with essential oils and to improve their bioavailability in fruits biopreservation. Nano-formulation as nanoemulsions has garnered significant attention in recent years owing to their functional and physicochemical features (de Oliveira et al., 2023). Low and high-energy methods are the most methods investigated in the preparations of nanoemulsions, for instance, ultrasonication is a high-energy method, widely applicated and known for its efficacy in the production of efficient nanoemulsions (Dhankhar et al., 2021).

Radi et al. (2018) showed that pectin coatings containing orange peel essential oil nanoemulsion effectively diminished the rate of microbial proliferation. Pullulan associated with nanoemulsified cinnamon essential oil coating of strawberries demonstrated the most substantial inhibition against molds at the end of the storage (Chu et al., 2020). Additionally, Dhanasekaran et al. (2024) indicated that essential oils encapsulated in polymer matrices effectively combat *Aspergillus carbonarius*, thereby prolonging the shelf life of grapes.

Coatings of cassava starch incorporated with clove and cinnamon EOs inhibited molds and yeasts in papaya fruits (do Nascimento et al., 2023).

Despite the demonstrated improvement of antifungal efficacy and stability in food systems by nanoemulsions of lemon, lemongrass, and marjoram essential oils (Abdallah et al., 2023 ; Boukhatem et al., 2024 ; Sellami et al., 2009), significant research gaps remain, none of the studies has performed a standardized, comparable application of these three nanoemulsions on Thomson orange fruits, specifically, marjoram has not been employed on oranges, despite its potential antifungal efficacy. Prior studies rarely investigate the optimal application strategy (immersion, coating, or spray) in Algerian post-harvest conditions, Furthermore, there is insufficient data in Algeria concerning the management of fungus and mycotoxins in citrus fruits, population exposure, and related health risks, which hinders prevention efforts. Moreover, deficiencies in effective pre- and post-harvest measures, insufficient citrus fruits handling, and a lack of awareness or knowledge on mycotoxins further increse these problems (Belasli et al., 2023)

Algeria is known by its wide diversity in medicinal plants, which is mainly due to different climates, soils, and altitudes. Numerous of these plants and their wastes are reservoirs of interesting bioactive compounds, unfortunately remain undervalued or unexploited (Sahraoui et al., 2007).

The primary aim of this study is the application of the nanoemulsions of lemon, lemongrass, and marjoram on Thomson orange fruits as natural post-harvest solutions to reduce agricultural and economic losses, considering the significant citrus production in Algeria. A further objective is highlighting the value of Algerian medicinal and aromatic plants by investigating their practical application in post-harvest preservation, therefore encouraging sustainable use of local plant biodiversity and eco-friendly practices within the citrus industry.

This study opens future perspectives to apply these nanoemulsions in edible coating films through the preliminary experiments performed on Thomson Navel oranges (*C. sinensis* (L.) Osbeck).

Literature review

Literature review

I. Fruits contamination

1. Introduction

Algeria is well known for its variety in terms of soil and temperature; particular areas with high humidity help molds to proliferate and mycotoxin production in fruits is facilitated (Belasli et al., 2023). The invasion of molds and their mycotoxins threatens food economy; so, the most importing nations enacted strict regulations addressing this risk (Moretti et al., 2017).

According to the Food and Agriculture Organization and The World Health Organization FAO & WHO, (2023) using unsuitable contaminated water for the irrigation of fruits and vegetables poses a significant risk for humans, animals, and the environment.

Apart from their impacts on consumer's health, the broad spread of toxigenic molds on fruits has resulted in significant economic losses, therefore influencing their import and export, commonly found in fruits and fruit-derived products (Figure 1), two particularly lethal mycotoxins are patulin (PAT) and ochratoxin A (OTA) (Pushparaj et al., 2023). Generally, the pH level rises through the matuasion process, and the outer layers of the fruit become softer, which reduces the defensive mechanisms later, rendering the fruits more prone to fungus contamination, moreover, fruits are high in carbohydrates, minerals, vitamins, and proteins, thereby providing essential resources to both consumers and pathogenic microbes which create the ideal habitat for the spread and survival of different saprophytic fungus in different stages (Zhao et al., 2022).

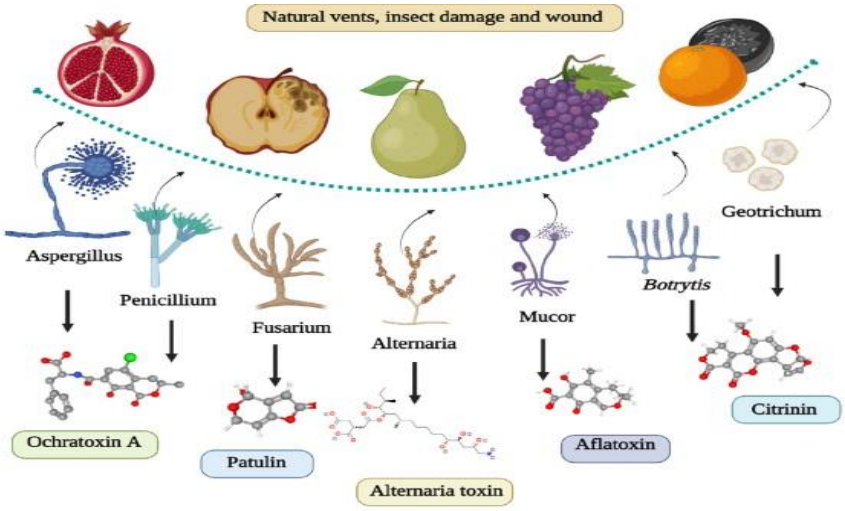


Figure 1. Usual fungi and mycotoxins found in frruits (Pushparaj et al., 2023)

1. Spoilage and quality degradation of citrus fruits

Citrus industry in Algeria faces several challenges, including water limit, pests and diseases, in addition to insufficient investment in post-harvest infrastructure, particularly cold-storage units, since the local production is destinated to fresh consumption, considerable quantities of citrus waste are generated (Kazi-Tani, 2024).

Citrus fruits are susceptible to contamination at any stage of the production chain. Contamination sources are classified into two primary categories : pre-harvest factors and post-harvest factors (Balali et al., 2020).

1.1. Physical disorders in citrus crops

In the postharvest period, citrus fruits encounter multiple abiotic stresses that may lead to significant physiological, biochemical, and molecular changes as part of their adaptive responses to unfavorable environments (Zacarias et al., 2020).

Improper storage temperatures and inappropriate handling procedures can significantly affect fruit quality, resulting in various exterior defects, particularly stem-end rind breakdown, peel pitting, peel injuries and mechanical abrasion are significant factors in postharvest spoiling, as they promote pathogen proliferation, enhance respiration, ethylene synthesis, and cause considerable water loss, resulting in fruit wilting (Strano et al., 2022).

1.2. Microbial contamination of citrus crops

Over 20 different postharvest diseases have been recognized in citrus, being the principal cause of fruit deterioration and leading to significant economic losses (Moraes Bazioli et al., 2019).

Citrus diseases are varied, caused by phytopathogenic agents such as fungi, bacteria, and viruses. Some pathogens result in severe infections that lead to loss of fruit, while others produce fewer symptoms (Berraf-Tebbal et al., 2020).

A. Bacterial contamination

Multiples kind of bacteria are responsible for citrus contamination, with the most often identified pathogens being *Candidatus liberibacter* and *Xanthomonas citri*, the latter serving as a primary causative agent of citrus canker and a significant pathogen of orange fruits (Mendonça et al., 2017).

Additional bacteria identified on citrus surfaces include those from the genera *Escherichia*, *Staphylococcus*, and *Salmonella*, however, their appearance on orange fruits is typically linked to substandard hygiene procedures among sellers (Amadi-Ikpa et al., 2023).

These pathogenic bacteria can pose harm to human health only if the proliferation is important within the consumed food product (Gallo et al., 2020).

B. Fungal contamination

Mycotoxigenic fungi are a much-diversified group and can grow within the food substrate under different situations; their prevalence is usually connected to insufficient food distribution (Moretti et al., 2017). While removing fungi from fruits does not guarantee the absence of mycotoxin contamination due of their microscopic character and resistance, the presence of fungus does not always imply the occurrence of mycotoxin contamination (Benedict et al., 2016).

The most common postharvest fungi impacting citrus fruits are from the genus *Penicillium*, specifically *Penicillium digitatum* and *Penicillium italicum*, which are responsible for green and blue mold, respectively (Figure 2), additional species comprise *P. fellutanum*, *P. expansum*, *P. crustosum*, and *P. ulaiense* (Coutinho et al., 2020). Other significant pathogens include those responsible for Citrus Black Spot (CBS), particularly *Guignardia citricarpa* (Kassim et al., 2020), *Diplodia natalensis*, *Alternaria citri*, *Dothiorella gregaria*, *Phytophthora* spp., along with *Geotrichum candidum*, *Colletotrichum gloeosporioides*, *Trichoderma viride*, and *Sclerotinia* spp.

Only a limited number of mycotoxins, for example those present in cereals, are likely to have health effects on humans, Algeria is emphasizing aflatoxins and undervaluation of other

imopratnt mycotoxins such OTA, DON, or ZEA; therefore, these mycotoxins need to be taken under account (Belasli et al., 2023).

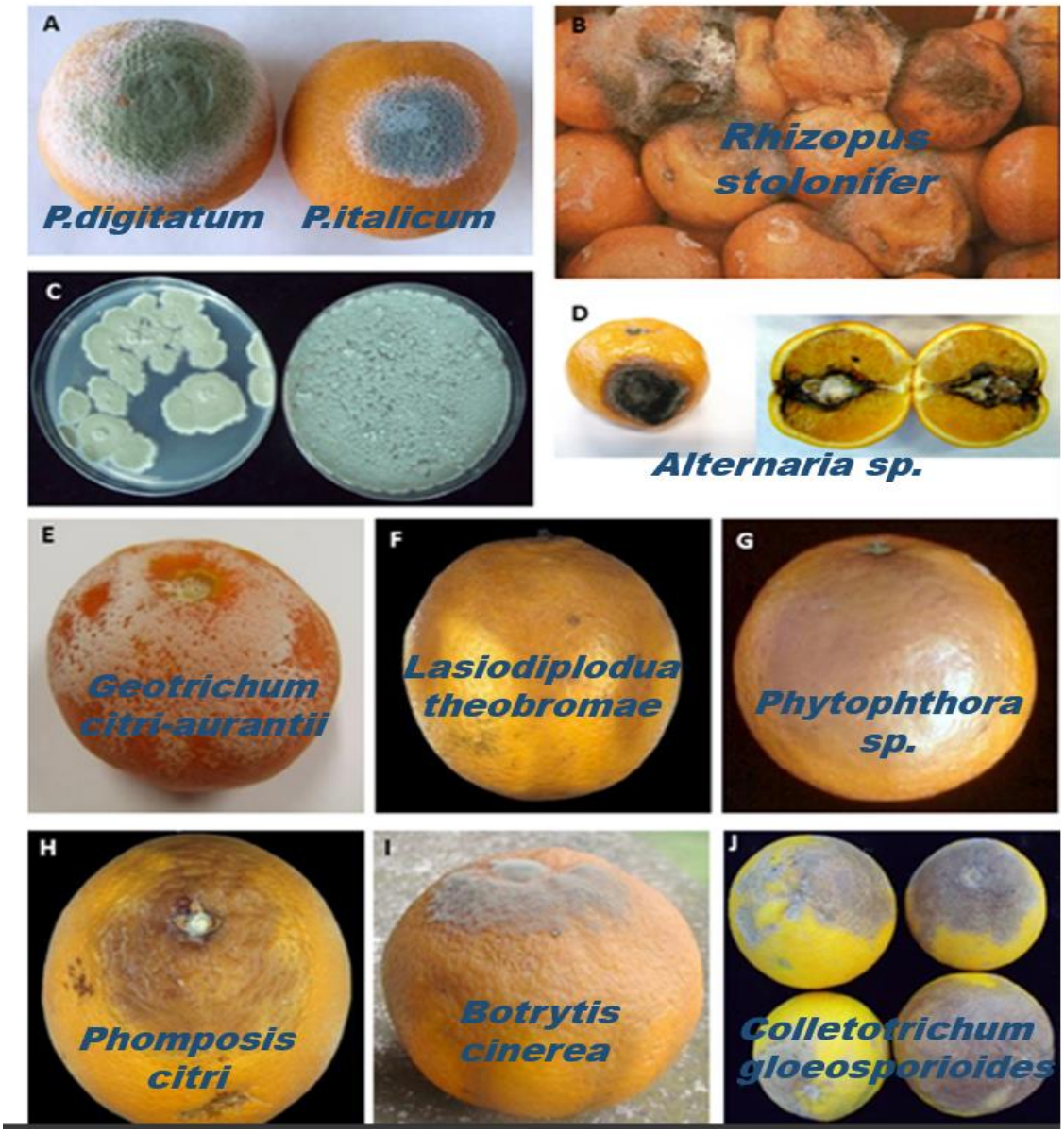


Figure 2. Some of the main pathogenic microoganisms of citrus fruits (Zacarias et al., 2020).

1.3. Chemical contaminants

Every stage of the food production process (Figure 3) is susceptible to chemical contamination. Substances thet can contaminate food include:

- Veterinary pharmaceuticals.
- Pesticides
- Cleaning agents and heavy metals (cadmium, tin, and mercury).

- Naturally occurring chemical toxins such shellfish, mycotoxins, and phytochemicals (Bartoletti & Theobald, 2011).

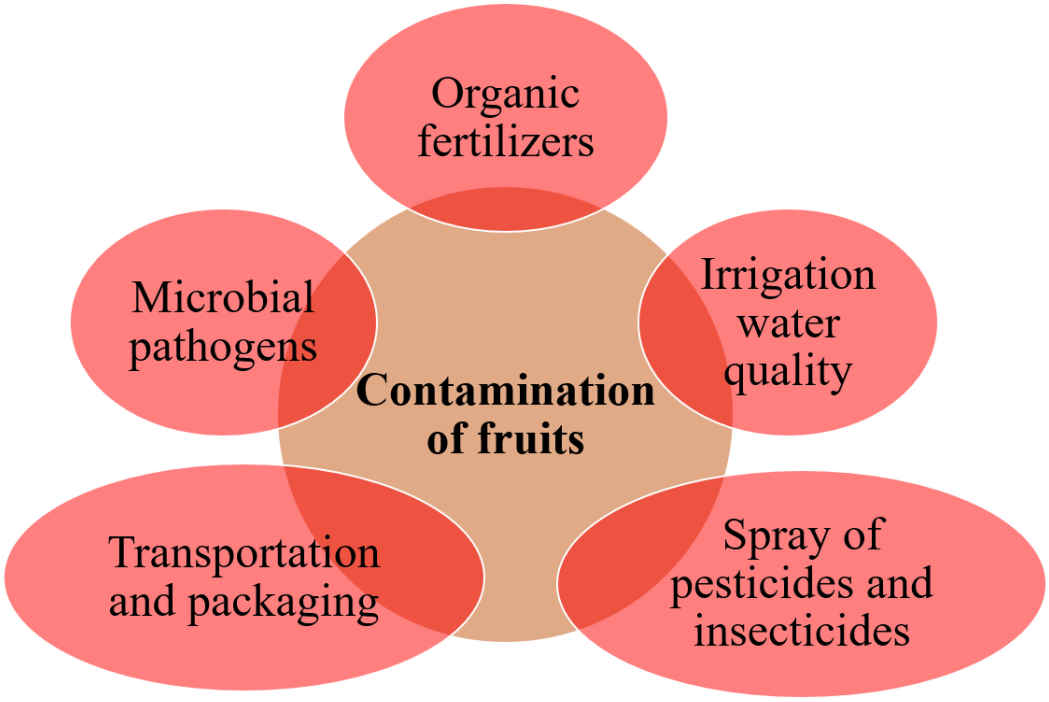


Figure 3. A number of factors influencing the contamination of citrus fruits (Balali et al., 2020).

II. Fruit Preservation and Biopreservation

1. Introduction

Unlike other food categories, fresh fruits are highly susceptible to deterioration due to their high moisture content, active metabolism, and sensitivity to environmental conditions, consequently, a critical assessment of both conventional and emerging preservation

technologies is essential to better understand their effects on product quality, sensory attributes, and overall consumer acceptability (Alquraishi et al., 2025).

These preservation strategies are designed to maintain the nutritional value, taste, texture, and color of fresh fruits, while limiting spoilage induced by microorganisms, enzymatic activity, and external environmental factors, preservation methods can be classified into three main groups: physical, chemical, and biological, physical methods include refrigeration, freezing, and drying, as well as heat treatments such as canning (Maurya et al., 2024).

A wide range of approaches is currently employed for the preservation of fruits, which can be classified into two main categories : synthetic and natural preservatives (Figure 4) (Sharif et al., 2017).

Numerous synthetic preservatives and additives, such as formaldehyde, sorbates, sulfites, and nitrates, are widely used in the food industry, however, these compounds are generally regarded as safe when applied within regulatory limits, their excessive or improper use has been associated with potential adverse effects on human health and the environment (Dwivedi et al., 2017 ; Farid et al., 2023).

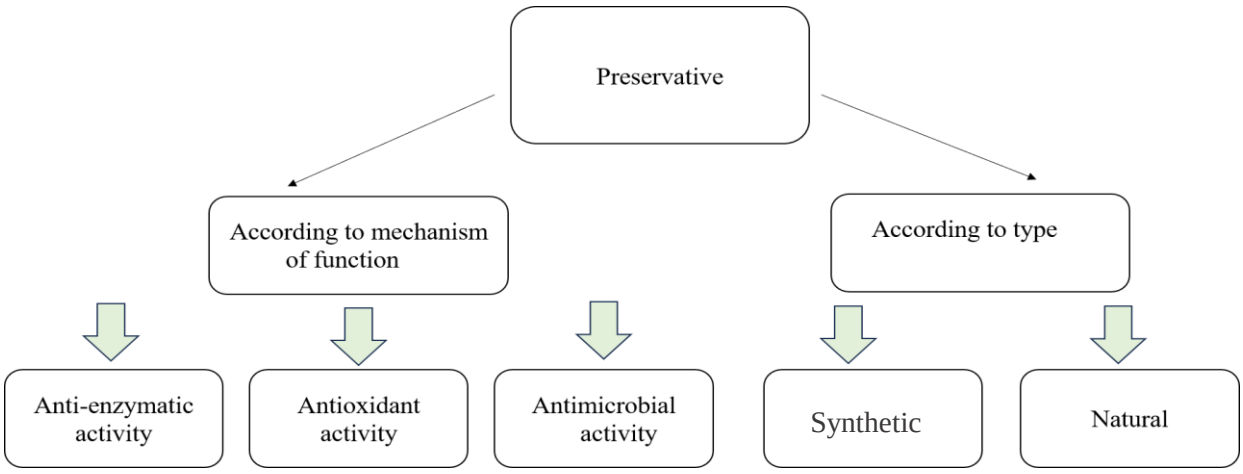


Figure 4. Classification of food preservative based on mechanism of action and kind (Rathee et al., 2023).

2. Methods used in citrus fruits preservation

The predominant treatment for managing citrus postharvest disease involves chemical fungicides derived from the imazalil and thiabendazole groups (Coutinho et al., 2020). Alongside these synthetic fungicides, physical methods such as heat treatment, irradiation, LED

blue light, and precooling are also employed (Strano et al., 2022), a large variety of pesticides is extensively employed to manage insects, fungi, and weeds at various stages of current agricultural production (Li et al., 2020).

The study by Youcefi & Mokhtari (2024), indicates that Algeria employs between 6,000 and 10,000 tons of chemical insecticides annually, the farmers generally use insecticides, followed by acaricides, herbicides, and ultimately fungicides.

Recently, natural and biopreservative agents (Figure 5), such as plant extracts, were used as preservative agents in citrus fruits (Kanashiro et al., 2020).

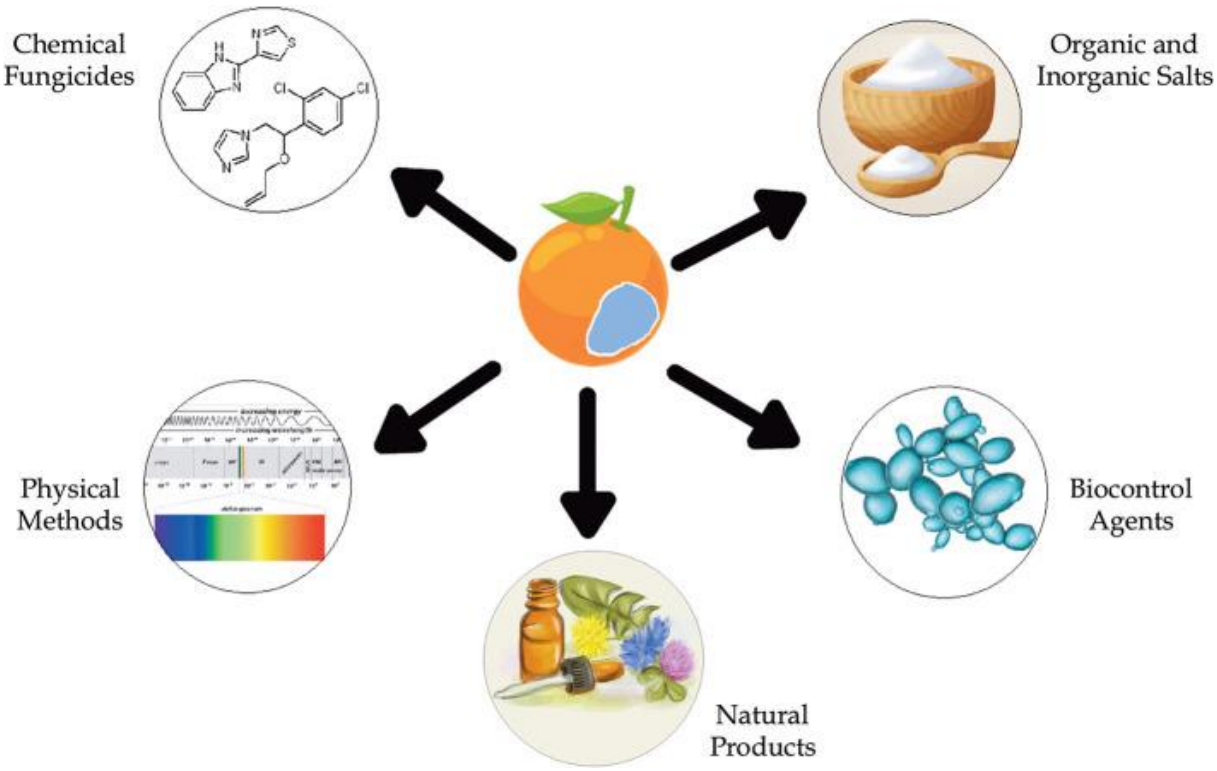


Figure 5. Different methods used for citrus fruits preservation (Kanashiro et al., 2020).

3. Negative effects of synthetic additives and preservatives

The excessive use of the aforementioned synthetic substances results in increased residual content and acute toxicity, prolonged decay time, potential adverse effects on human health, and particularly the emergence of resistant strains (Coutinho et al., 2020).

Pesticides and their metabolites may pose health risks, including respiratory issues, cancer, genotoxicity, neurotoxicity, and endocrine-disrupting toxicity, therefore, it is crucial to

carefully regulate pesticide application in agricultural fields and examine the risks of pesticide residues on human health (Radulović et al., 2023).

In Algeria, 50% of the citrus growers have experienced poisoning or respiratory issues due to pesticide overuse, and they are unable to accurately differentiate between the active ingredients and the commercial names of the pesticides employed (Youcefi & Mokhtari, 2024).

The food sector is increasingly confronted with a more informed and demanding consumer base, which drives the search for preservation strategies that align with expectations of quality, safety, and sustainability (Nunes et al., 2023). In this context, innovative preservation techniques such as biopreservation, nanoformulations, and active packaging have emerged as promising alternatives or complements to conventional preservation methods (Amiri et al., 2021).

4. Biopreservation

a. Definition

Biopreservation is the protection of food from deterioraion while maintaining its quality and safety, using biologically produced substances and processes (Garcia-Gutierrez et al., 2024).

b. Preservatives classification according to their origin

Biopreservation strategies (Figure 6), involve the application of antimicrobials or metabolites (Eg. Secondary metabolites) produced by certain strains of microorganisms or their metabolites, as well as secondary metabolites substances originating from biological organisms including bacteria, fungi, plants, or animals (Sionek et al., 2024).

i. Microbial origin

Microbial origin involves the use of controlled or natural microbiota, their antimicrobial substances or fermentations byproducts, in the control and the inhibition of the occurrence of pathogenic microorganisms in food products (Nusrat, 2024). Lactic Acid Bacteria are among

the most used microorganisms regarding to their preservative effect, these bacteria are known for the production of interesting compounds (Aguirre-Garcia et al., 2024).

ii. Animal origin

the most important natural compounds of animal origin are proteins and enzymes, including lysozyme, lactoperoxidase, and lactoferrin (Batiha et al., 2021), for example, chitosan, is a polysaccharide biopolymer, and is extensively utilized in the food sector (Sharif et al., 2017). Films based on such compounds could serve as food packaging (Muñoz-Tebar et al., 2023).

iii. Plant origin

Plant-derived bio-preservatives are classified as secondary metabolites, comprising tannins, alkaloids, terpenoids, phenols, and their oxygen-substituted derivatives (Othman et al., 2019). Herbs and spices possess antifungal, antibacterial, antiviral, and antiparasitic compounds (Ranathunga et al., 2023). Essential oils may be utilized independently or in combination with other oils or technologies to guarantee the microbiological stability of the product to which they are applied (Freitas & Cattelan, 2018).

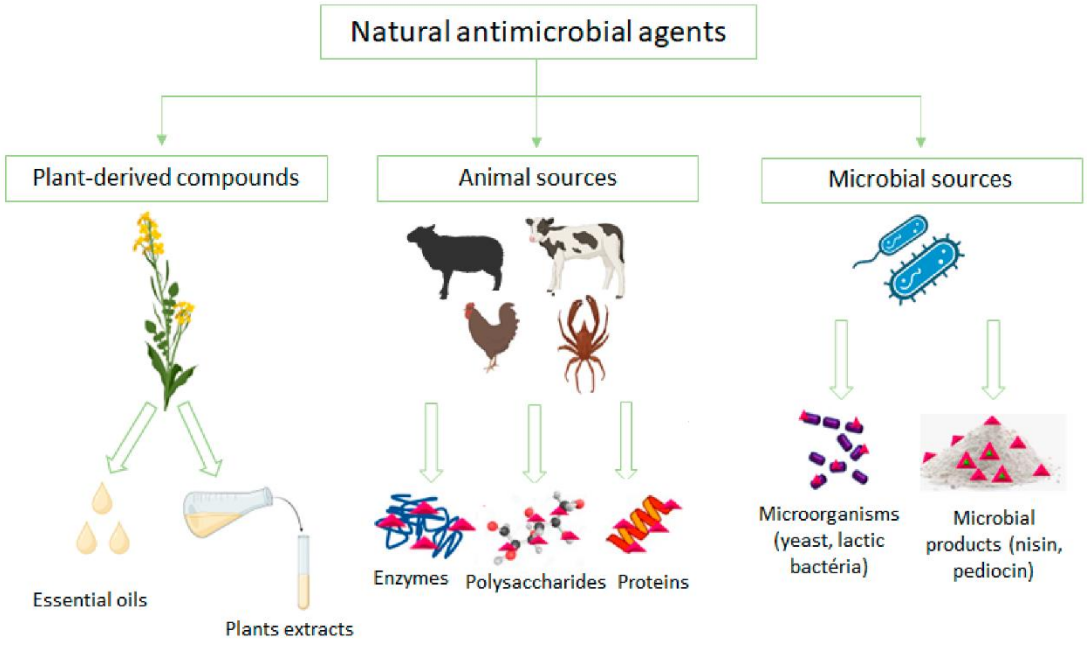


Figure 6. Natural antimicrobials and their origins (Nunes et al., 2023).

5. Essential oils in preservation

The excessive use of synthetic additives and preservatives in food sector is growing with time, as mentioned previously, consumers are concern about the negative effects of these synthetic compounds on health, this situation required natural alternatives in food sector such as essential oils (EOs) for the eradication of undesirable microorganisms, aromatic herbs, essential oils, and their constituents have been extensively employed as taste enhancers in culinary applications since ancient, it is widely acknowledged that numerous essential oils exhibit a diverse array of antimicrobial characteristics (Mwale, 2023). The European Commission (EC) and the United States Food and Drug Administration (FDA) have approved numerous essential oils (EOs) and EO components, as Generally Recognized as Safe (GRAS) for use as flavorings and preservatives in food products, however, the direct application of pure essential oils in food products is limited in some case due to toxicity of certain compounds within the oil (Falleh et al., 2020). Before applying essential oils for preserving any food product from spoilage, it is important to evaluate the antimicrobial activity of this oil against the pathogenic microorganisms present in the specific food item, and evaluating their effect on organoleptic properties of the product at the same time (Tiwari et al., 2009). EOs are commonly employed in food sector because to their biological properties, for example, carvacrol, thymol and eugenol, are among the most phenolic chemicals found in the EOs that have the greatest antimicrobial effects against foodborne pathogens (Hussein, 2022).

6. Edibles films and coatings

Edible coatings consist of soluble and bioactive ingredients **(**Nunes et al., 2024**)**. Edible coatings and films can be composed of lipids, polysaccharides, and protein **(**Jurić et al., 2024). Additional bioactive chemicals, notably, antioxidants, antibacterial and antifungal, can be added to enhance the activities of edible coatings against microorganisms (Martins et al., 2024). The application of this sort of method for the preservation of fruits and vegetables has shown considerable attention (Nusrat, 2024).

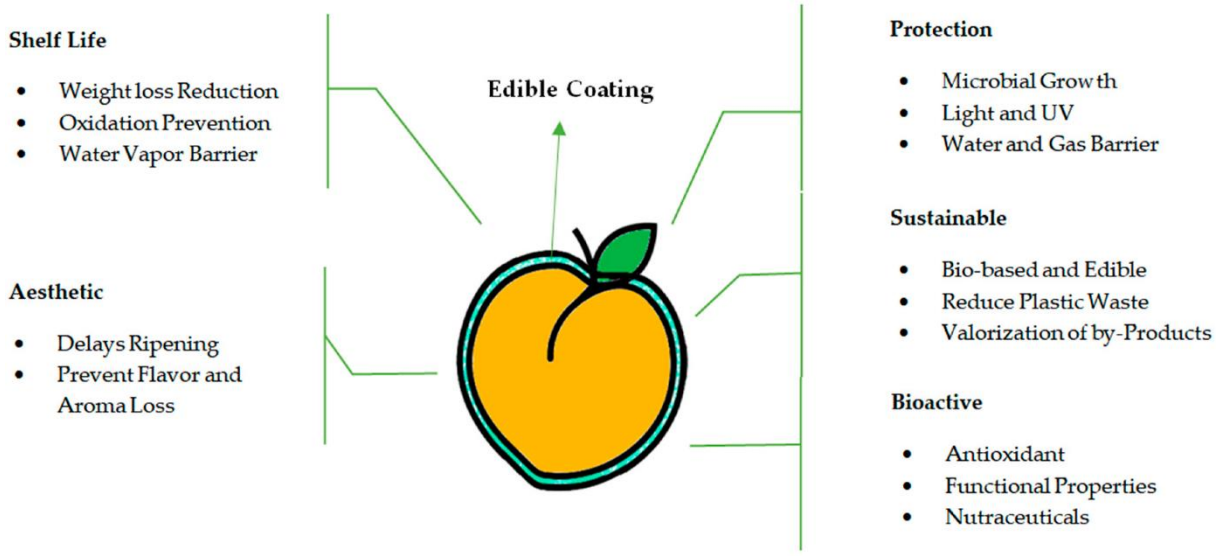


Figure 7. Some characterestics and advantages of esibles films and coatings (Aaqil et al., 2024).

a. Composition and application methods of edible films and coatings

The majority of edible coatings and films are derived from biological compounds, the bio macromolecules utilized for edible films and coatings formation can be classified into three primary categories **(**Figure 7) based on their physicochemical characteristics: proteins polysaccharides, and lipids (Liyanapathiranage et al., 2023).

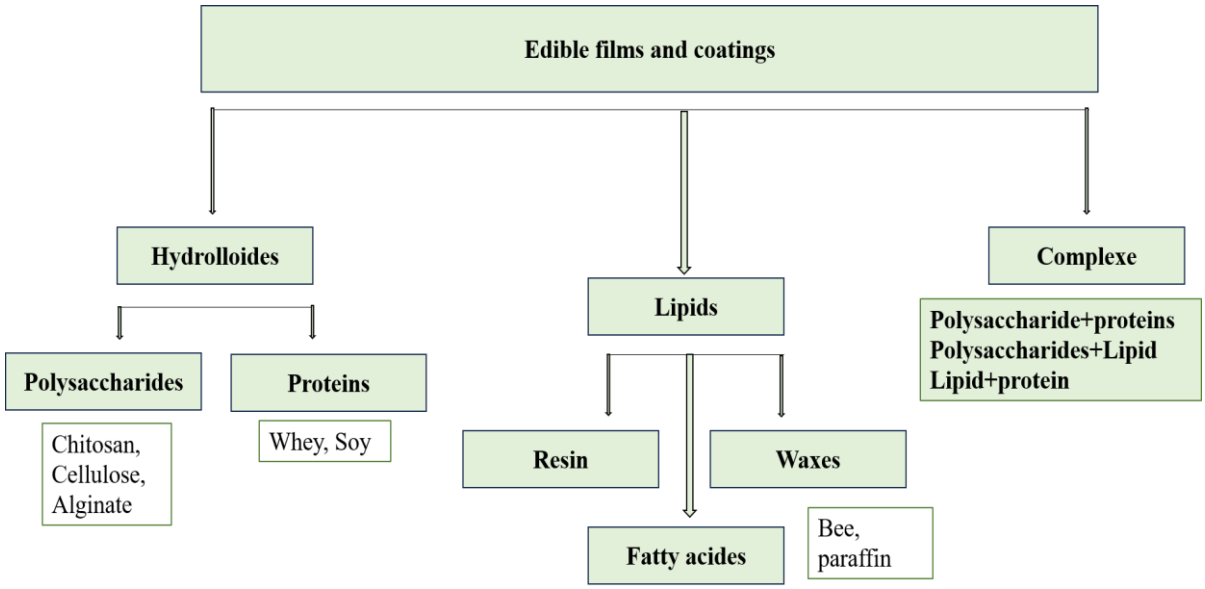


Figure 8. Composition of edible films and coatings (Poonia, 2018).

Moreover, edible films and coatings based on nanoemulsion are currently experiencing a growing utilization in the field of food preservation; the oil-in-water nano-emulsions are favored for use in edible films or coatings because of their capacity to effectively incorporate different lipophilic bioactive chemicals into the hydrophilic biopolymer substrate, Nanoemulsions are highly efficient carriers for bioactive substances such as natural extracts and essential oils. They show significant promise for use in edible films or coatings (Hashemi et al., 2023). Food can be coated by different methods **(**Figure 8) including, immersing (dipping) method, spraying and spreading (Antonino et al., 2024**)**.

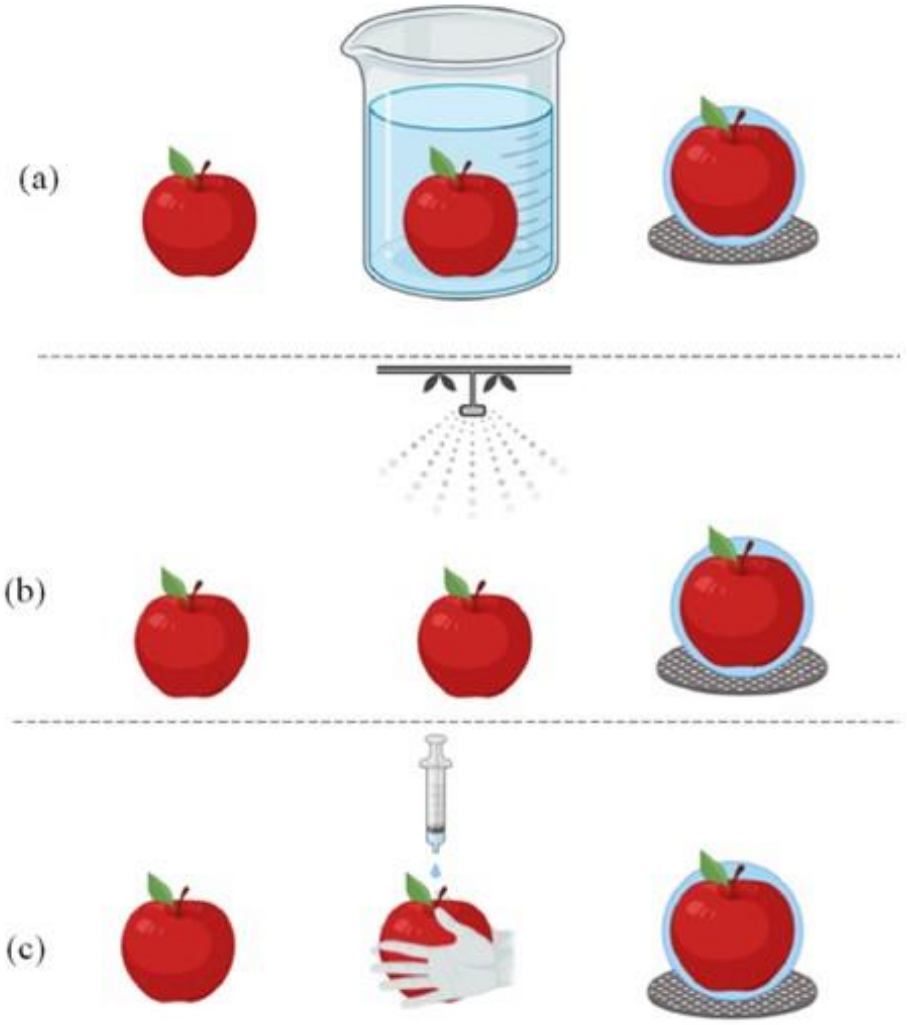


Figure 9. Different methods used for fruits coatings including, immersing (a), spraying (b) and spreading (c) (De Oliveira Filho et al., 2021).

b. Essential oils as additives in edible films and coatings

Currently, coating formulations commonly incorporate plant essential oils to effectively retard the deterioration of food as mentioned previously (Antonino et al., 2024). Additional compounds such essential oils can be directly added in coating or encapsulated, essential oils derived from various plants such as, lemongrass, lemon, eucalyptus, cinnamon, clove, thyme, have been commonly used in coating and packaging (El Ghorab et al., 2023).

These essential oils are commonly used as additives while their compounds can enhance the physical, antioxidant and biological activities of the coatings, and the organoleptic properties of the food products, since these essential oils are known for their pleasant and strong

aroma (Liyanapathiranage et al., 2023). Hence, the use of natural alternatives active biodegradable packaging that includes biological agents such essential oils can effectively reduce economic losses by minimizing the waste within both food and environmental sector, additionally; the integration of essential oils can avoid the disadvantages linked to synthetic additives while meeting consumer demands for natural and environmentally friendly packaging options (Tomić et al., 2023).

III. Aromatic plants and Essential oils

1. Aromatic plants

Aromatic plants have been cultivated in the Middle East since ancient time, they were utilized for several purposes such as conservation, and enhancing the organoleptic properties of food (Christaki et al., 2012).

According to the World Health Organization, (2001), "herbal medicines" are substances or products produced from plants that have therapeutic or other beneficial effects on humans.

The benefits of aromatic herbs, their extracts, and essential oils were analyzed in comparison to antibiotics as promoters of development, aromatic plants are free from any residues and are generally safe (Christaki et al., 2012). Algeria is well known for its climatic biodiversity, which serves as evidence of its rich variety of herbs and aromatic plants (Hamani et al., 2021).

1.1. *Cymbopogon citratus* (DC.)

Cymbopogon citratus (DC.), which is commonly referred to lemongrass, عشبة الليمون, Indian lemongrass, or lemongrass from Madagascar, is a fragrant herb belongs to the *Poaceae* family and it is a prevalent plant throughout Australia (Machraoui et al., 2018). Lawal et al. (2017) estimate that it consists of more than 180 species, subspecies, variants, and subvarieties, all of which are extensively dispersed worldwide in both temperate and tropical regions.

Table 1. Taxonomy and classification of *C. citratus* (Oladeji et al., 2019).

Kingdom	**Plantae**
Division	Magnoliophyta
Class	Liliopsida

Order	Poales
Family	*Poaceae*
Genus	*Cymbopogon* Spreng
Species	*citratus*

1.1.1. Botanical description

The perennial herb *Cymbopogon citratus* (DC.) (Figure 9) has green leaves with an upright orientation, flat structure, linear form, closed configuration at the base, it has a maximum length of 2 meters and a maximum width of 1.2 meters, and grows in massive masses without branching, the plant possesses reduced rhizomes, and flowering is infrequent in *C. citratus* (Machraoui et al., 2018).

Figure 10. Lemongrass (*C. citratus*) (Encyclopaedia Britannica, 2023).

1.2. *Citrus limon* (L.)

Lemon, (الليمون) known significantly as *C. limon* (L.), belongs to the *Rutaceae* family (Klimek-Szczykutowicz et al., 2020). Its origins are in South East Asia, more especially in India or Southern China (Pandey et al., 2011). Third in terms of economic value among all citrus

species, Lemon is one of the most significant crops cultivated worldwide, second after lime, with a total harvested area of 1.2 million hectares, and a total yield of 20.1 million tonnes (Catalano et al., 2021).

Table 2. Taxonomy and classification of *C. limon* (Rafique et al., 2020).

Kingdom	**Plantae**
Division	Eudicots
Class	Rosids
Order	Sapindales
Family	*Rutaceae*
Genus	*Citrus*
Species	*limon*

1.2.1. Botanical description

The lemon tree leaves are green all year long; the blossoms are bisexual, white, with a little reddish shading around the petal margins, they are either seen in small clusters or as individuals developing in the intervals between leaves. Lemon tree reaches 2.5–3 m in height. As it ripens, the green fruit becomes elongated, oval, pointed and turns yellow (Figure 10). Internally, the fruit has a soft flesh split into sections similar to those of an orange (Klimek-Szczykutowicz et al., 2020).

Figure 11. Lemon (*C. limon*) (Encyclopaedia Britannica, 2024).

1.3. *Origanum majorana* L.

Commonly known as marjoram, or البردقوش, *Origanum majorana* is a fragrant member of the *Lamiaceae* family (Yasar et al., 2022). One of the most aromatic species in the *Origanum* genus (Chenna et al., 2018), marjoram is extensively distributed globally, mostly in Morocco (Almasi et al., 2020), Egypt, and some areas of Algeria (Brada et al., 2013).

Table 3. Classification and taxonomy of *O. majorana* (Dhiman & Bhasin, 2020).

Kingdom	**Plantae**
Division	Tracheophyta
Class	Magnoliopsida
Order	Lamiales
Family	*Lamiaceae*
Genus	*Origanum* L.
Species	*majorana*

1.3.1. Botanical description

The marjoram plant is characterised by its dark green color, represented by globular leaves (Figure 11) with oval form that show branching from smallest to largest (Chenna et al., 2018), the plant can attain a height of 60 cm, and its flowers can come in red, purple, or white (Paudel et al., 2022).

Figure 12. Marjoram (*O. majorana*) (Encyclopaedia Britannica, 2025).

1.4. *Foeniculum vulgare* M.

The aromatic plant *Foeniculum vulgare* M. belongs to the *Apiaceae* family (Khammassi et al., 2018). This plant is almost widely dispersed over Algeria and shows great expansion, from food to cosmetics, it is used in many different fields owing to its great qualities, especially those found in the seeds (Zoubiri et al., 2014; Freitas & Cattelan, 2018).

Table 4. Taxonomy and classification of *F. vulgare* (Badgujar et al., 2014)

Kingdom	**Plantae**
Division	Tracheophyta
Class	Magnoliopsida
Order	Apiales
Family	*Apiaceae*
Genus	*FoeniculumM.*
Species	*vulgare*

1.4.1. Botanical description

F. vulgare is a straight, branching perennial herb characterised by delicate, feathery, nearly hair-like leaves, the leaves are light green, the blooms are small and yellow (Figure 12)

originating from multiple short branches, and the plant's seeds are flat and slightly curved at the end (Badgujar et al., 2014).

Figure 13. Wild fennel (*F. vulgare*) (Badgujar et al., 2014).

2. Essential oils

2.1. Definition

The notion of "essential oil" originates from Paracelsus, a 16th-century physician, who named a medicine Quinta essential; there is a huge variety of essential oils, where most of them are economically important (Burt, 2004). The French Agency for Normalisation, known as Agence Française de Normalisation (AFNOR), provides the following definition according to NF T 75-006: The essential oil is produced from a plant source, either through steam distillation or mechanical procedures, or through a process known as "dry" distillation (Dhifi et al., 2016).

2.2. Methods of extraction of essential oils

The extraction of essential oils from plants can be conducted using several ways; the choice of the suitable extraction process generally relies on the sorts of plants or fruits employed, along with the region and desired components, the selection of extraction method is crucial, as an inappropriate method may lead to the loss or modification of essential compounds in the plants, as well as a decline in the physical properties of the oils, thereby affecting the biological activities (Ni et al., 2021). Extraction methods can be categorized into traditional and advanced procedures.

2.2.1. Conventional and innovative methods of extraction

There are various methods available for extracting essential oils, conventional methods such as hydrodistillation, steam distillation, and cold expression are advantageous due to their

simplicity, ease of operation, and lower cost, however, to enhance the quality and yield of plant essential oils, advanced methods such as microwave-assisted extraction (MAE), ultrasound-assisted extraction, supercritical fluid extraction (SFE), supercritical water extraction, and pulsed electric field-assisted extraction (PEFAE) are employed, these advanced methods emphasize economic viability, ecological sustainability, high efficiency, and quality production, additionally, these techniques take less time and energy compared to conventional methods; however, the cold expression method remains the most suitable technique as it preserves the properties of the essential oil compounds without the influence of temperature (De Oliveira et al., 2022; Ni et al., 2021).

One of the traditional and widely used techniques for extracting essential oils from plants is hydrodistillation (Bolouri et al., 2022). This approach involves using a Clevenger equipment to extract essential oils (Mishra & Rathore, 2021). During the process of HD (Figure 14), the plant material is initially placed into a fixed compartment, followed by the addition of an adequate amount of distillated water, and ultimately brought to a boiling point, the primary variables that liberate bioactive chemicals from plant tissue are hot water and steam (Oreopoulou et al., 2019).

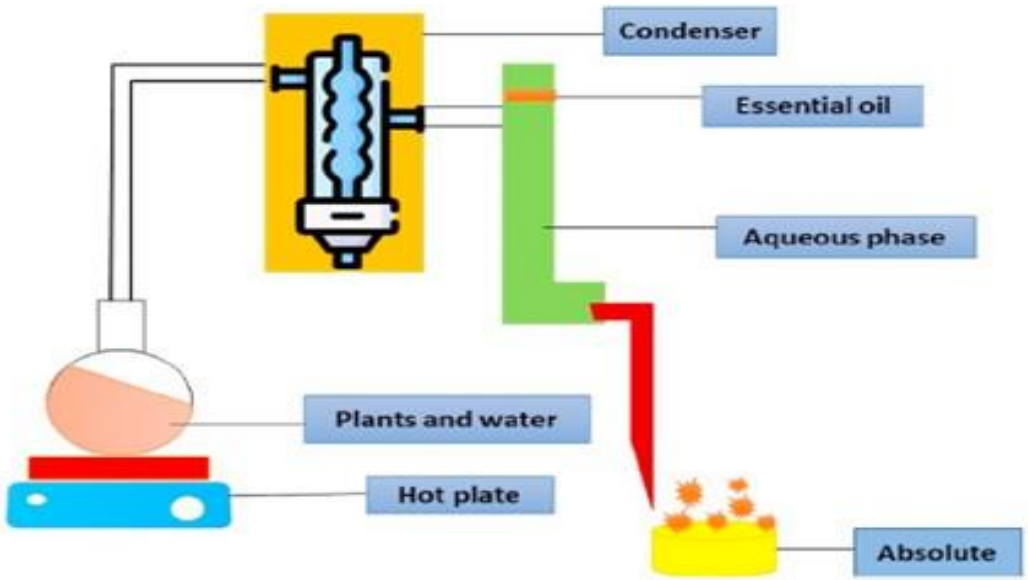


Figure 14. Hydrodistillation equipment (Pandey et al., 2024).

2.3. Chemical composition of essential oils

Essential oils (EOs) are complex combinations that consist of more than 300 distinct components; they consist of volatile chemical molecules, typically with a low molecular weight (Jugreet et al., 2020). It is imperative to ascertain the phytochemical constituents of the essential oil prior to conducting any subsequent investigations into their bioactivities, the chemical composition of essential oils is affected by several factors, including the growth environment, harvest season, and plant part used for essential oil extraction (Sun et al., 2022).

The primary constituents of essential oils are plant-derived secondary metabolites, which consist of hydrocarbons like terpenes and sesquiterpenes, as well as different oxygenated compounds such as phenols, lactones, ketones, aldehydes, ethers, esters, alcohols, and phenol ethers, a particular EO can consist of a minimum of 20 to 60 components with different concentrations, most oils are distinguished by two or three primary components that are found in large concentrations (20-70%), while additional components are present in very small proportions (Gupta & Variyar, 2016). Essential oils can be classified into two groups: (a) aromatic and aliphatic chemicals, and (b) hydrocarbon terpenes (isoprenes) and terpenoids (isoprenoids) (Figure 14), terpenes are a category of chemicals that are produced from isoprene and are found in essential oils, isoprene is an organic molecule consisting composed of five carbon atoms and one double bond, terpenoids are a diverse collection of substances that include terpenes (compounds containing double bonds) and their oxygenated derivative (Fokou et al., 2020). Generally, aromatic chemicals are less commonly found than terpenes, similar to phenylpropane (Eslahi et al., 2017).

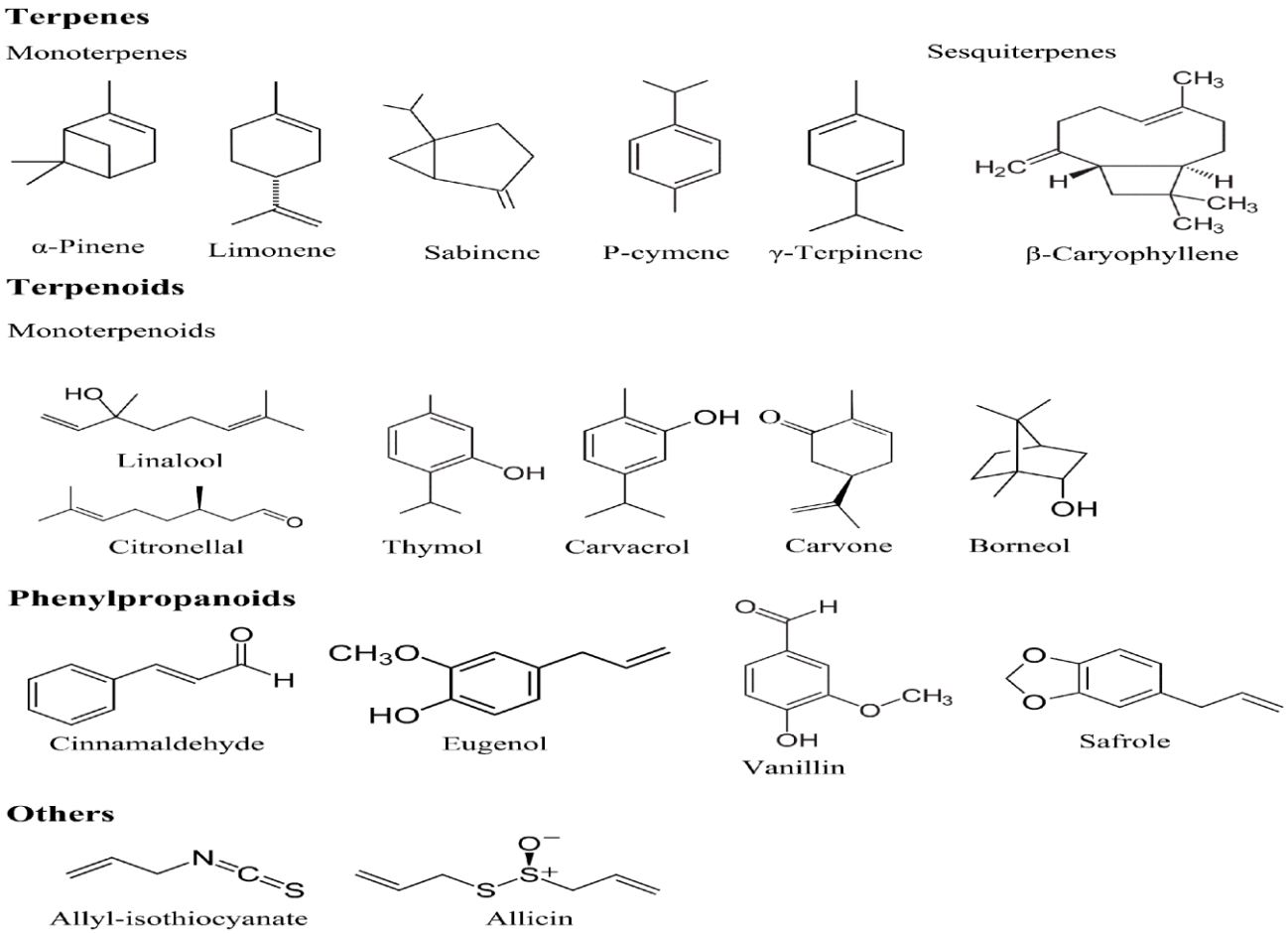


Figure 15. Chemical composition of essential oils (da Silva et al., 2021).

2.4. Mode of action

Historically, the mechanism by which essential oils exert their effects was mostly established through biochemical tests and their derivative compounds; however, the precise mechanisms of action of these oils against pathogenic microorganisms remain uncertain in certain instances (Yang et al., 2021).

2.4.1. Against bacteria

Studies showed that essential oils are influenced by the category and the shape of the microorganism's cells, where it was reported that rod-shaped cells are more susceptible compared to cells with a coccoid form, in addition Gram positive bacteria are more susceptible to essential oils than Gram negative bacteria (Mishra et al., 2020).

Nazzaro et al. (2017) reported that the primary target of essential oils is the membrane (Figure 15). It was reported that the main effect of essential oils is the increase of cellular

permeability, resulting from the disruption of cellular structural integrity, consequently, this action impacts crucial functions such as energy conversion, nutrient digestion, the synthesis of structural macromolecules, and the secretion of developing regulators, furthermore, essential oils facilitate the leakage and release of cellular contents, which subsequently influences vital activities including membrane transport, metabolic regulation, and energy production (Swamy et al., 2016; Marín et al., 2024).

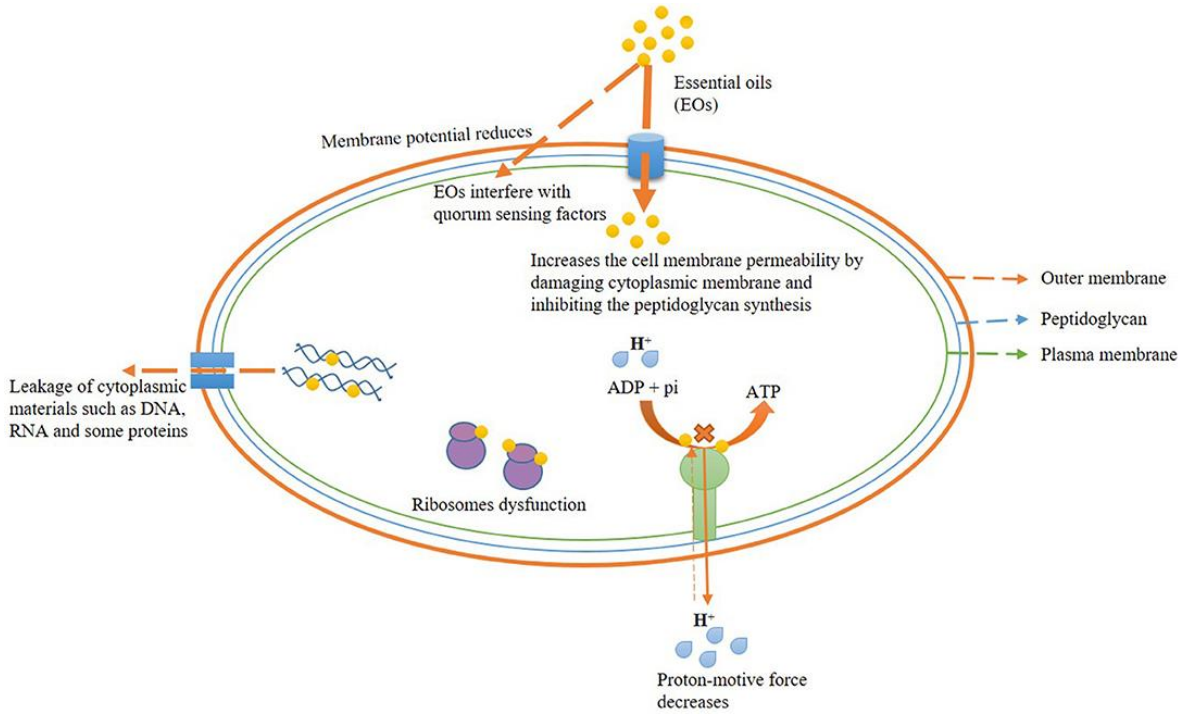


Figure 16. Mechanism of action of essential oils against bacteria (Maurya et al., 2021).

2.4.2. Against fungi

Similar to bacteria, essential oils influence fungal growth by damaging their cells through the disruption of both the outer and inner fungal membranes, leading to irreversible coagulation or leakage of cellular components (Figure 16), these mechanisms impact cellular contents such as proteins, lipids, and nucleic acids, while also affecting calcium ion concentration and increasing mitochondrial membrane permeability by interfering with the proton pump and ATP (Adenosine Triphosphate) pools, consequently, this results in a reduced membrane potential, the increased permeability obstructs cytochrome c pathways, inducing

either apoptosis or necrosis, ultimately resulting in cell death (Mani-López et al., 2021).

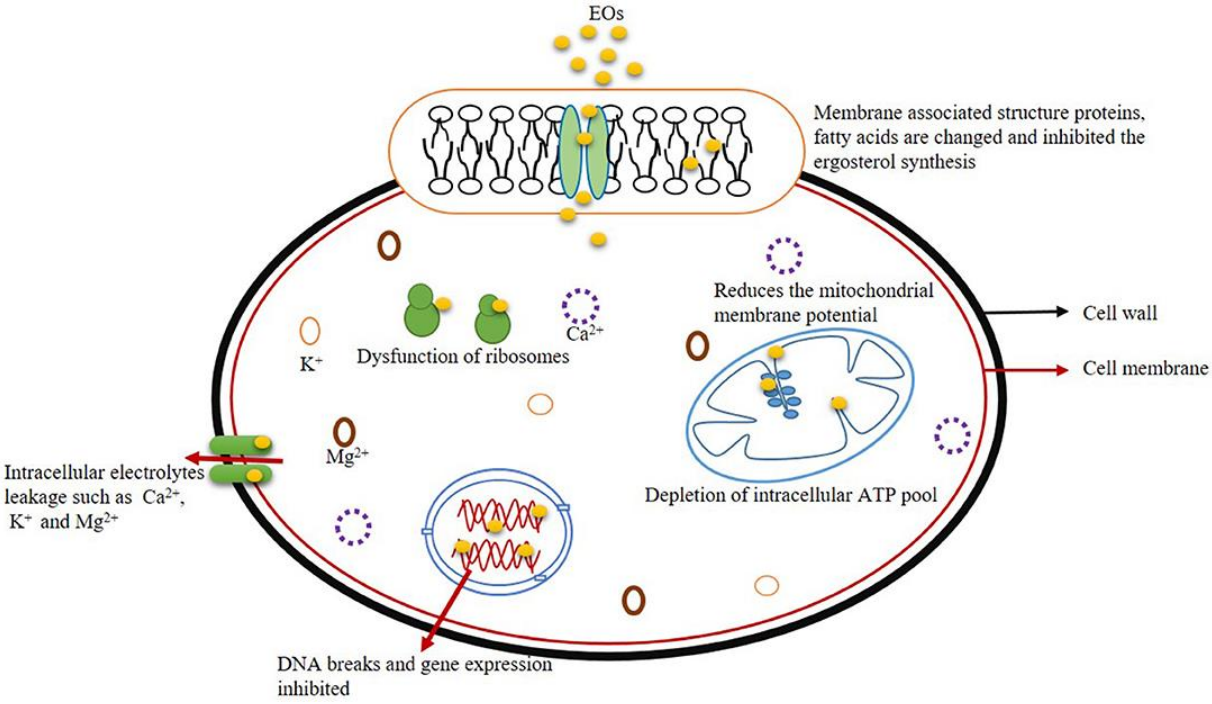


Figure 17. Mechanism of action of essential oils against fungi (Maurya et al., 2021).

2.5. Biological activities of essential oils

The biological properties of essential oils primarily relate to the characteristics of the aromatic plants from which they are obtained, especially their variety and chemical composition, the antimicrobial activity, for example, is particularly influenced by the chemical components found in the plant (Hammer & Carson, 2010)

Numerous studies have examined the antibacterial activity of essential oils and their constituents in comparison to their antifungal activity; however, current researchs are concentrating on the entirety of biological activities associated with essential oils (Mutlu-Ingok et al., 2020).

2.5.1. Antibacterial activity

Several studies showed the efficacy of essential oils against significant foodborne pathogens, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Campylobacter*, *Escherichia coli* O157:H7, and *Salmonella typhimurium* (Freitas & Cattelan, 2018). A study by Diniz et al. (2023) revealed that the essential oil of *Thymus vulgaris* shown significant antibacterial efficacy against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and

Staphylococcus saprophyticus. Likewise, Sateriale et al. (2022) demonstrated the potent antibacterial efficacy of thyme and clove essential oils against isolates of *S. aureus* and *E. coli*.

2.5.2. Antifungal activity

Essential oil antifungal activity is correlated with terpens, trans-anethole, a main component in anise oil, showed strong antifungal activity against *Mucor mucedo* in Yutani et al. (2011) research. Viuda-Martos et al. (2008) examined a collection of *Citrus* family essential oils comprising lemon, orange, mandarin, and grapefruit, *Penicillium chysogenum*, *Penicillium verrucosum*, *Aspegillus niger*, and *Aspergillus flavus* were considerably inhibited by these essential oils. While Cebi and Erarslan (2023) indicated that rising the doses (5, 10, 15, 20 μL) of *Citrus bergamia* essential oil resulted in decreased growth of both *A. niger* and *P. expansum*, Liu et al. (2009) found that thyme oil effectively inhibited the development of *Geothricum citri-aurantii*.

2.5.3. Antiviral activity

Current research is concentrating on the capacity of essential oils to inhibit the proliferation of several damaging viruses affecting humans and animals (Reichling, 2022). Essential oils have been assessed for their efficacy against several pathogenic viruses, including influenza and other respiratory viral diseases, the specific mechanisms via which essential oils exert antiviral effects are not yet well understood (Ma & Yao, 2020).

Oskuee et al. (2011) demonstrated that oil from *Carum copticum* directly eradicated viral particles, thereby inhibiting the attachment of the virion to host cells. Elaissi et al. (2012) demonstrated that the essential oils derived from *Eucalyptus bicostata* had notable antiviral properties.

2.5.4. Antioxidant activity

Researchers are progressively focussing their investigations on the use of essential oils with antioxidant properties to improve the oxidative stability of various products, with phytochemicals showing great potential as edible natural antioxidants, their identification is seen as good for health, principal antioxidants identified for their ability to neutralise free radicals and show antioxidant properties are phenolic chemicals derived from higher plants (Freitas & Cattelan, 2018). Research by Abdellaoui et al. (2017) and Khalid et al. (2014) showed that chemicals anethole, estragole, and fenchone obtained from *F. vulgare* provide great antioxidant potential.

IV. Nanoemulsion

1. Definition and composition

A nanoemulsion is a dispersion of nanometric particles within another material or liquid, with droplet diameters ranging from 10 to 200 nm, comparable to microemulsions or smaller in certain instances; these nanoemulsions are susceptible to physicochemical changes (Kumar et al., 2019).

There are various possible combinations of components within emulsions (Figure 17), including:

- Oil-in-water (O/W) nanoemulsions.
- Water in oil, W/O nanoemulsions.
- Oil in water in oil, O/W/O nanoemulsions.
- Water in oil in water, W/O/W nanoemulsions (Rehman et al., 2021).

Nanoemulsions consist of two primary phases: an oil or lipid phase and a water or aqueous phase, accompanied by a surfactant, the right combination and the physicochemical alterations of these chemicals are critical considerations since they are directly related to the stability of the nanoemulsion (Barradas et al., 2021).

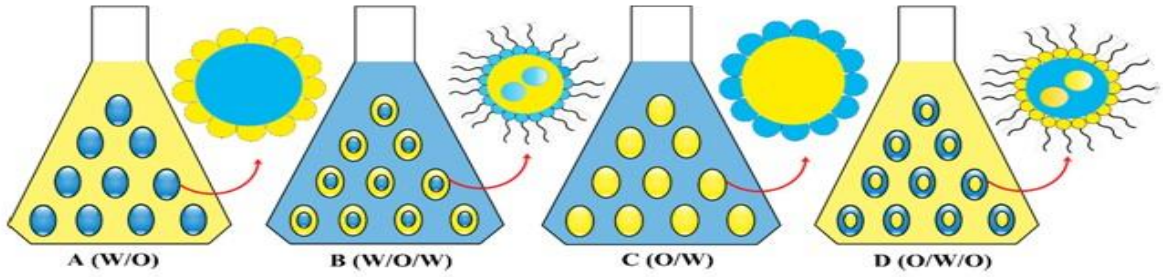


Figure 18. Possible combinations of nanoemulsions (Rehman et al., 2021).

2. Methods of nanoemulsion preparation

The high-energy and low-energy techniques are the two primary methods for the production of nanoemulsions, with high-energy methods being predominantly used on an industrial basis (Barradas et al., 2021).

2.1. High energy methods

High energy methods (Figure 18) are highly efficient techniques for the production of nanoemulsions, involving the conversion of high energy into mechanical force, which causes the dispersed phase to separate from the dispersion medium, allowing the rapid generation of nanoparticles. High energy approaches encompass:

- High-pressure homogenization
- Intense agitation with high shear forces.
- Ultrasonic waves for emulsion formation
- Microfluidizisation and membrane methodologies (Choradiya & Patil, 2021).

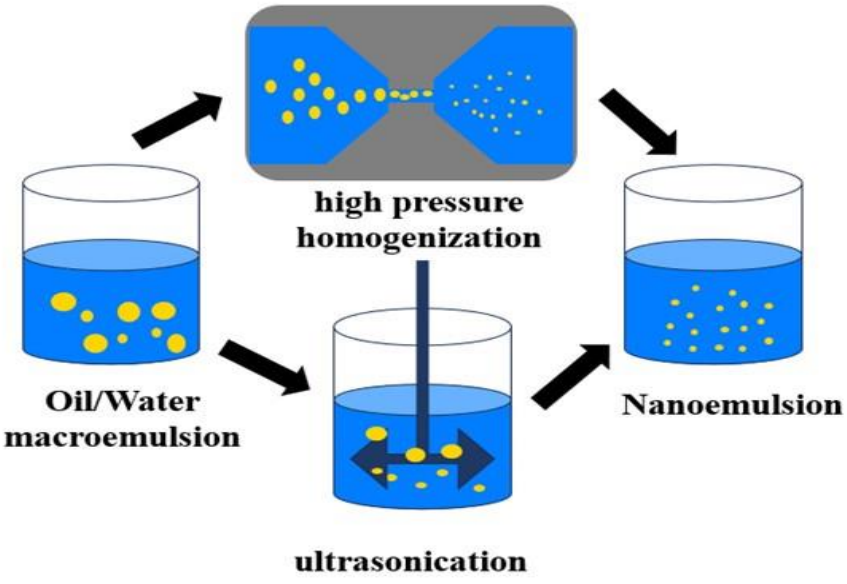


Figure 19. High energy methods (Gul & Riaz).

2.2. Low energy methods

The low energy methods (Figure 19) initially generate water-in-oil (W/O) macroemulsion at ambient temperature, subsequently; an emulsion inversion points or emulsion inversion temperature is applied, wherein the macroemulsion transitions to another phase

through dilution or temperature inversion, resulting in an oil-in-water (O/W) nanoemulsion, several low-energy approaches are available, primarily comprising: The Phase Inversion, Temperature (PIT) method, the Emulsion Inversion Point, often referred to as the Phase Inversion Composition method and Spontaneous nanoemulsification (Choradiya & Patil, 2021).

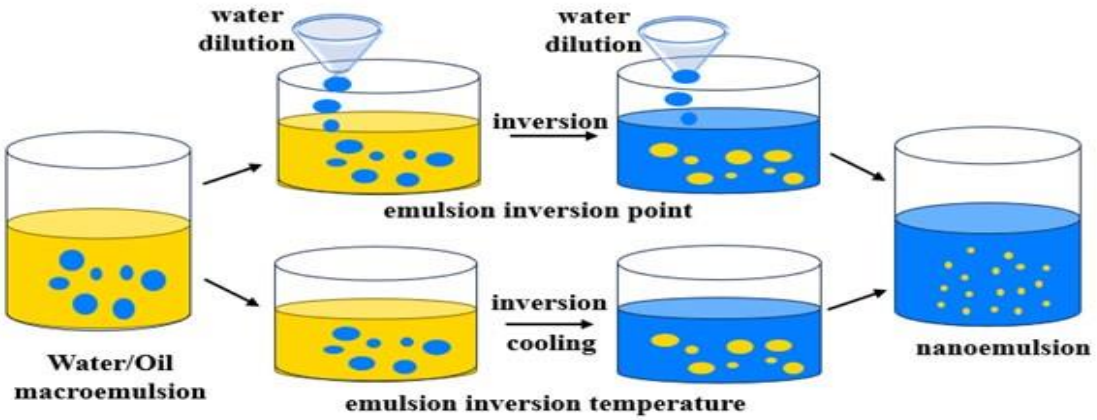


Figure 20. Low energy methods (Gul & Riaz, 2024).

3. Possible antimicrobial mode of action of nanoemulsions

Numerous studies demonstrate the antimicrobial efficacy of essential oil nanoformulations (Moradialvand et al., 2025; Basholli Salihu et al., 2025). Nevertheless, the precise mechanism of action remains ambiguous due to the complicated relationship of bioactive constituents, the principal target of essential oil nanoemulsions in bacteria is the phospholipids within cell membranes and mitochondria, leading to increased permeability, destabilization of cellular structure, and a reduction in proton motive force, electron flow, and active transport (Figure 20), similar to bacteria, the main target in fungus is the cell membrane (Figure 21) ,as essential oils are liophilic, they can penetrate the fungal cell membrane and cause alterations to the lipid bilayer structure, cellular integrity, and membrane permeability, moreover, nanoencapsulated essential oils restrict the mitochondrial electron transport chain, diminish membrane potential by inhibiting the proton pump, resulting in a reduction of the ATP lake, and eventually result in cellular apoptosis, notably, EO nanoemulsions exerted a profound effect on ergosterol, leading to the destabilization of membrane integrity and stability (Maurya et al., 2021).

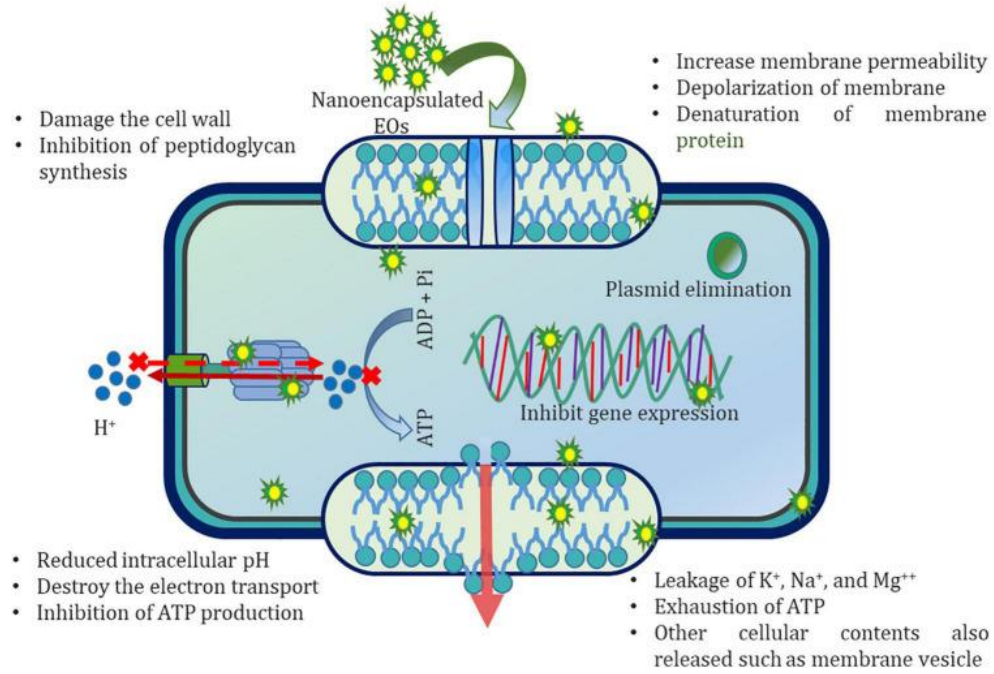


Figure 21. Mechanism of action of nanoemulsions against bacteria (Maurya et al., 2021).

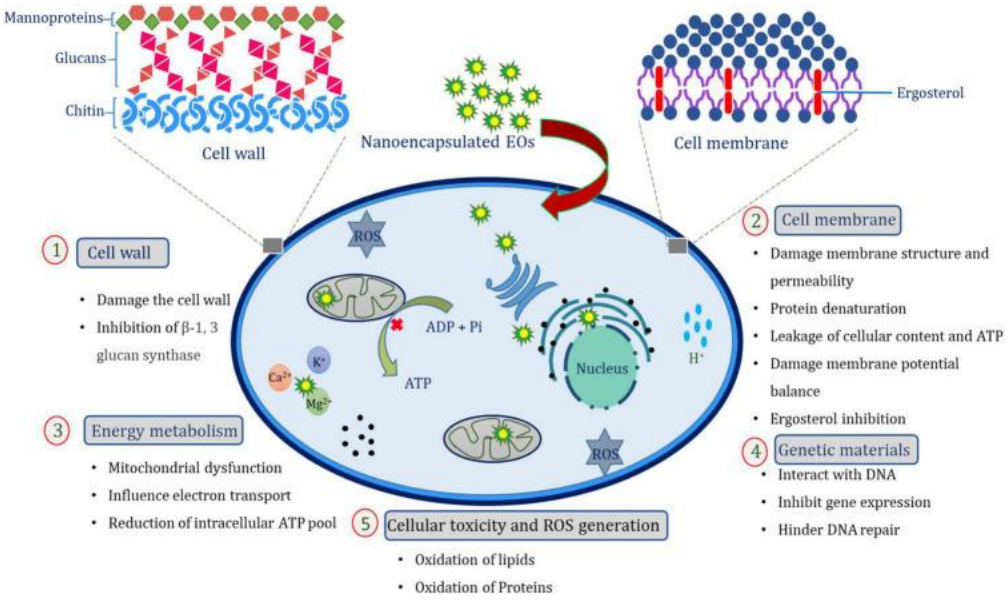


Figure 22. Mechanism of action of nanoemulsions against fungi (Maurya et al., 2021).

4. Nanoencapsulation and nanoemulsions in food biopreservation

Fruits and vegetables are prone to oxidation and microbiological deterioration from harvest to consumption; numerous methods are now utilized to prolong the shelf life of these items (Rawat, 2015). As previously stated, essential oils possess significant biological

activities; nevertheless, their application in preservation is limiting, diverse distribution systems utilizing innovative technology have been implemented to mitigate those drawbacks, nanoencapsulation of essential oils (EOs) is a contemporary method that enhances their antibacterial efficacy by improving stability, solubility, and controlled release of scent in food, this approach also guarantees the safety of essential oils; hence, encapsulated essential oils possess the potential to serve as better, non-toxic and environmentally sustainable alternatives to chemical preservatives in agriculture and the food sector, edible films and coatings enriched with essential oils have been recognized as an effective means to create a protective barrier against microbial contamination and mycotoxin production, hence enhancing food shelf life, challenges can be overcome by utilizing nanoemulsions to enhance the bioavailability of essential oils (Aswathanarayan & Vittal, 2019; Tomić et al., 2023).

Experimental part

Material and Methods

Material and Methods

1. Material

1.1. Chemicals and Microorganisms

All chemicals employed in this study were procured from the laboratory of Applied Microbiology at Ferhat Abbas University in Setif, Algeria.

The antibacterial activity was conducted on referential Gram-positive and Gram-negative strains obtained from the Laboratory of Applied Microbiology at Ferhat Abbas University in Setif; the bacterial strains include:

- *Staphylococcus aureus* (ATCC 25923).
- *Bacillus subtilis* (ATCC 6633).
- *Escherichia coli* (ATCC 25922)
- *Salmonella enterica* (ATCC 14028).
- *Klebsiella pneumoniae* (ATCC 13883).
- *Pseudomonas aeruginosa* (ATCC 27853).

The antifungal assessment was conducted on referential fungal strains obtained from National Research Centre, Egypt, in addition, to two isolated strains obtained from decaying orange fruits in Setif. The fungal strains include:

- *Aspergillus niger* (ATCC 16888).
- *Aspergillus flavus* (ITEM 698).
- *Fusarium culmorum* (KF91).
- *Candida albicans* (ATCC 1023).
- *Penicillium expansum* GY1 (isolate).
- *Penicillium digitatum* GY2 (isolate).

1.2. Plant material

In this study, marjoram (*Origanum majorana*), lemongrass (*Cymbopogon citratus*) and wild fennel (*Foeniculum vulgare*) plants were used along with lemon fruits (Figure 22), scientifically referred to *Citrus limon*. The lemons were acquired from a local market in Setif, Algeria. *C. citratus* was harvested from the wilaya of Bousaada, Algeria, marjoram was recolted from Hassi El Garaa in Mniaa wilaya, Algeria, with latitude of 30.54491° N and longitude 2.86047°E, while fennel was obtained from Guidjel region, Setif, Algeria.

Cymbopogon citratus had already been taxonomically identified in the study of Boudechicha et al. (2024), where it was deposited under the voucher number CAS28/06/21. The other plant materials, and lemon fruits, were also identified by the Department of Biology and Plant Ecology, (Faculty of Life and Natural Sciences, Ferhat Abbas University, Setif 1, Algeria). The voucher numbers MAS.58.29/03, F.W.S.19.19/09, and CAS09/01/23 were attributed to marjoram, lemongrass, and lemon, respectively.

a **b**

c **d**

Figure 23. Wild marjoram (a), lemongrass cultivated (b), wild fennel seed (c) and lemon fruits (d).

1.3. Fruits

Medium-sized Thomson Sweet oranges (*Citrus sinensis* (L.) were purchased in December 2023 from a local market in the Sétif region (Algeria). All fruits originated from the same batch (same arrival/lot).

2. Methods

2.1. Essential oils extraction

The hydrodistillation method using a Clevenger-type apparatus (See Figure A1.1, Appendix 1) was employed to extract volatile oils from the air-dried leaves of marjoram and lemongrass, fennel seeds, and the fresh zest of lemon fruits. For marjoram and lemongrass, the aerial parts were air-dried in the dark at ambient temperature and then directly used for extraction, together with the dried fennel seeds. while, lemon peels were processed fresh for essential oil extraction.

200 g of each sample was placed in a round-bottom flask containing a sufficient amount of distillated water, this mixture was heated until boiling for about 3 hours, two phases were obtained, hydrosol and the crude oil, this oil was separated from the other phase and dehydrated with anhydrous sodium sulphate, then placed in airtight glass vials covered with aluminum foil at refrigerator temperature until further use (Tran et al., 2023).
The experiment was conducted thrice, and the yields of the obtained essential oils were calculated with the following formula:

$$Y(\%) = \frac{\text{Weight of the essential oil (g)}}{\text{Weight of the plant (g)}} \times 100$$ (Mahboub & Slimani, 2021).

2.2. Nanoemulsions preparation

A nanoemulsion was formulated by mixing essential oil and Tween 80 at a 4:1 (v/v) ratio and using deionized water as the aqueous phase to reach a final volume of 100 mL, the procedure, slightly modified from Patel and Ghosh (2020), was repeated in several independent preparations. The selected concentration was based on preliminary screening, where lower tested concentrations did not exhibit activity against the microbial strains.

First, a coarse emulsion was prepared by gradually introducing the organic phase, which consisted of the oil and the surfactant (Tween 80), under magnetic stirring at ambient

temperature for 2 h. The second step (15 min) consisted of high homogenization of the coarse emulsion by probe ultrasonication (See Figure A1.2, Appendix 1) at a power of 250 W (Ultrasonic Microprocessor VCX 500, Fisher Scientific, Loughborough, UK), while keeping the sample on ice (4 °C) to avoid overheating (Patel & Ghosh, 2020).

2.3. Nanoemulsion characterization

The stability of the nanoemulsions was assessed using centrifugation at 3500 rpm for 30 minutes to evaluate the separation of phases (Sigma 3-18 KS, Osterode am Harz, Germany). The separation percentage is determined by the formula:

$$\text{Separation \%} = \frac{\text{weight of separated aqueous phase}}{\text{total weight of nanoemulsion}} \times 100$$ (Cecchini et al., 2021)

The particle size distribution and despersity index of the nanoemulsion samples was assessed using a Zetasizer Nano ZS (Nano-S90, Malvern Panalytical Ltd., UK) at a temperature of 25 ± 0.1 °C. Deionized water was utilized for dilution to minimise various scattering effects. The morphology of nanoemulsions was examined via transmission electron microscopy (TEM) at an operational voltage of 160 kV (JEOL Ltd., Tokyo, Japan) (Boudechicha et al., 2023).

2.4. Gas Chromatography-Mass Spectrometry (GC–MS)

GC-MS study demonstrates the chemical compounds of the essential oils and the impact of emulsification on the composition and components of the pure essential oils. The nanoemulsion was transferred to a screw-cap vial for measurement after being treated with diethyl ether, vortexing, and drying over anhydrous sodium sulphate. The extraction procedure is conducted three times. The separation of oils and nanoemulsion volatiles was conducted using a GC (Agilent 8890 System) connected with an MS (Agilent 5977B GC/MSD), as depicted in Figure A1.3, (Appendix 1), and equipped with an HP-5MS capillary column (30 m, 0.25 mm i.d., 0.25 mm film thickness).

The injected sample volume was 1 µL at 230°C in split mode (1:50). The oven initially set to 50°C, increased at a rate of 5°C/min to 200°C, then elevated from 200°C to 280°C at a rate of 10°C/min, and maintained isothermally for 7 minutes. Mass spectra in electron impact (EI) mode were acquired at 70 eV, covering the m/z range from 39 to 500

amu. Peaks were recognized through comparison with NIST standards and available data. The percentages of identified chemicals were determined using the peak regions from gas chromatography. The Kovats index for each chemical was calculated using the retention durations of C6-C26 n-alkanes and compared to literature values (Adams, 2017; Himed et al., 2019).

2.5. Morphological, molecular and phylogenetic analysis of the isolated fungal strains

The *Penicillium* strains were cultivated on Potato Dextrose Agar (PDA) medium at 25°C for 5 days. Following purification, macroscopic and microscopic identification was conducted at the Laboratory of Applied Microbiology in Setif, Algeria. The fungus was subsequently identified using molecular techniques by Gene Life Science in Algeria in Sidi Bel Abbès. Genomic DNA was isolated from fungal mycelium using the NucleoSpin Plant II kit (Macherey-Nagel, Germany). PCR amplification focused on the ITS, Ef1 rDNA regions. For ITS, primers ITS1 and ITS4 were utilized at a temperature of 55°C, yielding a fragment size of 600 bp (Table 05) in accordance with Gardes & Bruns (1993). For Ef1, primers EF-728F and EF-2 were utilized at a temperature of 52°C, yielding a fragment at the expected size of 450 bp, according to Carbone & Kohn (1999). The PCR mixture comprised ultra-pure water, Taq buffer (Promega), MgCl2 (25mM), dNTPs (25mM), forward and reverse primers (10µM each), and Taq polymerase (Promega), along with the addition of genomic DNA. Thermal cycling included an initial denaturation at 95°C for 5 minutes, after by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55-52°C for 30 seconds, and extension at 72°C for 45 seconds, culminating in a final extension at 72°C for 7 minutes. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide (GelRed, Biotium, USA) and scanned under ultraviolet light using a Biorad Gel Doc system (USA). The PCR products were purified utilizing the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany) and subsequently sequenced employing the Sanger method (Sanger et al., 1977) with the BigDye v3.1 kit. The sequences were analyzed using CHROMAS PRO software and compared to the GeneBank database via the NCBI BLAST program for identification purposes.

Phylogenetic trees for the isolated fungal strains *Penicillium expansum* (GY1) and *Penicillium digitatum* (GY2) were constructed and analyzed utilizing MEGA 12 software, employing both Internal Transcribed Spacer (ITS) and Elongation Factor 1-alpha (EFA1)

markers, with sequences sourced from the NCBI database. The alignment of the selected strains was conducted using ClustalW, accompanied by a visual inspection and trimming of the alignment's ends. The development of inter- and intra-strain interactions was evaluated using the Neighbor-Joining approach. The bootstrap test comprised 1000 replicates (Nakbi et al., 2024).

The ITS and elongation factor (TEF1-α) gene sequences of both fungal isolates were deposited in the NCBI database to obtain accession numbers.

Table 5. Primers used in molecular identification.

Description	Type	Sequence 5'-3'	Temperature	Expected Fragment size	Reference
ITS	**ITS1**	CTT GGT CAT TTA GAG GAA GTA A	55	**600bp**	Gardes& Bruns (1993
	ITS4	TCCTCCGCTTATTGATATGC	55		
EF1	**EF-728F**	CAT YGA GAA GTT CGA GAA GG	52	**450bp**	Carbone &Khon (1999)
	EF-2	GGA RGT ACC AGT SAT CAT GTT	52		

2.6. *In vitro* activity

2.6.1. Antimicrobial assay of essential oils and their nanoemulsions

The disk diffusion method in solid media and the microdilution method in liquid media were employed to asses the antibacterial and antifungal efficacy of both the pure essential oils and their nanoformulations, The major aim of this study is to investigate fungi; however, antibacterial activity was also evaluated in order to broaden the scope of the research.

A. Disk diffusion assay

A slightly modified disk diffusion method outlined by (Yadegarinia et al., 2006 and Micić et al., 2021) was used, first the bacterial suspension was prepared and adjusted to a concentration of 10^8CFU/ml using Spectrophotometer, while the fungal suspension was adjusted to 10^5spores/ml using a hemocytometer (Neubauer counting chamber), these suspensions where then distributed in petri plates containing Muller Hinton Agar (MHA) and Sabouraud Dextrose Agar for bacteria and fungi respectively, sterile paper disks each measuring 6mm were soaked with different doses of the essential oils and their nanoemulsions and placed on the surface of the plates. As standards inhibitors, ceftriaxone (30µg) and amphotericin B (100µg) were used against bacteria and fungi, respectively. The inhibition zones were measured in (mm) after incubation of 24 hours at 37°C for bacteria and 5-7 days at 25°C for fungi. The evaluation was conducted in triplicate.

B. Determination of Minimum Inhibitory Concentrations (MIC)

The microdilution assay methodology used is detailed by Taleb et al. (2018), a 96-well micro plate was utilized, wherein essential oils were initially dissolved in DMSO, followed by two-fold serial dilution in micro plates containing Mueller Hinton broth for bacteria and Sabouraud broth for fungi. The resulting concentrations ranged from 0.01% to 4% v/v. on the other hand, the nanoemulsions were only dissolved in the liquid media. Similar to the disk diffusion assay, ceftriaxone and amphotericin B served as positive controls for bacteria and fungi, respectively, while DMSO incorporated into broth media served as the negative control. The microplates were incubated at 37°C for 24 hours for bacterial cultures and at 25°C for 72 hours for fungal cultures. The minimum inhibitory concentrations (MICs) were assessed using Tetrazolium chloride (2, 3, 5-triphenyl-2H-tetrazolium chloride, TTC) as a bacterial viability indicator.

C. Determination of Minimum Bactericidal (MBC) and Fungicidal (MFC) Concentrations:

To determine the Minimum Microbicidal Concentration (MBC and MFC), from wells with no visible growth, aseptically, an aliquot (loopfull) was subcultured in agar media. The plates were incubated for 24 hours at 37°C for bacteria and for 5 days for fungi, all the tests were performed in triplicates (Boudechicha et al., 2023).

2.7. Antifungal effect of nanoemulsions as coating on oranges

Marjoram (*O. majorana*), lemongrass (*C. citratus*) and lemon (*C. limon*) nanoemulsions were selected as separated coatings on Thomson oranges, which were procured from a local market in Setif, Algeria. The oranges were chosen based on their uniformity in size and color, as well as the minimal presence of blemishes and injuries. Prior to treatment application, the oranges were disinfected with a 2% sodium hypochlorite solution for 2 minutes, washed twice with sterile distilled water, and then allowed to air dry overnight, the duration of immersion and disinfection was adjusted according to the protocol of OuYang et al. (2020). The fruits were organized into three treatments: the first treatment (T1) involved orange fruits treated with *O. majorana*, the second treatment (T2) consisted of fruits treated with *C. limon*, the third treatment included fruits treated with *C. citratus*, in addition to a group treated with distilled water, serving as the control. The coating method employed was the dipping method (See Figure A1.4, Appendix 1), the fruits were immersed for 2 minutes. In terms of *in vivo* antifungal activity, the fruits were wounded on two sides and subsequently inoculated with 15 µl of a sporal suspension of *P. digitatum* and *P. expansum* at a concentration of 10^5 spores/ml (OuYang et al., 2020). Following inoculation, the fruits were immersed in the specified nanoemulsions and allowed to dry overnight. A separate group of fruits was also wounded and inoculated with the same concentration of the fungal strain but without the application of nanoemulsion, serving as the control group. Each group consisted of five oranges. All oranges were then stored in clear plastic boxes for 21 days at room temperature.

2.8. Physicochemical parameters and fruits quality evaluation

2.8.1. Weight loss

The weight loss of orange fruits during storage was measured by weighing orange fruits at the first day of storage W1 and at the end of the storage period (21 days), W2, following the formula reported by Motamedi et al. (2018), the weight loss is calculated as a percentage.

$\text{weight loss (\%)} : \frac{\sum_{i=1}^{n} \frac{w1-w2}{w1}}{n} \times 100$, where: n: number of fruits; W1: weight of the fruit on the first day; W2: weight of the fruit on the last day of the storage.

2.8.2. Firmness

The Fruit firmness was assessed using a Brookfield CT3 Texture Analyser (AMETEK Brookfield, Middleboro, MA, USA) equipped with a 3R probe (Motamedi et al., 2018).

2.8.3. Measurement of Total Soluble Solids (TSS), Titratable Acidity (TA), Ascorbic Acid content (AA) and pH

From each replication, orange fruits were selected and juiced using a hand press juicer, regarding all the chemical parameters, the measurements were done on the first day of the storage (1) and the last day of the storage (21) (Niu et al., 2023).

The ascorbic acid content (vitamin C) was determined by iodometric titration, represented as mg of ascorbic acid per 100 mL of juice (Motamedi et al., 2018).

The TSS content of the juice was determined using a handheld manual refractometer, measuring in degrees Brix; pH was measured using PH-meter, while TA was expressed as a percentage (%) of citric acid, measured via titration with 0.1 N sodium hydroxide to achieve pH 8.2, after diluting 10 mL of juice with 50 mL of distilled water, according to the following formula:

$$\text{TA}(\%) = \frac{(0.0064)(\text{volume of NaOH in ml})}{10\text{ml (juice)}} \times 100$$ (Ncama et al., 2017).

2.9. Fruit sensory analysis

On both day 1 and day 21, four coated oranges from each treatment, along with oranges from the control group that exhibited no fungal growth, were selected and cut into many parts for sensory evaluation. A cohort of twelve panelists, consisting of six males and six females, was chosen to assess the quality aspects of the fruits, including color, scent, flavor, and overall acceptance. The assessment utilized a scale with endpoints of 0 and 5, where color was evaluated from pale yellow (0) to dark orange (5), and scent and flavor were assessed from weak (0) to powerful (5). A hedonic scale was utilized to assess overall preference, ranging from extreme dislike (0) to extreme liking (5) (Radi et al., 2018).

2.10. Molecular docking

Following the assessment of the efficacy of essential oils and their nanoemulsions (both *in vitro* and *in vivo*), an *in-silico* investigation of marjoram and fennel was conducted

to ascertain the precise activity and interaction between the essential oil and the nanoemulsion compounds against the tested fungi. Additionally, the study aimed to identify the most potent antifungal molecules within the examined essential oils and their nanoemulsions, as well as to evaluate their strongest affinities. Five crystalline proteomic enzymes were acquired from the Protein Data Bank (PDB) (https://www.rcsb.org, retrieved on September 29, 2024). Including:

- Fungal non-reducing polyketide synthase (PDB ID: 8CG4).
- Aldehydedehydrogenase 12 (PDB ID : 6D97).
- Serine hydrolase (PDB ID: 1VKH).
- Sterol 14-α demethylase (PDB ID: 4UYM).
- Patulin synthase (PDB ID: 8BXL).

PyMOL version 2.5.1 was utilized to prepare the selected proteins by eliminating extraneous chains, removing water and co-crystallized chains, and adding protons for enhanced molecular recognition. Subsequently, Avogadro version 1.2.0 was employed to minimize and optimize the structures of ligands utilizing the MMFF94 force field for stable molecular conformations.

Blind docking with CB-DOCK2 software (September 29, 30, and October 1, 2024, http://clab.labshare.cn/cb-dock/php/) was employed to investigate potential interaction sites and identify the five principal cavities, which were subsequently analyzed using the AutoDock Vina algorithm (Liu et al. 2022). The Discovery Studio program version 24.1.0.23298 was responsible for the visualization of the interaction results.

3. Statistical analysis

Statistical analysis and graphical presentations were conducted using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) and Excel softwares. Results are expressed as means ± standard deviations (SD) from three replicates. Analysis of variance (ANOVA) was employed to assess the significance of differences between mean values, followed by Tukey's multiple range tests, with $P < 0.05$, indicating statistically significant difference.

Results and Discussion

Results and Discussion

1. Chemical composition

The essential oils from *O. majorana*, *C. citratus* and *C. limon*, extracted with a Clevenger equipment, yielded 1.25 ± 0.08 % (w/w), 1.45 ± 0.04 % (w/w), and 1.25 ± 0.03 % (w/w), respectively, with fennel seed essential oil exhibiting the highest yield at 3.90 ± 0.15%. These findings align with a study conducted in Morocco by Abdellaoui et al. (2020) which reported a yield of 3.67 ± 0.13 % for wild fennel seed. In contrast, other studies in Algeria by Zoubiri et al. (2014) and Zheljazkov et al. (2013) reported yields of 0.93 ± 0.07 % and 0.68 %, respectively, additional studies in Algeria by Chenna et al. (2018) and in Tunisia by Hadjlaoui et al. (2018) reported different yields of marjoram with 0.61 % and 1.85 %, respectively. Numerous investigations in Algeria about lemongrass and lemon essential oils align with our findings, Himed et al. (2019) and Boudechicha et al. (2023). Boukhatem et al. (2014) documented a yield of 0.6 % for *C. citratus* from the Blida region in Algeria, whereas Himed et al. (2016) observed a yield of 0.81 % for *C. limon*. The discrepancies in these results can be ascribed to the period of the recolte, the age and the genetic of the plant, the method and duration for the extraction, the storage conditions, and climatic and environmental factors, which are pivotal in influencing both the chemical composition and yields of the essential oils (Mohanty et al., 2023).

The chemical composition of essential oils and their nanoemulsions is a critical standard for selecting suitable plants for application in various fields, such as food, as the chemical compounds provide insight into the efficacy of essential oils or their nanoemulsions in combating pathogenic microorganisms, thereby indicating their economic value (Salih & Çelikbıçak, 2012).

The present study assessed the chemical composition of four essential oils and their nanoemulsions. Marjoram essential oil contains 30 compounds (Table 6), with Terpinen-4-ol, *γ*-Terpinene, *cis-β*-Terpineol, and *α*-Terpinene as the predominant constituents, comprising 17.97%, 11.12 %, 9.80 %, and 8.54 %, respectively. This aligns with the findings of Paudel et al. (2022) which identified Terpinen-4-ol as the principal compound. Conversely, studies conducted in Algeria revealed differing chemical compositions; for instance, research in the El Oued region by Chenna et al. (2018) identified borneol and eucalyptol as the major compounds, while a study in Khemis-Miliana by Brada et al. (2013) reported β-caryophyllene, followed by α-terpinolene and λ-terpinene, as the primary constituents, along with various other compounds.

Concerning lemongrass essential oil, 21 compounds were found (Table 7) with geranial, neral, and β-Myrcene as the predominant constituents, achieving significant percentages of 31.7%, 27.97 %, and 9.64 %, respectively; these findings align with the research of Boudehicha et al. (2023) and Rhimi et al. (2022), where geranial and neral were also predominant compounds. Conversely, a study by Moutassem et al. (2024) indicated that geranial was the principal compound 20.86 %, followed by limonene 10.5 %, additionally, research by Benoudjit et al. (2022) demonstrated that LGEO comprises neral 43.75 % and isogeranial 41.77 % as its major constituents.

GC-MS analysis of *C. limon* revealed 14 compounds (Table 8) with D-Limonene as the predominant component at 61.8%, followed by geranial and neral, which constituted 8.75 % and 7.89 %, respectively. These results correspond with those documented in Algeria by Djenane (2015), Hamdani et al. (2013), Boughendjioua and Djeddi (2017), and Himed et al. (2016), wherein limonene appears as the predominant chemical, with concentrations of 51.40%, 36.10 %, 61.647 %, and 66.75 %, respectively. The predominance of neral, geranial, and γ-terpinene, together with the reduction of β-pinene and the occurrence of p-cymene, constitutes the primary distinctions documented for the first time in the literature. Related to fennel essential oil, eighteen compounds were identified, with estragole, anethole, and L-fenchone as the predominant constituents, exhibiting percentages of 38.07 %, 29.47 %, and 11.47%, respectively (Table 9), in comparison to studies conducted in Algeria by Ouis et al. (2014) and Zoubiri et al. (2014), estragole and fenchone were similarly identified as major compounds, corroborating our findings.

The chemical composition of the nanoemulsified essential oils was significantally ($P < 0.0001$) influenced by the ultrasonication method, which notably altered the concentrations of various compounds, some compounds increased, while others decreased, resulting in the absence of several compounds. In the marjoram nanoemulsion, only 21 compounds were identified (Table 6), with the previously mentioned compounds dominating at higher percentages compared to the crude essential oil; for instance, terpinen-4-ol increased from 17.97 % to 33.91% in the nanoemulsion. The analysis of fennel revealed only six compounds, with estragole 49.94 % and anethole 41.61 % predominating over oil monoterpenes, which were also present in higher concentrations than in the pure essential oil, similar to marjoram. In the nanoemulsified lemongrass, geranial and neral were the predominant compounds contained in the nanoemulsion, comparable to the pure essential oil; however, β-Myrcene greatly diminished

to 1.29%. Overall, the lemongrass nanoemulsion included 14 compounds. The results correspond with those of Boudechicha et al., 2023 who employed microfluidization for the homogenization of *C. citratus* oil. Regarding the *C. limon* nanoemulsion, ten chemicals were identified, including α-Pinene and β-Myrcene, which are components of the chemical structure present in the nanoemulsions. Furthermore, certain molecules demonstrated an increase, whereas others indicated a drop, as shown with the lemongrass nanoemulsion. For instance, geranial and neral increased to 27.15 % and 24.77 %, respectively; these two chemicals, in conjunction with D-Limonene at 35.59 %, are the most significant, as shown in the raw oil. Nevertheless, there is insufficient information regarding the impact of homogenization on the composition of *C. limon* oil. The high-pressure homogenization of Algerian *Satureja hortensis* L. oil significantly elevated the carvacrol concentration from 45.15 % to 94.51 %, while simultaneously lowering the levels of γ-terpinene and p-cymene (Boudechicha et al., 2024). In a study conducted by Mohtashami et al. (2018), it was shown that throughout the preservation of summer savory at varying temperatures and durations, γ-Terpinene underwent transformation into carvacrol via aromatization and hydroxylation activities. This study examined the effects of the intensive-energy technique, specifically ultrasonication, on the composition of essential oils for the first time, highlighting the potential influence on the volatile profile and, consequently, the biological activities, while considering the benefits of nanotechnology.

Table 6. Chemical composition of *O. majorana* essential oil (OMEO) and its nanoemulsion (NEOM).

	Compound	RI [a]	LRI [b]	Area (%)		Identification method[c]
				OMEO	NEOM	
1	*α*-Thujene	926	930	1.19	-	RI, MS
2	*α*-Pinene	935	939	1.32	-	RI, MS, STD
3	Sabinene	971	975	6.4	1.74	RI, MS, STD
4	*β*-Pinene	974	979	0.67	-	RI, MS
5	*β*-Myrcene	988	990	2.00	0.36	RI, MS, STD
6	*α*-Phellandrene	996	1002	0.89	-	RI, MS

7	***α*-Terpinene**	1014	1017	**8.54**	**4.13**	RI, MS, STD
8	*p*-Cymene	1023	1024	3.69	2.36	RI, MS, STD
9	*D*-Limonene	1027	1029	4.77	1.66	RI, MS, STD
10	Eucalyptol	1032	1031	0.34	-	RI, MS
11	***γ*-Terpinene**	1061	1059	**11.12**	**8.46**	RI, MS, STD
12	Sabinene hydrate	1067	1070	4.90	7.74	RI, MS, STD
13	Terpinolene	1090	1088	4.41	2.47	RI, MS, STD
14	***cis*-*β*-Terpineol**	1139	1144	**9.80**	**17.04**	RI, MS, STD
15	Camphor	1148	1146	1.98	2.63	RI, MS, STD
16	*trans*-*β*-Terpineol	1165	1163	1.56	2.13	RI, MS
17	**Terpinen-4-ol**	1178	1177	**17.97**	**33.91**	RI, MS, STD
18	*α*-Terpineol	1187	1188	4.63	7.05	RI, MS, STD
19	*cis*-Piperitol	1192	1196	0.70	0.74	RI, MS
20	Dihydrocarvone	1204	1200	0.27	-	RI, MS
21	*trans*-Piperitol	1213	1208	1.09	1.43	RI, MS
22	Citronellol	1229	1225	0.30	1.20	RI, MS
23	Linalyl acetate	1260	1257	3.15	2.56	RI, MS, STD
24	*trans*-Ascaridol glycol	1274	1269	0.36	-	RI, MS
25	4-Terpinenyl acetate	1303	1300	0.45	0.49	RI, MS
26	Geranyl acetate	1388	1381	0.43	0.57	RI, MS
27	*β*-Caryophyllene	1422	1419	4.18	0.79	RI, MS, STD
28	*α*-Humulene	1455	1454	0.30	-	RI, MS

29	Bicyclogermacrene	1501	1500	2.26	0.55	RI, MS
30	Spathulenol	1580	1578	0.35	-	RI, MS
	Total			100	100	-

RI[a]: Retention indices were calculated using the DB-5 column using alkane standards. **LRI**[b]: Retention indices according to literature. [c] Confirmed by comparison with the retention indices, the mass spectrum of the authentic compounds, and the NIST mass spectra library data.

Table 7. Chemical composition of *C. citratus* essential oil (CCEO) and its nanoemulsion (NECC).

	Compound	RI [a]	LRI [b]	Area (%)		Identification method[c]
				CCEO	NECC	
1	6-Methyl-5-heptene-2-one	983	985	1.27	0.77	RI, MS
2	***β*-Myrcene**	992	991	**9.64**	1.29	RI, MS, STD
3	*Z*-*β*-Ocimene	1040	1037	0.25	-	RI, MS
4	*E*-*β*-Ocimene	1051	1050	0.57	-	RI, MS
5	Rosefuran	1063	1065	0.42	-	RI, MS
6	Linalool	1100	1096	2.46	2.31	RI, MS, STD
7	Isocitral (exo-)	1147	1144	0.31	-	RI, MS
8	Isoneral	1171	1170	2.63	0.85	RI, MS
9	Rose furan oxide	1180	1177	1.64	1.48	RI, MS
10	Isogeranial	1189	1185	3.45	1.79	RI, MS, STD
11	Estragole	1199	1196	0.63	2.92	RI, MS, STD
12	Rose ether	1221	1223	-	1.79	RI, MS
13	Citronellol	1228	1225	0.64	0.63	RI, MS

14	**Neral**	1240	1238	**27.97**	**33.58**	RI, MS, STD
15	Geraniol	1258	1255	8.49	8.36	RI, MS, STD
16	**Geranial**	1270	1267	**31.7**	**38.47**	RI, MS, STD
17	Anethole	1279	1283	0.26	1.73	RI, MS, STD
18	Nerylformate	1284	1284	0.68	-	RI, MS
19	Nerolic acid	1337	1340	0.47	-	RI, MS
20	Geranyl acetate	1384	1383	5.25	4.03	RI, MS
21	Caryophyllene oxide	1582	1583	0.3	-	RI, MS
22	Selin-6-en-4α-ol	1633	1636	0.31	-	RI, MS
22	Total	-	-	99.34	100	-

RI[a]: Retention indices were calculated using the DB-5 column using alkane standards. **LRI**[b]: Retention indices according to literature. [c] Confirmed by comparison with the retention indices, the mass spectrum of the authentic compounds, and the NIST mass spectra library data.

Table 8. Chemical composition of *C. limon* essential oil (CLEO) and its nanoemulsion (NECL).

	Compound	RI [a]	LRI [b]	Area (%)		Identification method[c]
				CLEO	NECL	
1	α-Pinene	942	939	1.88	-	RI, MS, STD
2	Sabinene	978	975	0.92	-	RI, MS
3	*β*-Myrcene	992	991	3.46	-	RI, MS, STD
4	Octanal	1001	998	0.42	-	RI, MS
5	3-Carene	1013	1011	0.29	-	RI, MS
6	*p*-Cymene	1025	1024	4.27	1.29	RI, MS, STD

7	**D-Limonene**	1031	1029	**61.8**	**35.59**	RI, MS, STD
8	γ-Terpinene	1058	1059	**7.77**	1.74	RI, MS, STD
9	Linalool	1100	1096	0.75	1.42	RI, MS, STD
10	*cis*-Limonene oxide	1136	1134	0.66	1.45	RI, MS
11	trans-Limonene oxide	1143	1142	0.33	0.63	RI, MS
12	Rose ether	1221	1223	-	3.81	RI, MS
12	Decanal	1203	1201	0.38	-	RI, MS, STD
13	**Neral**	1240	1238	**7.89**	**24.77**	RI, MS, STD
14	Carvone	1245	1243	-	1.43	
15	**Geranial**	1258	1255	**8.75**	**27.15**	RI, MS, STD
	Total	-	-	99.57	99.28	-

RI[a]: Retention indices were calculated using the DB-5 column using alkane standards. **LRI**[b]: Retention indices according to literature. [c] Confirmed by comparison with the retention indices, the mass spectrum of the authentic compounds, and the NIST mass spectra library data.

Table 9. Chemical composition of *F. vulgare* essential oil (FVEO) and its nanoemulsion (NEFV).

	Compound	**RI** [a]	**LRI** [b]	**Area (%)**		**Identification method**[c]
				FVEO	NEFV	
1	*α*-Pinene	935	939	5.73	-	RI, MS,STD
2	Sabinene	971	975	0.42	-	RI, MS,STD
3	*β*-Pinene	974	979	0.70	-	RI, MS
4	*β*-Myrcene	988	990	1.59	-	RI, MS
5	*α*-Phellandrene	996	1002	1.33	-	RI, MS

6	*p*-Cymene	1023	1024	0.86	-	RI, MS,STD
7	*D*-Limonene	1027	1029	2.95	-	RI, MS, STD
8	Eucalyptol	1032	1031	0.82	-	RI, MS
9	*trans*-β-Ocimene	1053	1050	0.36	-	RI, MS
10	*γ*-Terpinene	1061	1059	0.99	-	RI, MS,STD
11	***L*-Fenchone**	1084	1086	**11.47**	3.68	RI, MS,STD
12	Linalool	1099	1096	1.93	1.23	RI, MS, STD
13	Camphor	1148	1146	0.74	-	RI, MS, STD
14	Terpinen-4-ol	1178	1177	0.54	-	RI, MS,STD
15	**Estragole**	1198	1196	**38.07**	**49.94**	RI, MS, STD
16	Rose ether	1223	1221	-	2.35	RI, MS
17	Anisaldehyde	1246	1242	0.60	-	RI, MS
18	**Anethole**	1289	1284	**29.47**	**41.64**	RI, MS
19	Methyl eugenol	1407	1403	1.46	1.17	RI, MS
	Total			100	100	-

RI[a]: Retention indices were calculated using the DB-5 column using alkane standards. **LRI[b]**: Retention indices according to literature. [c] Confirmed by comparison with the retention indices, the mass spectrum of the authentic compounds, and the NIST mass spectra library data.

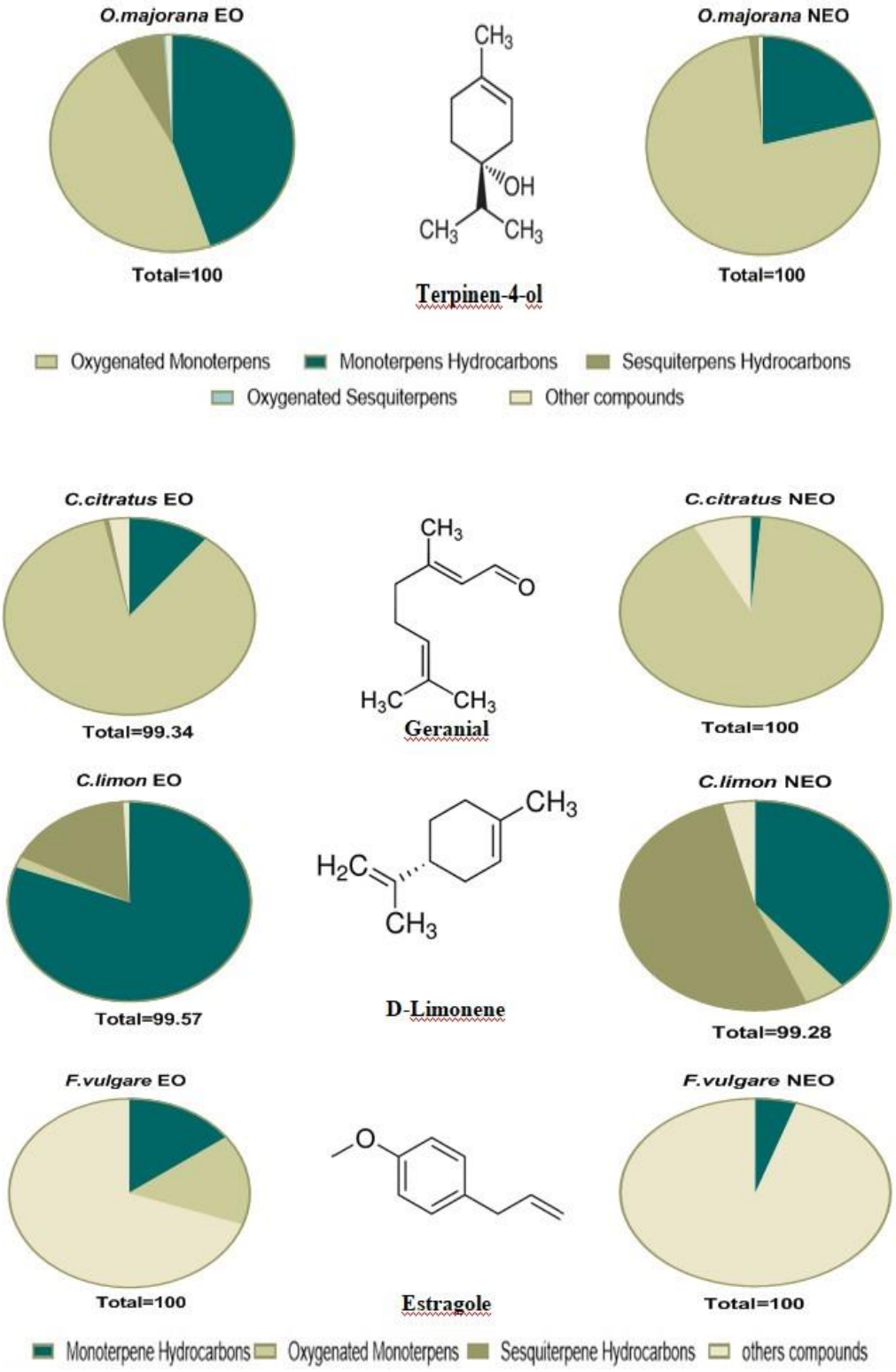


Figure 24. Different percentages of chemical groups in tested EOs and their NEs.

2. Parameters of the nanoemulsions

Concerning the physicochemical parameters of the tested nanoemulsions, *O.majorana*, *C.citratus*, *C. limon* and *F.vulgare* nanoemulsions showed values of 1.34, 1.33, 1.47 and 1.34 as refractive index respectively, these results indicates the isotropic nature of these nanoemulsions (Laxmi et al., 2015), the density and the pH values are shown in Table 10, *C.citratus* and *F.vulgare* showed a density of 0.96 g/mL and *O.majorana* showed a density of 0.92 g/mL inferior than *C.limon* 1.13 g/mL. The pH values were 5.84, 5.01 5.47 and 5.40

For *O. majorana*, *C. citratus*, *C. limon* and *F. vulgare* respectively, these values indicat that these nanoemulsions could be important as coating since the pH could influence the antimicrobial activity (Wiegand et al., 2015)

The droplet sizes (nm) and the polydispersity index values are in table (10) were the particular size is under 200nm which indicate a monomodal pattern, however higher PDI values for *F. vulgare* implies more variability that could alter its physiochemical characteristics and efficacy, in a study by Dhanasekaran et al. (2024), the nanoemulsion containing lemon essential oil showed a particle size of 130 nm, these physical parameters are influenced by several factors such time of homogenization, temperature, pH, and the concentration of the essential oils within the nanoemulsions (Karimirad et al., 2019). Other studies by (Sabry et al. 2022 and Patel & Ghosh, 2020) revealed partial size ranging from 129 nm to 137 nm, in addition particle size of 83 nm was shown in ultrasonic emulsification with Tween 80 to create thyme essential oil nanoemulsion, respectively. The greater stability is indicated by PDI values ≤0.2, which means that oil droplet sizes are consistent or have monomodal distributions (Li et al., 2021; Patel & Ghosh, 2020).

Figure 25, shows the results of the morphological examination of nanoemulsification samples using the transmission electron microscope. Emulsion drying during preparation is the likely cause of the discrepancy between the particle sizes determined by TEM and the DLS equipment, according to the literature (Filippov et al., 2023). In a comparable manner, Aouf et al. (2020) produced well-dispersed, spherical nanoparticles with a narrow size distribution by encapsulating *Saccocalyx satureioides*, an Algerian species. Overall, there was a global significant difference ($P < 0.05$) between the tested nanoemulsions in term of physicochemical parameters.

These physicochemical parameters are important and must be taken in consideration since they are related to homogeneity, stability and the potential application of these nanoemulsions as coating in orange fruits.

Table 10. Physicochemical parameters of *O. majorana*, *C. citratus*, *C. limon* and *F. vulgare* nanoemulsions.

Property	**Value**			
	NE*O.majorana*	NE*C. citratus*	NE*C. limon*	NE*F.vulgare*
Refractive Index	1.34±0.006	1.33±0.004	1.472±0.005	1.34±0.001
Density (g/mL)	0.92±0.01	0.96±0.002	1.136±0.005	0.96±0.08
pH	5.84±0.01	5.01±0.008	5.47±0.009	5.40±0.06
Stability (%)	92.41±1.05	ND*	ND	89.17±2.56
Particle size (nm)	121.26±8.37	74.12 ± 2.33	103 ± 3.72	115.33±5.77
PDI	0.27±0.02	0.19±0.04	0.22±0.07	0.31±0.05

ND:Not Determined

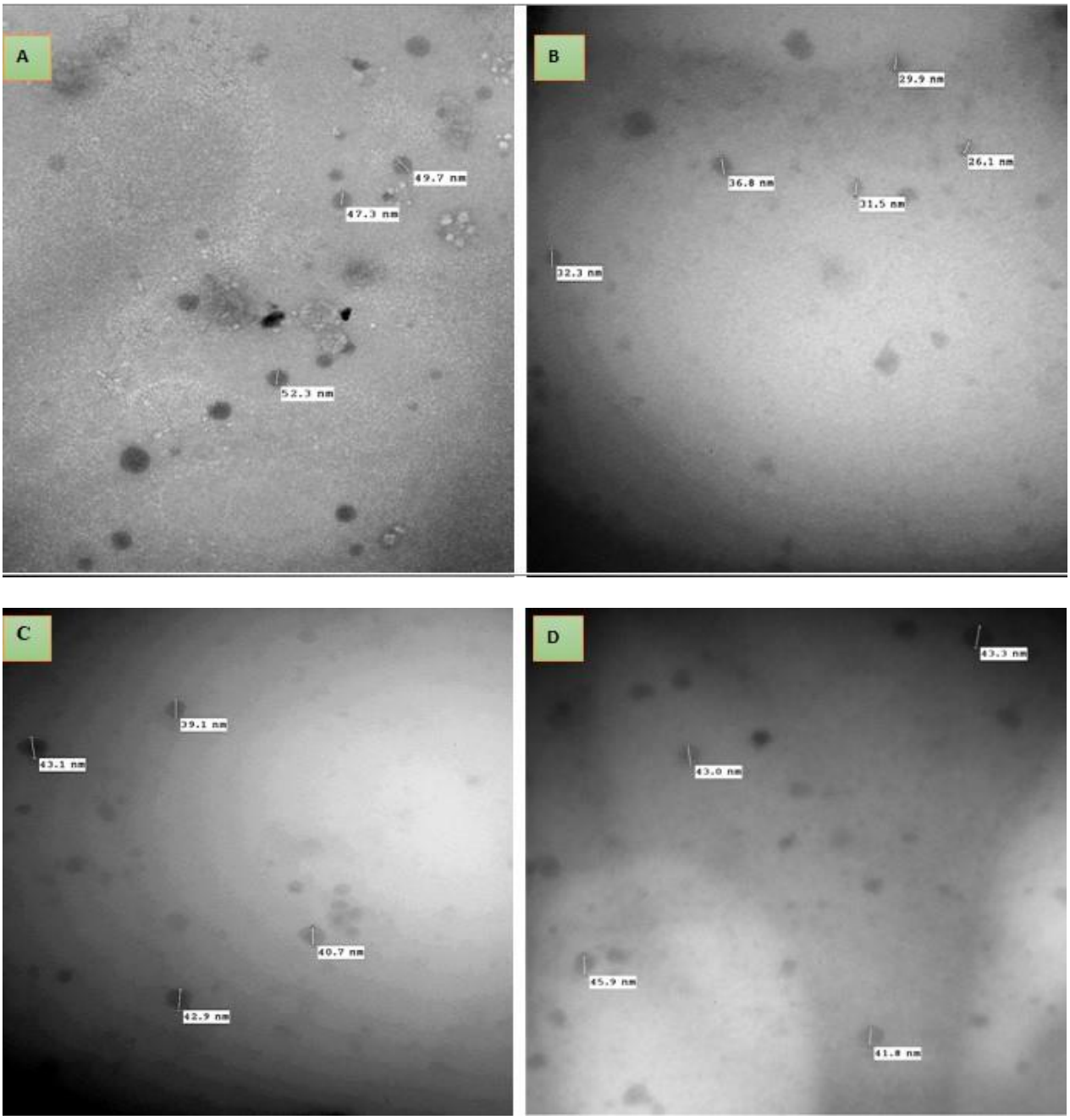


Figure 25. *O. majorana* (A), *C. citratus* (B), *C. limon* (C) and *F. vulgare* (D) nanoemulsions droplets under TEM microscope.

3. Morphological, molecular and phylogenetic features

The classification and identification of *Penicillium* species is challenging because to their structural similarities, which exhibit only minor variances (Gardoso et al., 2007). In terms of macroscopic characteristics, the first fungus (GY1) exhibited white mycelia at the colony's periphery, with a predominant blue-green color of conidia at the centre for both media (Figure 26, a1, a3) consistent with the findings of Abastabar et al. (2016). The reverse side displayed creamy to brown and orange colors for Sabouraud and PDA (Figure 26, a2, a4) respectively. Regarding the second fungus (GY2), the color was grey with white mycelia on Sabouraud media at the periphery (Figure 27, a1), similar to (GY1), while on PDA it exhibited an olive-green color, with a grey reverse for both media (Figure 27, a2, a4), these results align with those of Desouki et al. (2023). The colonies of (GY2) on Sabouraud medium were narrow and spread across the plate (Figure 27, a1), and the texture of both fungi was velutinous.

Concerning the microscopic characteristics of both fungi (GY1 and GY2), the conidiophores exhibit a Bruch-like appearance, with (GY1) displaying a more complex structure (Figure 26, b1, b3) in comparison to the second fungus (GY2). The spores of (GY1) are spherical to ellipsoidal and separated, whereas the second fungus presents oval, ellipsoidal spores in chains. These findings confirm that both species belong to the *Penicillium* genera; however, precise species identification is challenging due to their shared morphology, necessitating the use of modern molecular tools, which are highly effective in fungal classification (Allawi et al., 2022).

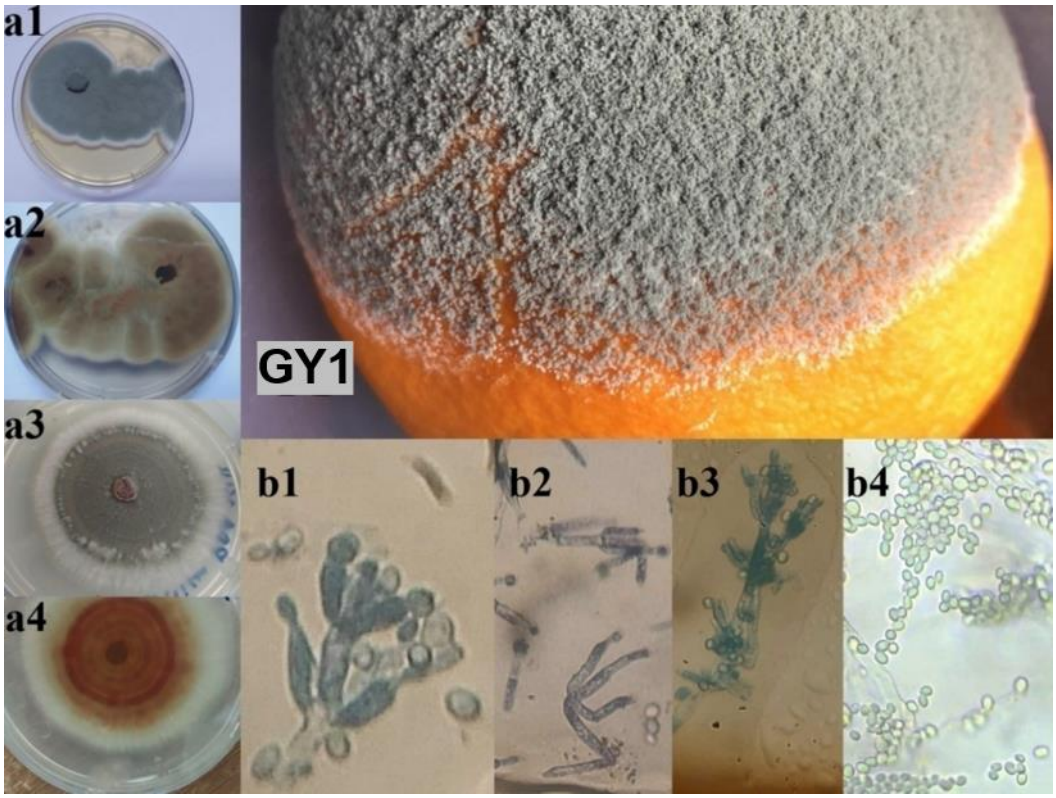


Figure 26. Macroscopic (a), and microscopic observation (b), of the isolated fungi (GY1) from decaying orange fruits, where (a1 and a2), and (a3 and a4) represent the aspect on PDA and Sabouraud media, respectively.

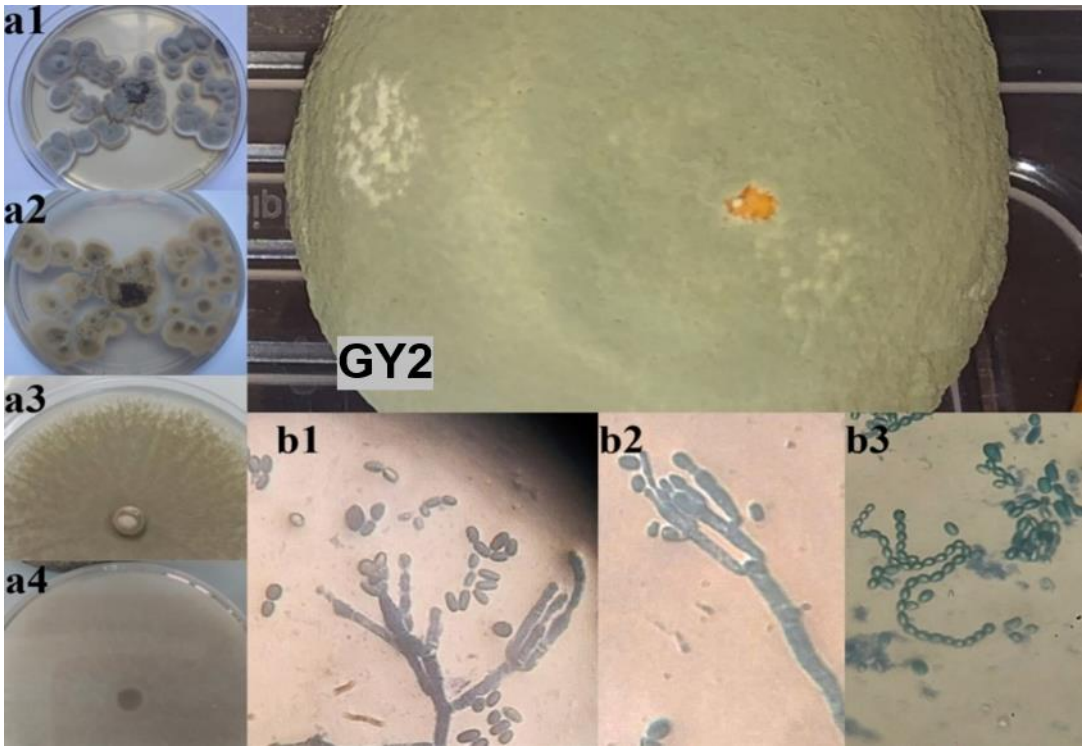


Figure 27. Macroscopic (a), and microscopic observation (b), of the isolated fungi (GY2) from decaying orange fruits, where (a1 and a2), and (a3 and a4) represents the asaspect on PDA and Sabouraud media, respectively.

The genomic analysis of the two fungal isolates (GY1) and (GY2), was conducted using a BLAST search against the GenBank database as part of the identifying process.

According to ITS sequencing, the first fungus (GY1) exhibited similarities to both *P. italicum* and *P. expansum*, while the second fungus shown 99% similarity to *P. digitatum*. The two related species in the genera *P. italicum* and *P. expansum* exhibited 100 % ITS sequence identity; hence, the ITS region was insufficient for differentiation. Introns in the TEF1 region contribute to its increased variability and enhanced species-level selectivity (Haouhach et al., 2020). Supplementary BLAST analysis confirmed that (GY1) corresponded to *P. expansum* 100 % match, and (GY2) corresponded to *P. digitatum* 100 % match. Results align with those of Louw and Korsten (2015), who established that *P. digitatum* was among the principal pathogens responsible for substantial lesions on contaminated citrus fruits. It is widely recognized that *P. digitatum* is the most damaging fungus affecting orange fruits (Palou, 2014; Platania et al., 2012).

A research by Vilanova et al. (2012) indicated that *P. expansum* is rarely encountered in citrus fruits; however, there are specific factors that may facilitate the infection of oranges by *P. expansum*. In addition, Veljović et al. (2017) indicated that *P. expansum* is able to infect orange fruits only under particular conditions, in contrast to *P. italicum*, which is one of the primary pathogens associated with decaying citrus fruits; however, Moosa et al. (2019) and Khokhar et al. (2021) documented its presence for the first time in *Citrus reticulata* and *Citrus limon*, respectively, El-Dawy et al. (2021) determined that *P. expansum* exhibited the highest prevalence at 38.2 % and a notable frequency of 16.7 %. Moreover, limited studies have recognized *P. expansum* as a pathogen affecting citrus crops.

Furthermore, our study documented the presence of *P. expansum* in orange fruits for the first time

Fungi constitute a highly diverse category, complicating the distinction between closely related taxa; species within genera such as *Penicillium* and *Aspergillus* display considerable physical similarities, complicating their differentiation, this situation requires the application of molecular and phylogenetic methodologies, incorporating several genetic markers with conserved sequences for the expedited classification of fungus (Ezeonuegbu et al., 2022).

This study employed Internal Transcribed Spacer (ITS) and Elongation Factor 1-alpha (EFA1) to investigate and analyze the evolutionary relationships among *Penicillium* species. Multiple isolated strains from various sources, as well as reference strains of *Penicillium*, were

chosen, with *Fusarium oxysporum* serving as an outgroup. The results presented in Figure 28 indicate that the phylogenetic analysis confirmed the classification of the two fungal isolates GY1 and GY2 as *Penicillium expansum* and *Penicillium digitatum*, respectively. They formed a sister group with other reference and isolated strains within the same genus and species. However, there is a lack of elongation factor sequences related to the *Penicillium* genus in GenBank databases, which is attributed to the significant intraspecific variation observed in this genus, necessitating the use of more reliable markers such as BenA, which is recommended for the identification of *Penicillium* species (Seo et al., 2022).

Furthermore, it was revealed that the *P. expansum* and *P. digitatum* groupings were situated inside the same clade, exhibiting genetic coherence; this confirms our prior findings about the *in vivo* pathogenicity of *P. expansum* on orange fruits. As previously indicated, *P. digitatum* is a significant pathogenic agent in *Citrus sinensis*; this correlation between our isolates confirms the pathogenic potential of *P. expansum* in Thomson orange fruits.

A bootstrap score range of 82 to 100 among the fungal strains in both trees (Figure 28, A, B) demonstrates the tree's robustness.

The NCBI database assigned the accession numbers PX680652 and PX680653 to the ITS sequences of the fungal isolates GY1 and GY2, respectively; however, these accession numbers are not yet publicly available. In addition, the translation elongation factor 1-alpha (TEF1-α) gene sequences of both isolates were also deposited in the NCBI database.

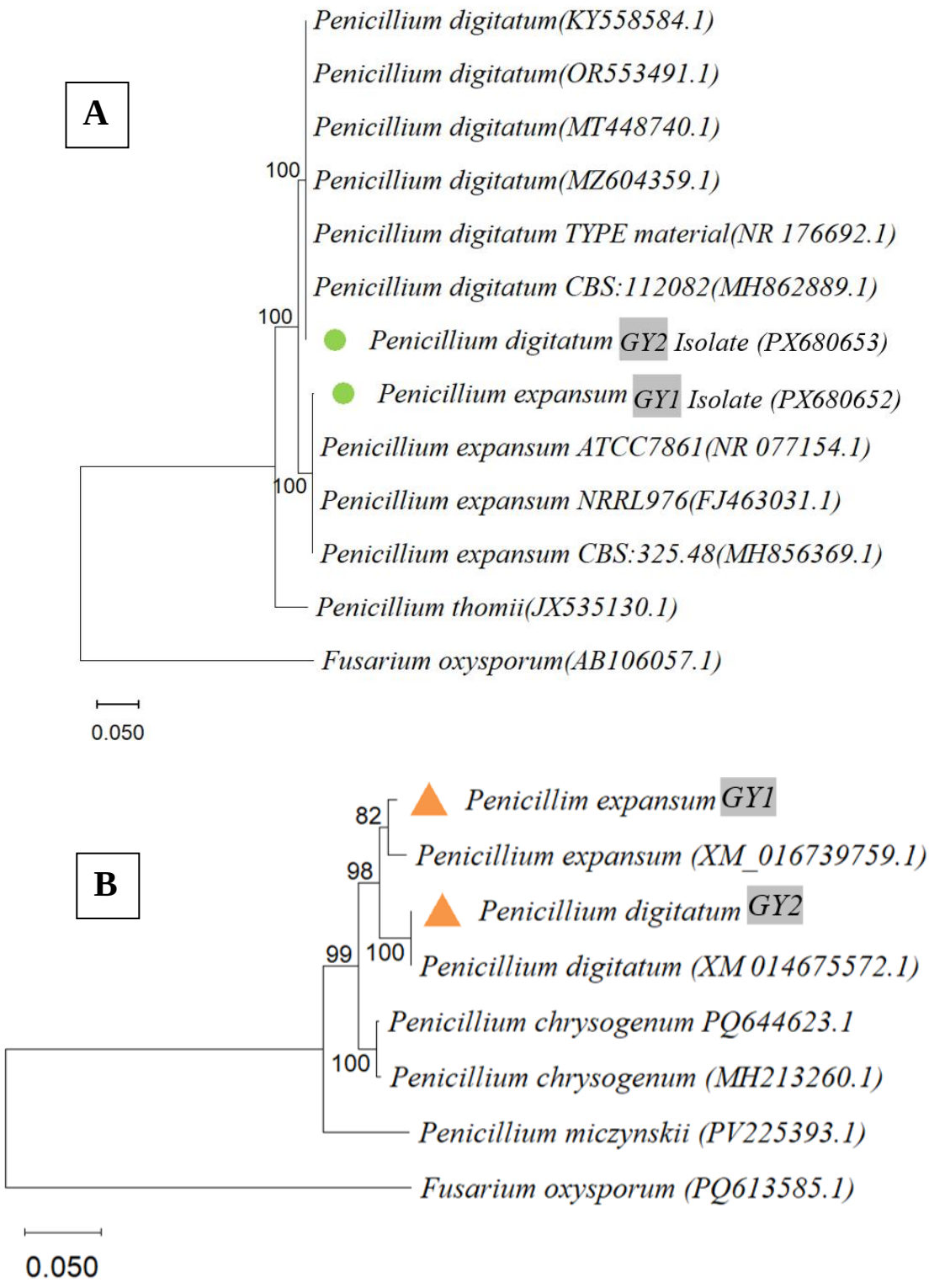


Figure 28. Phylogenetic trees of the isolated fungi (GY1 and GY2), constructed using (A) ITS sequences and (B) elongation factor alpha sequences.

4. Antibacterial and Antifungal activities

In the present study, the antimicrobial efficacy of both free and nanoemulsified essential oils was assessed employing the Disc diffusion and Microdilution methods. The diameters of inhibition zones obtained from the disc diffusion method (Figure 29) revealed variable results against Gram-negative and Gram-positive bacteria. Furthermore, it was observed that the antimicrobial activity of both essential oils and their nanoemulsions is dose-dependent (Figure 29). Previous research indicated that the primary target of essential oils against bacteria is their cellular membrane, where they enhance permeability, resulting in the leakage of cellular contents and disrupting membrane functions (Saad et al., 2013).

O. majorana EO, was not active at a concentration of 5 µL against *P. aeruginosa*, while it showed moderate inhibition with 12 ± 1.63 mm and 14.66 ± 0.94 mm at 10 and 15 µL respectively (P<0,0001), in studies by Ouedrhiri et al. (2016) and Ghazal et al. (2022), the activity was weak against *P. aeruginosa* at 10 µL with 9.66 ± 0.57 mm and 8 mm as inhibition diameters respectively. In contrast, OMEO showed significant activity against *K. pneumoniae*, *E.coli*, *S aureus*, and *B. subtilis* with inhibition zones ranging from 23.66 ± 3.21 mm to 34.33 ± 0.58 mm, these results were in line with Raouafi et al. (2021); and Ibrahim et al. (2017) who reported potent activity with inhibition zones of 20 ± 1.7 mm against *K. pneumoniae*, 20 ± 1.2 mm and 17.5±0 mm against *E.coli*, 39 ± 1.41 mm against *S.aureus*, and 22 ± 1.4 mm against *B. subtilis*.The strong antibacterial activity of *O. majorana* essential oil is mostly associated to terpinen-4-ol, γ-terpinene, α-terpinene, α-terpineol, and p-cymene, while these compounds are known to provide significantly to its efficacy (Ghazal et al., 2022; Raouafi et al., 2021). In contrast, weak activity against *K. pneumoniae* was observed by Ghazal et al. (2022), with diameter of inhibition of 10 ± 0.3 mm, while Ramos et al. (2011) reported moderate effectiveness against *K. pneumoniae* with inhibition zone of 13mm. *O. majorana* EO showed moderate to strong inhibition against *S.enterica* with inhibition zone ranging from 12 ± 0.81 mm to 23± 1.41 mm, which was in agreement with Amor et al. (2019), who found diameters of inhibition ranging from 14 ± 1.7 mm to 17.7 ± 0.6 mm at 10 µL and 15 µL respectively.

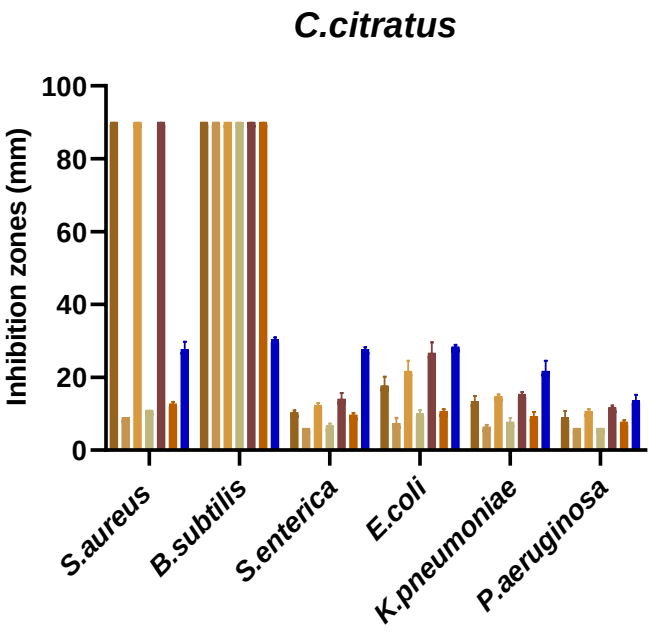
C. citratus EO exhibited the strongest antibacterial activity especially against the Gram-positive bacteria (Figure 29) where it totally inhibits the growth of both *B. subtilis* and *S. aureus* at all the tested concentrations (90 ± 0) mm, with no significant difference (P >0,9999), this confirm the results obtained by Shendurse et al. (2021) and Olaiya et al. (2016) who reported strong activity against *B.subtilis* and *S.aureus* with inhibition zones of 32 ± 0.75 mm and 48.0

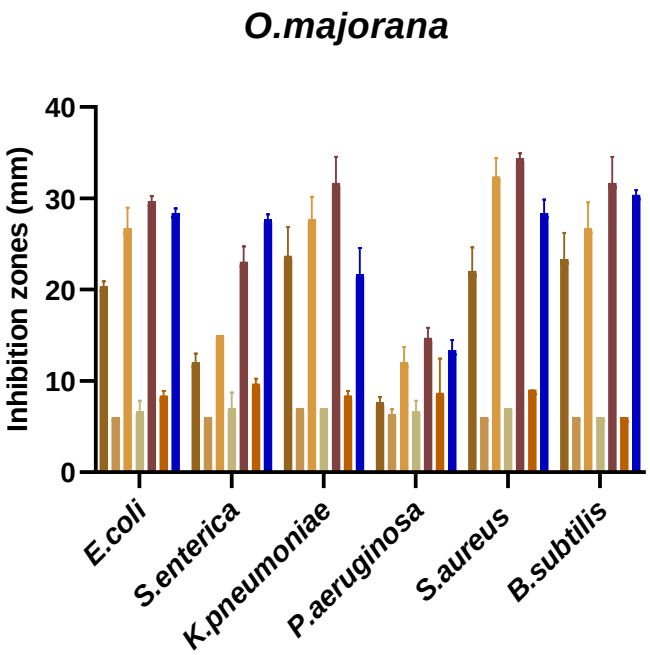
± 1.05 mm; 20 mm, respectively, in contrary, Stoica et al. (2019) reported reduced activity against *S.aureus* with inhibition zone of 12 mm, additionally, lemongrass EO showed strong activity against the Gram negative bacteria *E.coli* with inhibition zone diameters varying from 17.67 ± 2.51 mm to 26.67 ± 2.89 mm, this is comparable to the study by Partovi et al. (2019) who reported strong activity with diameter of 20.16 ± 0.28 mm, the strong potency of *C.citratus* is likely attributed to 1, 8- cineole, p-cymene, α- and β-pinene, limonene, α-terpineol and camphene, besides neral and geranial known as citral (Machraoui et al., 2018; Mukarram et al., 2021). *C. citratus* showed moderate to strong activity against *K. pneumoniae* and *S. enterica*, with inhibition zone diameters of 15.33 ± 0.57 mm and 14 ± 1.73 mm at 20 µL, respectively, better results were established in the findings of Ali et al. (2017) who reported strong activity against *K. pneumoniae* and *Salmonella*, with inhibition zone diameters of 30 mm and 23 mm, respectively. However, despite the significant activity of lemongrass against Gram-positive bacteria, a notable difference (P<0,0001) remains when compared to Gram-negative bacteria. Though it is among the most resistant bacteria, the evidence of this is the limited efficiency against *P. aeruginosa*. The cellular structure most certainly explains the variations in efficiency against the two types of bacteria (Chao et al., 2000).

At 15 µL, *citrus limon* essential oil had notable efficacy against *S. aureus*, with an inhibitory zone of 28.33 ± 2.89 mm. These findings accordance up with those of Aruna et al. (2022) who found a 22 mm inhibitory zone against *S. aureus*, Gupta, (2022) hypothesised that the presence of bioactive compounds such limonene, β-myrcene, and α-pinene could be related to this significant activity. On the other hand, *C. limon* essential oil showed insignificant action against *B. subtilis* at 15 µL (P<0,0001) providing an inhibition zone of 13 ± 1 mm, however no activity was shown against *S. enterica* and *B. subtilis* at 5 µL, so providing an inhibition zone of 13 ±1 mm. This result supports the findings of a 13.63 mm inhibition zone against *B. subtilis* observed by Mehmoud et al. (2019). Li et al. (2021) reported 12.33 mm inhibitory zones against *S. typhimurium*. *E. coli* and *P. aeroginosa* were resistant in the studies by Kehal et al. (2023) and Ibrahim et al. (2024), these results are consistent with our study, in which lemon oil exihibited weak action against *P. aeroginosa* at 15 µL with an inhibition zone of 11.66 ± 0.57 mm while no activity was observed at 5 µL and 10 µL (P <0,0001), Himed et al. (2019) carried research in Algeria showed weak activity against *K. pneumoniae*.

Concerning *F. vulgare*, the essential oil displayed the weakest antibacterial activity compared to the other tested essential oils (Figure 29), it was in particularly not active against *P. aeruginosa*. *F. vulgare* EO showed weak to moderate activity, with inhibition zones ranging

from 9.67 ± 0.58 mm to 13 ± 2 mm and from 12.67 ± 0.47 mm to 15.33 ± 0.47 mm against *K. pneumoniae* and *E. coli*, respectively, these results are comparable to those obtained by Gulfraz et al. (2008) who reported inhibition zones of 14 ± 0.5 mm and 16 ± 0.4 mm against *K. pneumoniae* and *E. coli*, respectively. However, *F. vulgare* essential oil presented moderate to strong activity against *S. enterica* and *B. subtilis*, with inhibition zones of 11.33 ± 1.53 mm to 20.33 ± 0.47 mm respectively. This was proch to the results reported by Kazemi et al. (2012) who reported potent activity against *Salmonella* and *B. subtilis* with 15 mm and 30 mm inhibition zones respectively. This activity is likely associated to estragole, the main compound of *F. vulgare* essential oil, which increases the non-specific permeability and destruct the bacterial cell membrane, especially in Gram-negative bacteria, in addition, compounds like fenchone and limonene from *F. vulgare* essential oil have been found to protect against bacterial infections (Rashid et al., 2023). The effectiveness of FVEO against *S. aureus* was significant at higher doses with inhibition zones of 21.67 ± 2.89 mm, in comparaison to the dose of 5 µL, however with no significant difference (P= 0,9995), these results are consistent with Upadhyay, (2015) who reported inhibition zone of 21.60 ± 0.28 mm against *S. aureus*. Another study by Khalid et al. (2014) demonstrated strong activity against *S. aureus* 28 ± 1.5 mm to 37.4 ± 0.7 mm and weak to moderate activity against *P. aeruginosa* 9.8 ± 1.2 mm to 13.2 ± 1.2 mm.





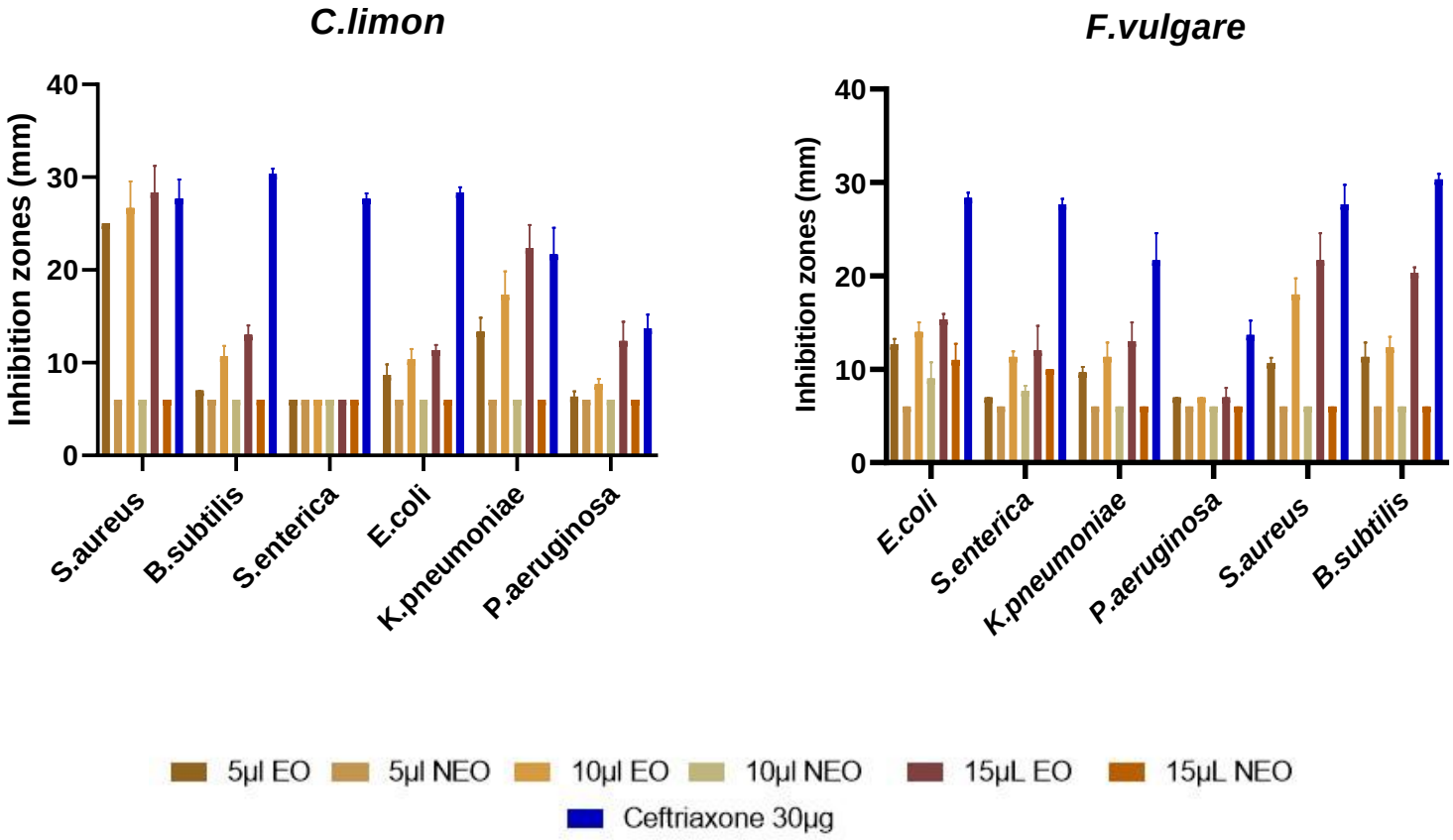


Figure 29. Inhibition zones exerted by *O. majorana*, *C. citratus*, *C. limon* and *F. vulgare* EOs and their nanoemulsions against bacteria with ceftriaxone as the standard inhibitior, shown with error bars indicating ± standard diviation (n = 3).

Consistent with previous results, the microdilution method (Figure 30) showed *C. citratus* being more potent regarding efficacy, with minimum inhibitory concentration (MIC) values of 0.25 % (v/v) against *S. aureus*, *B. subtilis*, and *P. aeruginosa*; 0.12 % (v/v) against *E. coli* and *K. pneumoniae*; and 0.5 % (v/v) against *S. enterica*, these results are in line with Subramaniam et al. (2020), who showed notable activity of lemongrass essential oil against *K. pneumoniae* and *E. coli*. Furthermore, supporting these results are those of Naik et al. (2010) who recorded activity of 0.06 % (v/v) against *S. aureus* and *B. subtilis* and 0.12 % (v/v) against *E. coli*. In addition, observed by Ahmad and Viljoen (2015) the MIC values were of 0.06 mg/mL and 0.12 mg/mL against *E. coli* and *S. aureus* respectively.

O. majorana EO showcase MIC values of 0.25 % (v/v) against *K. pneumoniae* and *P. aeruginosa*, 0.5 % (v/v) against *S. enterica*, *E. coli*, and *S. aureus* and 1% (v/v) against *B. subtilis*. Ouedrhiri et al. (2016) reported MIC and MBC values of (0.25/2 %, 0.125/2 %, 0.25/0.25 %) (v/v) against *S. aureus*, *B. subtilis*, and *E. coli*, respectively.

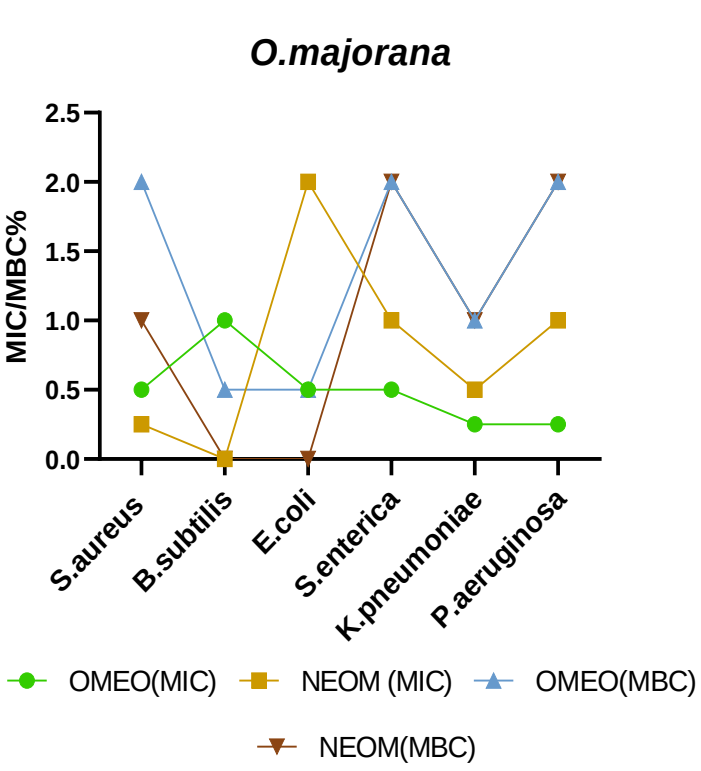
Concerning lemon EO, the MIC values were 0.5 % (v/v) against *S. aureus*, 1 % (v/v) against *E. coli* and *K. pneumoniae*, 2 % (v/v) efficacy against *B. subtilis* and *S. enterica*; inferior results were obtained by Galgano et al. (2022) who reported a minimum inhibitory concentration (MIC) of 1.25 % (v/v) against *S. aureus*, while AL-deen et al. (2021) identified MIC values of 4 µL/mL and 40 µL/Ml against *Bacillus* and *Pseudomonas*, respectively. Clearly, the essential oils proved to be more effective against Gram-positive bacteria, however, Frassinetti et al. (2011) showed, that the MIC values for Gram-negative bacteria were more favorable, implying that Gram-positive bacteria showed increased resistance to lemon essential oil.

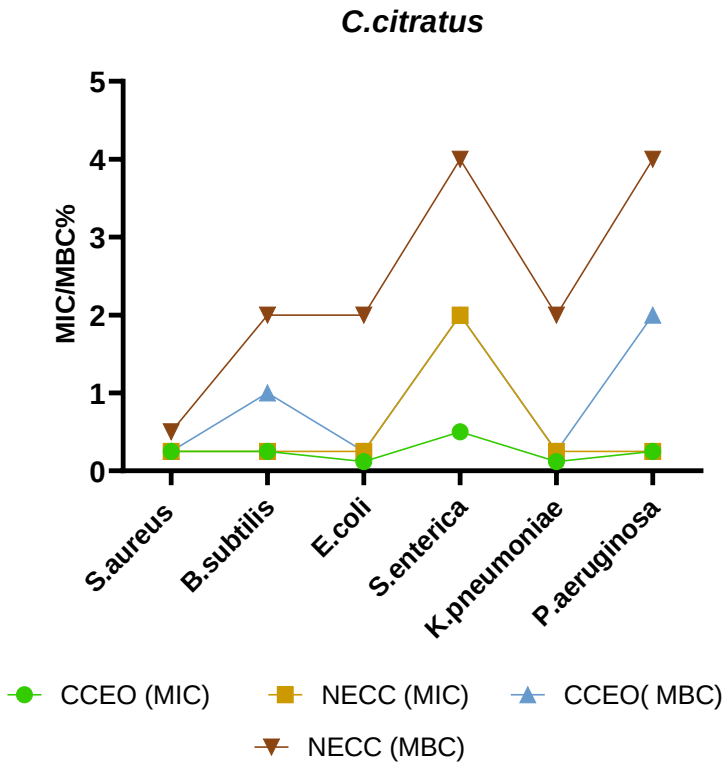
F. vulgare essential oil was the weakest exhibiting MIC values of 2 % (v/v) for all bacterial strains, with the same inhibition as *C. limon* against *B. subtilis* and *S. enterica* ($P > 0.05$), these results are comparable to the study by Mohsenzadeh (2007) who found MIC and MBC values of 2 ± 0.13 %; 4 ± 0.26 % (v/v) against *S. aureus* and 1 ± 0.03; 2 ± 0.05 % (v/v) against *E. coli*. Conversely, Bisht et al. (2014) showed better activity with MIC values at 0.062 % (v/v) and 0.031 % (v/v) against *E. coli* and *Salmonella*, respectively.

Numerous studies have elucidated the mechanism of action of essential oils; however, it remains incompletely understood in the context of nanoemulsions (Khairan et al., 2024). Comparatively to the pure essential oils, the nanoemulsified ones showed less potency (Figure 33, 34). It was observed that the efficiency of lemongrass essential oil matched well the efficiency of its nanoemulsion. Still, this does not always apply to all essential oils, showing inhibition zones of 90 mm (total inhibition), *Cymbopogon* NE displayed significant variation ($P < 0,0001$) in term of activity against *S. aureus* in comparaison to the pure essential oil, another hand, with an inhibiting zone of 9.33 ± 1.15 mm, *K. pneumoniae* had the lowest activity observed. The minimum inhibitory concentration (MIC) values of lemongrass nanoemulsion (Figure 30) were 0.25 % (v/v) for all strains, except for *S. enterica*, which showed an MIC of 2 % (v/v). on the other hand, *O. majorana* and *F. vulgare* nanoemulsions showed weak activity, in comparaison to *C. citratus* nanoemulsion ($P < 0,0001$), ranging from 9.6 mm to 11 mm respectively, besides, *F. vulgare* was not active against *S. aureus*, in contrast, Almasi et al. (2019) reported better activity of *O. majorana* nanoemulsion with 14.32 ± 1.02 mm and 12.65 ± 0.94 mm inhibition zones against *S. aureus* and *E. coli*, respectively. Moreover, FV and OM nanoemulsions were not active against *B. subtilis* and *K. pneumoniae*, with insignificant activity showed by *O. majorana* against *K. pneumoniae* at 15 µL. the minimum inhibitory concentrations of marjoram nanoemulsion varied from 0.25 % (v/v) to 2 % (v/v), while the

nanoemulsified *F. vulgare* was not active. Rasti et al. (2023) indicated that *O. majorana* nanoemulsion showed lower MIC value against *S. aureus* in comparison to *E. coli*, a different study by Venkadesaperumal et al. (2015) indicated that *F. vulgare* nanoemulsion showed an MIC value of 80 µL/mL against *K. pneumoniae*, *E. coli* and *salmonella*.

Lemon nanoemulsion (Figure 29) was ineffective against all tested strains (P >0,9999), in contrast to the findings of Yazgan et al. (2019) who reported moderate to strong activity of *C. limon* nanoemulsion with inhibition zones of 14.38 ± 0.63 mm, 17.88 ± 0.48 mm, and 16.63 ± 0.48 mm against *K. pneumoniae*, *S. paratyphi*, and *S. aureus*, respectively. Additionally, Ambrosio et al. (2020) demonstrated that the microemulsion of citrus essential oil enhanced the antibacterial activity, potentially due to the potent effects of chitosan present in the emulsion. Furthermore, this variation in results could be assigned to various factors, including various strains of bacteria tested and methodological variations (Gago et al., 2019).





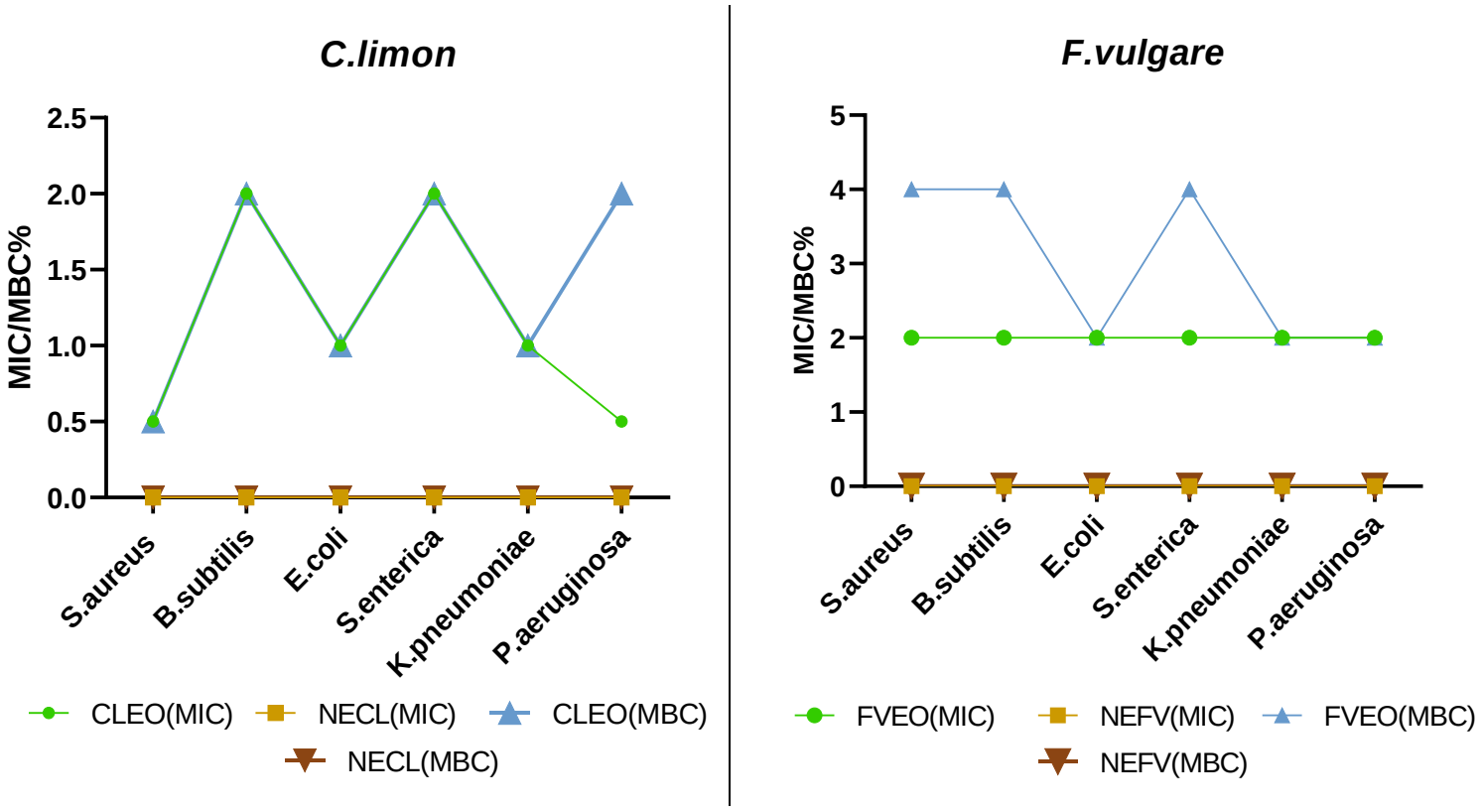


Figure 30. MIC and MBC determination of *O. majorana*, *C. citratus*, *C. limon* and *F. vulgare* EOs and their nanoemulsions against bacteria.

Fungal infections and mycotoxin production are increasing over time. Moreover, these fungi are challenging to eliminate due to their eukaryotic nature, and the excessive use of synthetic fungicides has resulted in the development of resistance (Nazzaro et al., 2017; Mutlu-Ingok et al., 2020). However, essential oils are recognized for their potent antifungal and anti-mycotoxigenic properties, attributed to their active compounds (Tian et al., 2022).

In the current research, the antifungal efficacy was assessed against the most harmful pathogens, including *Aspergillus*, *Penicillium*, and *Fusarium* species, in addition to *Candida albicans*. The results indicated that all the essential oils tested (marjoram, lemongrass, lemon and fennel) exhibited superior antifungal activity compared to antibacterial activity (Figure 35). This phenomenon may be attributed to various factors, including the type, structure, and resistance of the microorganisms, as well as the physicochemical conditions such as temperature, pH, and oxygen, which may also influence the antimicrobial effects of essential oils in general (Seow et al., 2014). In this study, Lemongrass essential oil demonstrated impressive level of activity (Figure 31), achieving total inhibition against *A. flavus*, *A. niger*, *F. culmorum*, *P. expansum*, and *C. albicans* with no significant difference ($P<0.001$).

Additionally, it displayed significant activity against *P. digitatum*, with inhibition zones ranging from 78.66 ± 0.94 mm to 90 ± 0.00 mm. These findings are consistent with prior research by Tyagi and Malik (2010), Hassan et al. (2022) and Boukhatem et al. (2014) which reported complete inhibition of various fungal strains, including *A. flavus*, *A. niger*, *F. oxysporum*, *Penicillium* sp., and *C. albicans*. In contrast, studies by Da Silva et al. (2008) and Ali et al. (2017) indicated diminished activity against *C. albicans*, with inhibition zones measuring 27.4 mm and 29 mm, respectively. Monoterpenes such as geraniol, α-Citral, and β-Citral are responsible for the pronounced antifungal activity of lemongrass essential oil (He et al., 2023; Rhimi et al., 2022).

Besides *C. citratus*, *O. majorana* and *C. limon* essential oils showed great antifungal activity than *F.vulgare* EO (P <0,0001), marjoram EO exhibited inhibition zones ranging from (33.3 ± 2.88 mm to 89.33 ± 1.15 mm with the most potent activity shown against *P.digitatum* (Figure 35), Some studies reported the antifungal activity of *marjoram* EO, Raouafi et al.(2021) reported complete inhibition of *F. oxysporum* by *O.majorana* essential oil, Helal et al.(2006) showed inhibition zones of 29 ± 3 mm, 27 ± 2 mm, 28 ± 3 mm, and 23 ± 2 mm mm against *A. flavus, A. niger, P. digitatum* and *C.albicans*, respectively. In addition, Nikkhah et al., (2017) reported 23.7 ± 0.1 mm inhibition zone against *P. expansum*. Better results were found in this study with inhibition zones of 37.5 ± 2.04 mm to 66.66 ± 3.11 mm against *A. niger*.

Lemon EO completely inhibits the growth of *A. niger*. These results align with a study conducted in Algeria by Hamdani et al. (2015) and Hernawan et al. (2015), where lemon oil was demonstrated to completely inhibit the growth of *Fusarium*, *Penicillium* species, and *Candida albicans*, exhibiting remarkable efficacy against other strains. The most potent activity was recorded against *Penicillium expansum*, with an inhibition zone of 79.33 ±1.15 mm, while the least activity was noted against *Candida albicans*, with an inhibition zone of 38.33 ± 2.88 mm (Figure 31), conversely, no activity was detected against *Penicillium digitatum* in a study by Vitoratos et al. (2013). It is believed that the antifungal properties of *Citrus limon* are attributed to terpenes such as limonene (Viuda-Martos et al., 2008).

F. vulgare essential oil showed great antifungal activity against *A. niger* and *P. digitaum* in comparison to the other strains with in inhibition zones of 61.66 ± 4.71 mm to 90 ± 0 mm and 53.16 ± 2.35 mm to 71.66 ± 2.35 mm respectively, this was aligned to Singh et al. (2006) who reported a total inhibition of *A. niger* by fennel EO. It was noted that *P. digitatum* and *A. niger* were the most sensitive strains to both essential oils and their nanoemulsions, these results

are better than studies conducted in Algeria, who reported inhibition zones of 22.67± 0.57 mm against *Penicillium* sp and 21.67± 2.08 mm against *Fusarium* sp. (Barkat & Bouguerra, 2012) and 19 mm and 15 mm against *C. albicans* and *A. flavus*, respectively (Belabdelli et al., 2020).

The minimum inhibitory concentration of the evaluated essential oils demonstrated significant efficacy (Figure 32), with lemongrass exhibiting 0.06 % (v/v) against *F. culmorum*, *P. expansum*, and *C. albicans*, 0.03 % (v/v) against *A. niger* and *P. digitatum*, and 0.12 % (v/v) against *A. flavus*. These results correspond the findings of Bansod et al. (2008) who reported an MIC value of 0.06 % (v/v) against *A. niger*. lemon EO had comparable MIC values against *F. culmorum* and *P. digitatum*, recorded at 0.06 % (v/v) and 0.03 % (v/v), respectively, with no significant difference (P>0,9999); these results are superior to Viuda-Martos et al. (2008) who observed minimum inhibitory concentration (MIC) values of 0.94 % (v/v) for CLEO against *A. niger*, *A. flavus*, and *P. chrysogenum*. Other investigations yielded various outcomes. Rhimi et al. (2022), Sawadogo et al. (2022), and Matasyoh et al. (2011) found minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values for *C. citratus* of 2.5 µL/mL against *C. albicans*, 1.50 ± 0.12 and 2 ± 0.22 against *A. flavus*, and 15 mg /mL against *A. niger*, respectively. Ben Miri et al. (2018) and Ben Hsouna et al. (2017) determined the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values for *C. limon* as follows: 1.50 ± 0 / 1.75 ± 0 mg/mL against *A. niger*, 1.75 ± 0 mg/mL / 2 ± 0 mg/mL against *Fusarium* sp., 1.5 ± 0.43 mg/mL/ 1.75 ± 0 mg/mL against *Penicillium* sp., 0.625 ± 0.4 mg/mL against *A. niger*, 0.312 ± 0.6 mg/mL against *A. flavus*, and 0.312 ± 0.8 mg/mL against *F. culmorum*, respectively

Marjoram EO showed MIC values of 0.12 % (v/v) against *A. flavus*, *F. culmorum* and *C. albicans*, 0.25% against *P. expansum* and *A. niger*, the most potent activity of OMEO was shown against *P. digitatum* with MIC value of 0.03 % (v/v). In a study by Souza et al. (2010) the MIC value of *O. majorana* EO against *A. flavus* and *C. albicans* was 160 µL/mL; while Prakash et al. (2012) found MIC and MFC values of 3 µL/mL and 7 µL/mL of OMEO against *A. flavus*. In contrast, *F. vulgare* EO presented the weakest activity with MIC values of 2 % against *P. expansum*, *P. digitatum*, *C. albicans* and 1% against *A. niger* and *A. flavus* (P<0.001). Belabdelli et al. (2020) and Upadhyay (2015) showed MIC values by *F. vulgare* EO of 0.2 mg/mL against *A. flavus*, 0.25 mg/mL against *A. niger* and 0.16 mg/mL against *C. albicans*, 24 µg/mL against *A. niger* and 6 µg/mL against *C. albicans*, respectively.

The activity of nanoemulsified essential oils was inferior to that of pure essential oils (Figure 31), *C. citratus* exhibited superior activity compared to the other plants, demonstrating the strongest efficacy against *A. niger* with total inhibition 90 mm, Aguiar et al. (2014) mentioned that citronellal is the main compound responsible for the antifungal activity of *Cymbopogon* species, while the weakest activity was observed against *P. expansum* at 15.66 ± 1.5 mm, being the most resistant fungal strain, Cafelike et al. (2021) observed that the nanoformulation of *Cymbopogon narduns* inhibits the growth of both *P. expansum* and *A. niger*.

The activity of the nanoemulsions of *O. majorana*, *F. vulgrae* and *C. limon* against *P. digitatum* varied from weak to strong with inhibition zones of 13 ± 0 mm to 16 ± 2.82 mm, 10 ± 0 mm to 20 ± 2.82 mm and 7 ± 0 mm to 21.66 ± 2.35 mm respectively (Figure 35), in addition, it was noted that the efficacy of *C. limon* nanoemulsion increased significantly with the increase of the dose (P<0,0001), no activity was excerced by *C. limon, O. majorana* and F. *vulgrare* nanoemulsions against *P. expansum, F. culmorum, A. flavus* and *A. niger*, in the exception of *O. majorana* nanoemulsion which exhibited inhibition zones ranging from 15 ± 0 mm to 20 ± 0 mm against *A. niger*. This diminished activity observed in the nanoemulsion, as compared to the free essential oil (P<0,0001), is likely attributable to the absence of specific bioactive compounds such as terpenes, including α-Pinene and β-Myrcene, as reported by Nóbrega et al. (2021), who highlighted the significant antifungal properties of α-Pinene, alongside a reduction in D-Limonene concentration.

The MIC values of marjoram, lemongrass and lemon nanoemulsions ranged from 0.25 % (v/v) to 1 % (v/v), 0.03 % (v/v) to 1% (v/v), and 0.12 % (v/v) to 4 % (v/v), however fennel nanoemulsion was active only against *P. digitatum* with MIC value of 2 % (V/V), showing that fennel nanoemulsion is the weakest in comparison to the other tested nanoemulsions. However, no effect was observed against *C. albicans*. It is evident that the results obtained via microdilution are superior to those from the disc diffusion assay, likely due to the direct contact with microorganisms in the liquid medium, as previously mentionned.

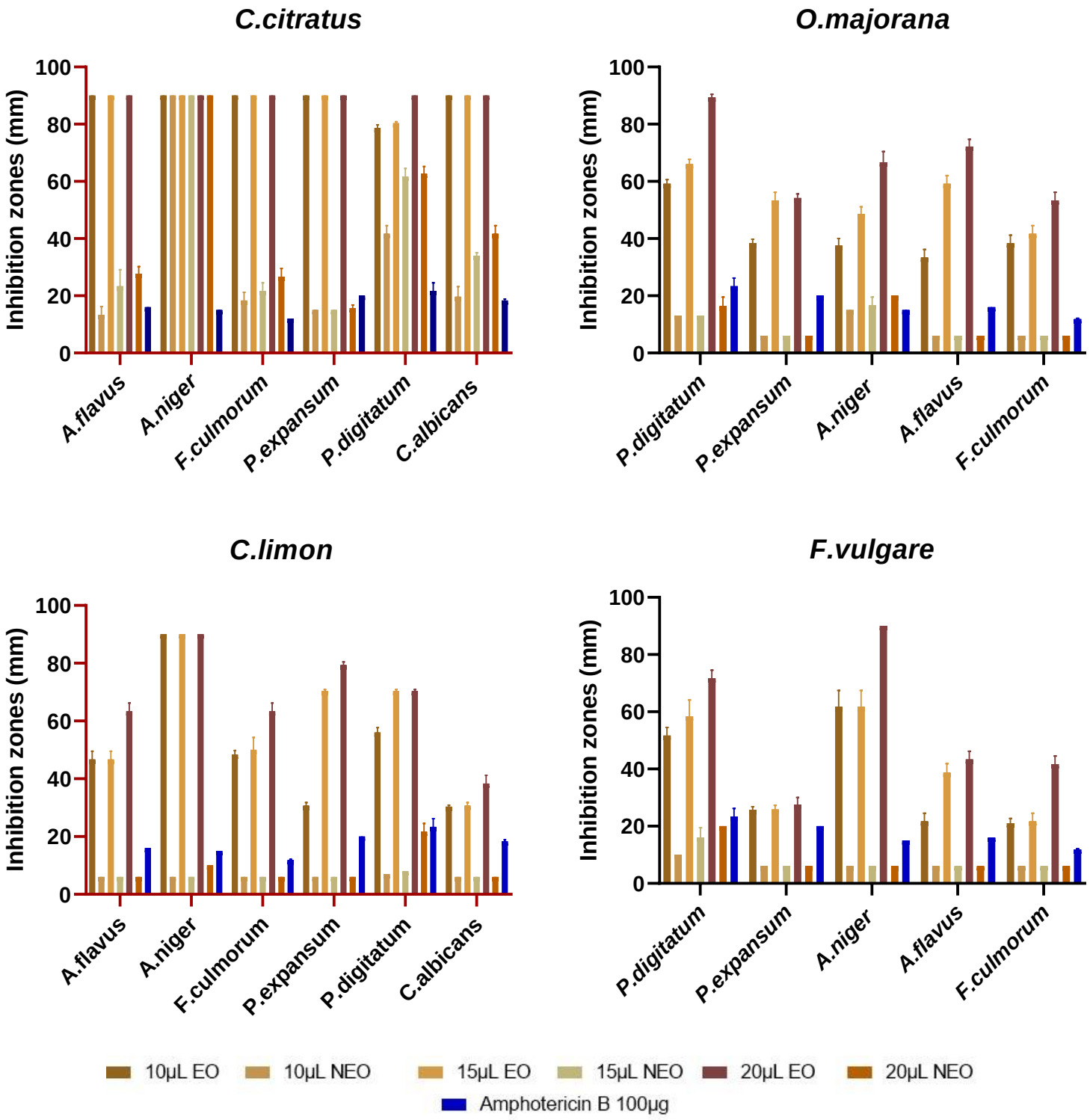


Figure 31. Inhibition zones excerted by *O. majorana*, *C. citratus*, *C. limon* and *F. vulgare* EOs and their nanoemulsions against fungi with amphotericin B as the standard inhibitor, show with error bars indicating ±standard deviation (n=3).

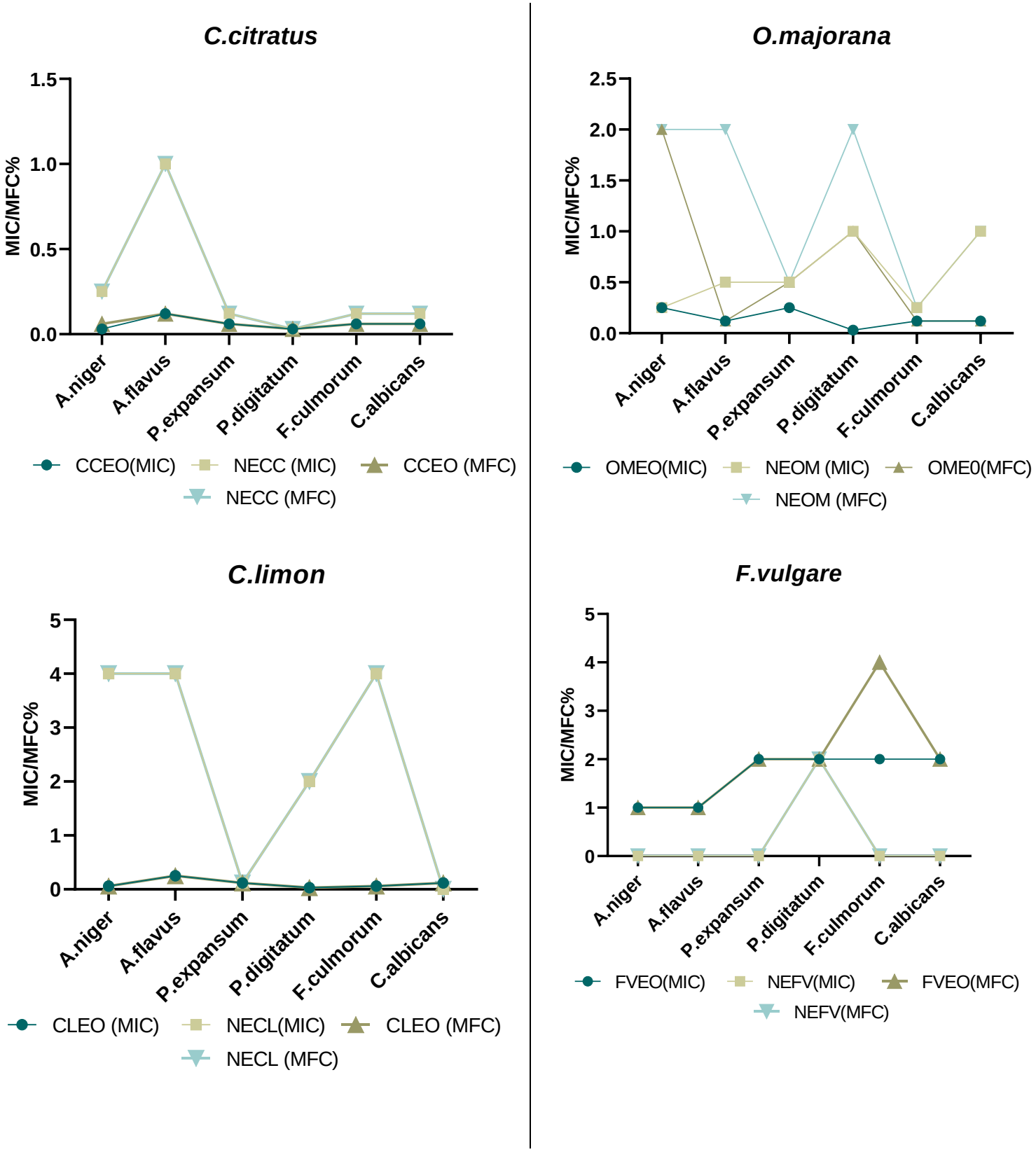


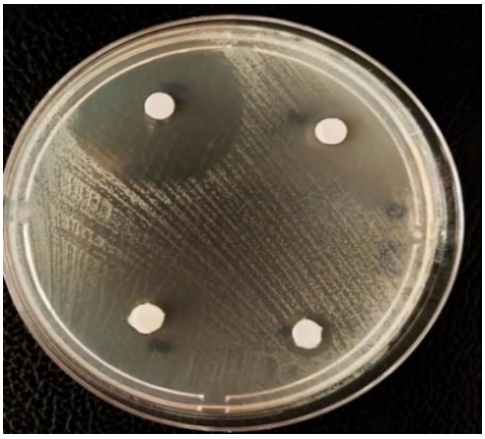
Figure 32. MIC and MFC determination of *O. majorana*, *C. citratus*, *C. limon* and *F. vulgare* EOs and their nanoemulsions against fungi.

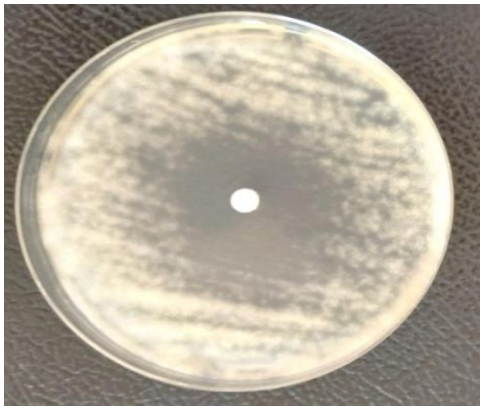
For the free essential oils and their nanoformulations, the minimum bactericidal concentration (MBC) values ranged from 0.25 % to 2 % (v/v) (Figure 30), the results revealed that the bactericidal activity of the essential oils was not significantly enhanced by the nanoemulsification process.

The minimal fungicidal concentration (MFC) produced somewhat variable results. Showing values between 0.03 % and 4 % (v/v) for the free essential oils and from their nanoemulsions (Figure 32), this variation suggests that nanoemulsions may greatly affect the fungicidal effect of essential oils more than their bactericidal action. The differences in MBC and MFC values between free and nanoemulsified essential oil imply that their antibacterial activity may be differently affected by the nanoemulsification technique.

This study indicated that, despite the advantages of nanoemulsification for essential oils, it does not necessarily enhance their antimicrobial efficacy.

a **b**





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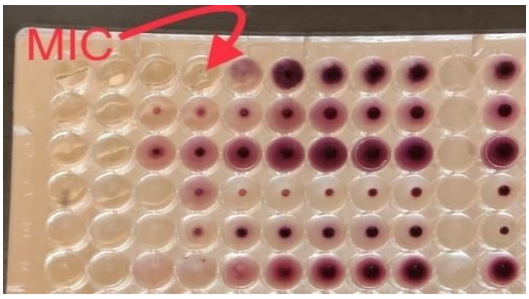

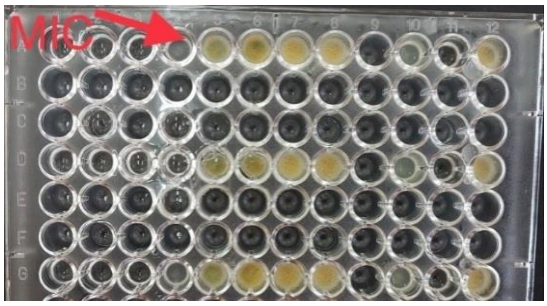

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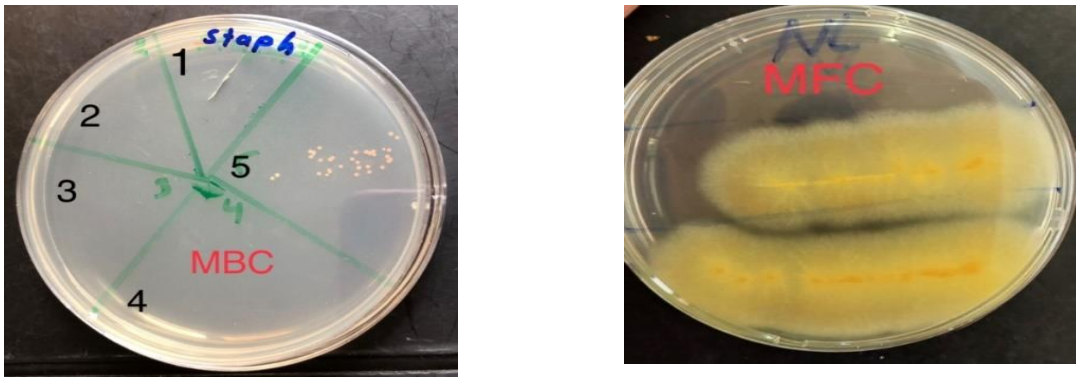


Figure 33. Antimicrobial activity results, with (a and b) representing inhibition zones, (c and d) respresent MIC s, while (e and f) represent MBC and MFC respectively.

5. Antifungal activity and coating application

Fruits are abundant in minerals, vitamins, organic acids, and various bioactive substances, rending this food products health beneficial for humans and at the same time, a favorable environment for the proliferation of harmful microorganisms (Liu, 2013). Postharvest disease losses exhibit considerable variability influenced by various factors, including climatic circumstances, fruit damage caused during harvesting, and post-harvest procedures (Zacarias et al., 2020).

Citrus fruits are susceptible to infections from harvesting to consumption, *P. digitatum* and *P. italicum*, known as green and blue molds respectively, are the most destructive spoilage diseases, particularly in oranges (Blasco et al., 2016).

In the present study, *O. majorana* (marjoram), *C. citratus* (lemongrass) and *C. limon* (lemon) nanoformulations were employed as coatings on orange fruits to inhibit the proliferation of pathogenic fungi, while simultaneously controlling the dissemination of *P. expansum* and *P. digitatum* within the fruits. Although *P. expansum* does not typically contaminate orange fruits, it was considered essential to examine the possibility of its involvement in spoilage, considering its isolation from the orange fruits.

The results from day 7, showed no fungal growth in fruits treated with *O. majorana* (T1), *C. limon* (T2), and *C. citratus* (T3) coatings besides the control group, however, the control group displayed some dark spots in the outer peel (Figure 34, A), these results align with Motamedi et al. (2018) where the control fruits displayed signs of dehydration and dryness during storage, in addition to some brown spots on the fruit peel, After 15 days of storage, no

fungal growth was observed within the treated fruits , this is explained by the fact that when combining essential oils with other technologies such nanoemulsions results in important antimicrobial activity (Freitas & Cattelan, 2018), however the control group displayed fungal growth in some areas (Figure 34). Beside the control group, lemongrass nanoemulsion treatment (T3) displayed some changes in peel color. By day 21, the fruits treated with *O.majorana* (T1) and *C.limon* (T2) nanoemulsions were intact in term of peel disorders and fungal growth (Figure 34) in comparison with the control group where the fungus spread in the same areas ,these results are consistent with various studies that indicated that coating containing essential oils reduced fungal growth in many types of fruits, Chen et al. (2016) and El-Mohamedy et al. (2015) reported that CMC+clove essential oil effectively control the fungal growth of *P.italicum* in mandarin fruits, additionally, orange fruits (*C.sinensis*) coated with lemongrass EO+chitosan coating had an extended shelf life in comparison with the control group where the coating prevent the growth of both molds (*P.italicum* and *P.digitatum*) respectively. Another study by Abdul-Rahaman et al. (2023) demonstrated that coatings containing orange and eucalyptus essential oils prolong the shelf life of orange fruits compared to the control group.

Fruits treated with *C. citratus* (T3) did not exhibit any signs of mold occurrence; nonetheless, they had some brown areas, additionally the peel color changes to dark orange, and noticeable water loss in the skin to some extent, for the control group as well (Figure 34). This phenomenon can be ascribed to the presence of essential oils in the oil glands of the fruit peel, which are susceptible to oxidation, citrus fruits exhibit cell darkening and browning when subjected to stressful conditions or injury, compounded by different environmental factors before and after harvesting which affect the outer layer of the fruit, iddentifying the particular origins of these defects can be challenging, as they can originate from various reasons (Zacarias et al., 2020).

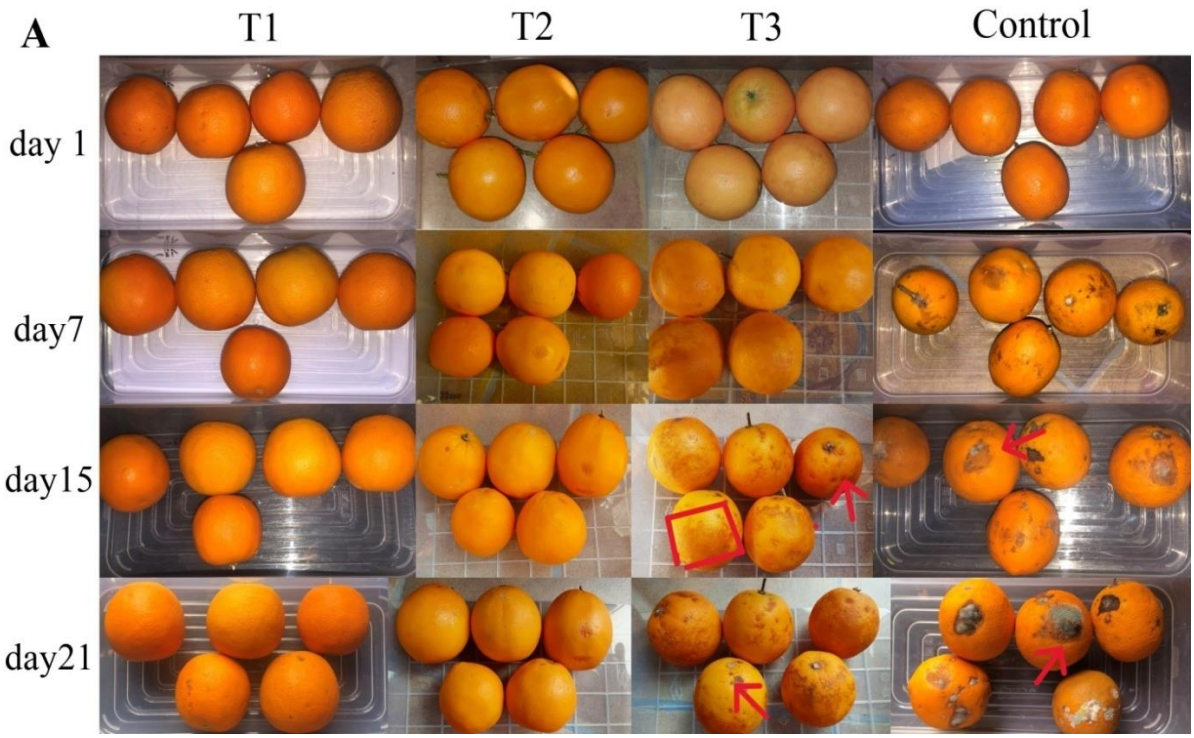


Figure 34. Application of nanoemulsions of *O. majorana* (T1), *C. limon* (T2), and *C. citratus* (T3) on orange fruits, alongside a control group treated with distillated water, during 21 days of storage.

After seven days of storage (Figure 35, B), *O.majorana* nanoemulsion showed no fungal growth in the fruits inoculated with *P. digitatum*, in contrast, *C.citratus* (T3) , *C.limon* (T2), beside the control group exhibited fungal growth at the inoculated sites, albeit with varying degrees of dissemination (Figure 35, B), Fruits treated with *C. citratus* (T3) nanoemulsion demonstrated less growth than those treated with *C. limon* nanoemulsion (T2) while the control group exhibited significant growth across nearly all fruits (Figure 35, B). This observation corroborates the findings of Rafiee et al. (2022) and Aminifard et al. (2018) who noted that control orange fruits displayed the highest level of decay compared to those treated with chitosan containing denak essential oil after 7 days of storage, as well as oranges treated with Anise and Caraway essential oils, respectively. Additional studies conducted on artificially inoculated fruits with *P. digitatum* by Alshallash et al. (2022) and Kharchoufi et al. (2018) demonstrated that treating infected oranges with chitosan nanoparticles significantly inhibited the growth of *P. digitatum* compared to untreated samples, which exhibited substantial fungal invasion within just four days, furthermore, oranges treated with chitosan and locust bean gum markedly suppressed the growth of *P. digitatum*, respectively. By day 15, *P. digitatum* proliferated extensively in the affected fruits except for those treated with marjoram nanoemulsion, where the proliferation was not important. After 21 days of storage, fungal

growth remained constant across all fruit samples, with complete coverage of the affected fruits (Figure 35, B).

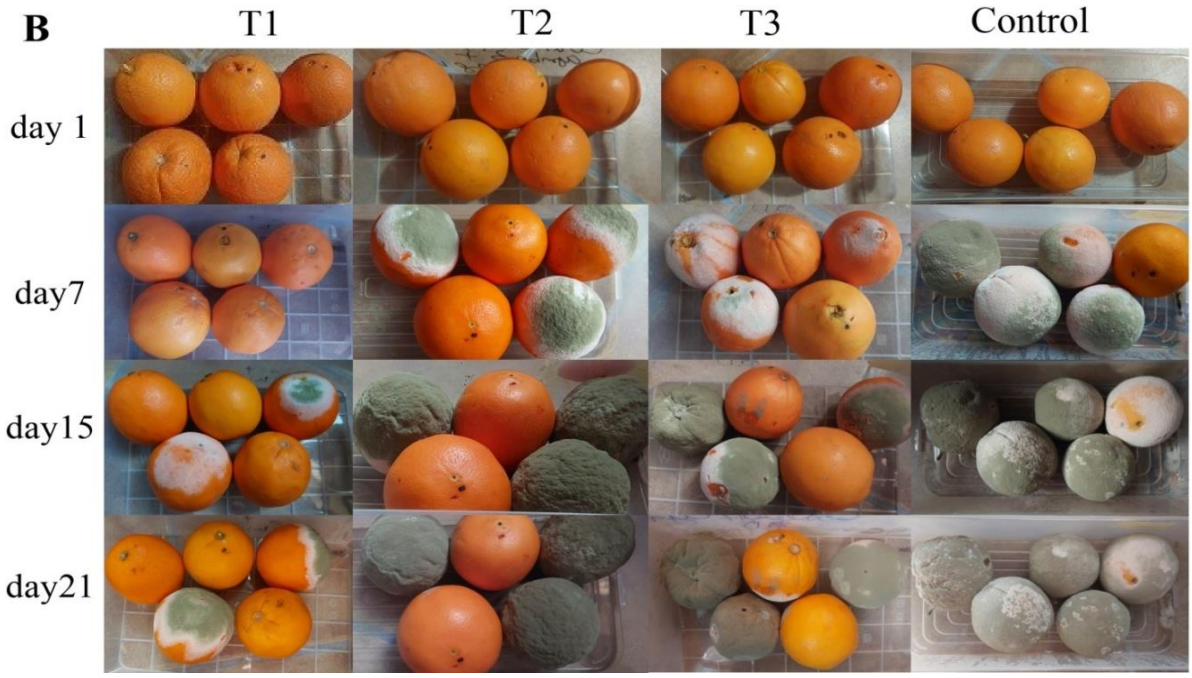


Figure 35. Antifungal activity of *O. majorana* (T1), *C. limon* (T2) and *C. citraus* (T3) nanoemlsions against *P. digitatum* compared to a control group over 21 days of storage.

The proliferation of *P. expansum* in the wounded regions of orange fruits was less significant than that of *P. digitatum* across all samples (Figure 36, C). By day 7, some peel defects were evident in fruits treated with *C. citratus* nanoemulsion (T3), additionally, the control group plus marjoram treatment exhibited minimal fungal growth in certain samples. Conversely, no fungal growth was detected in samples treated with *C. limon* nanoemulsion (T2). However, by day 15, *P. expansum* growth was noted in the wounded areas for all the treatments, the control group demonstrated substantial fungal growth. At the end of the storage period (21 days), fungal growth increased significantly, with the least growth observed in fruits treated with *C. citratus* nanoemulsion (T3). In contrast, the control group exhibited the most growth, accompanied by notable peel disorders (Figure 36, C). In a study conducted by Das et al. (2023), fungal growth was observed to be invasive in both the control group inoculated with fungus and the uninoculated fruits, unlike those coated with a nanoemulsion of *Valeriana officinalis* essential oil. *P. expansum* exhibited notable fungal activity on the fruits and proliferated rapidly within them; nevertheless, *P. digitatum* was more relevant for the decay and invasion of citrus fruits, highlighting the risk of cross-contamination among fruits. *P.*

digitatum have the ability to traverse the oil glands through pores where nutrition is available (Costa et al., 2019). These fungal species exhibit a tendency for rapid growth at temperatures between 20 and 25°C (Hocking, 2014).

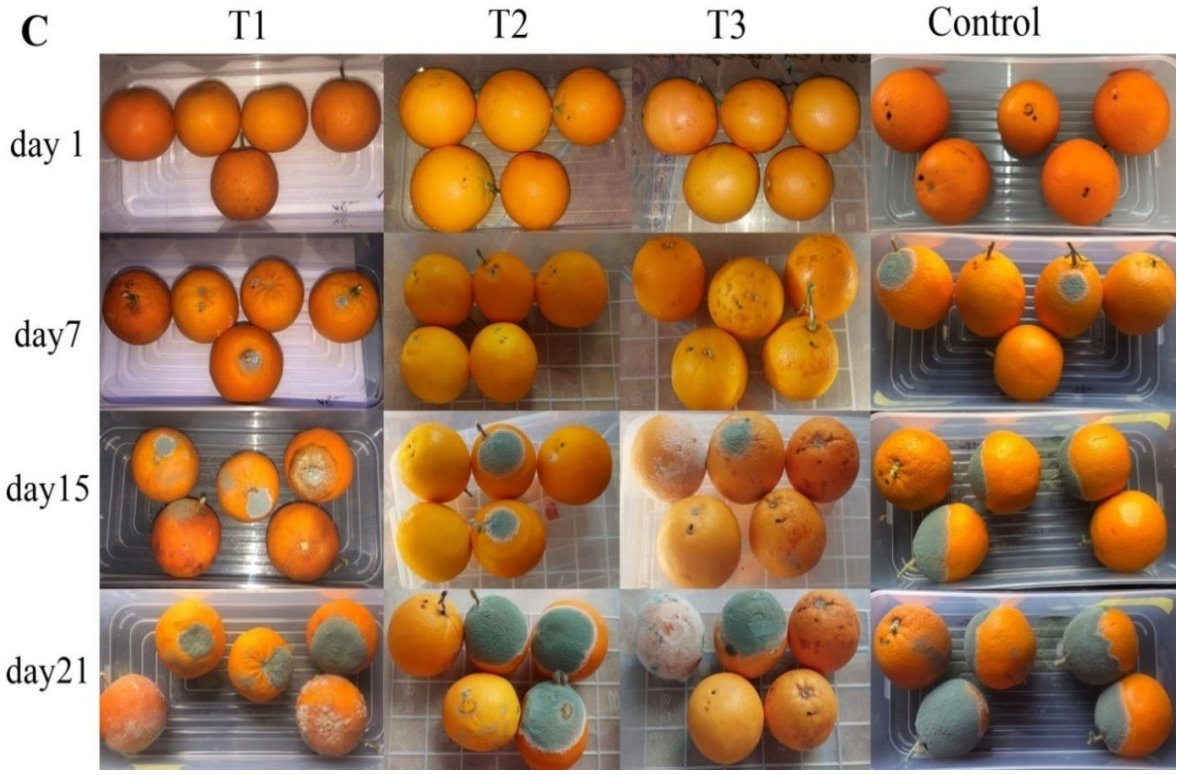


Figure 36. Antifungal activity of *O. majorana* (T1), *C. limon* (T2) and *C. citratus* (T3) nanoemulsions against *P. expansum* compared to a control group over 21 days of storage.

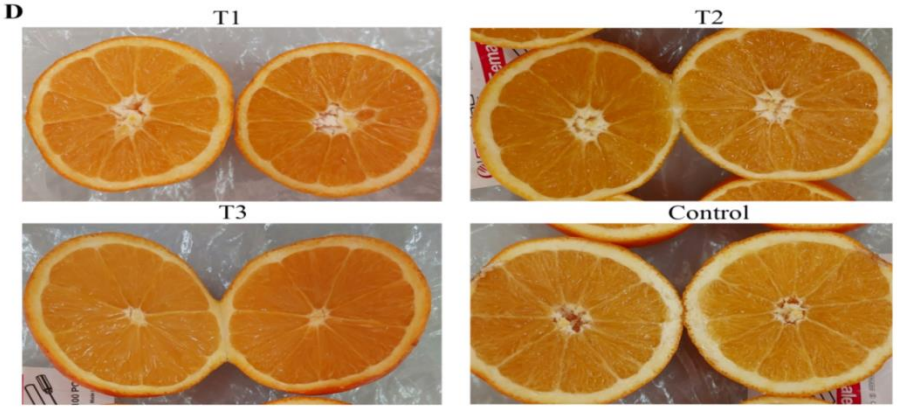


Figure 37. Cross section of orange fruits coated with *O. majorana* (T1), *C. limon* (T2) and *C. citratus* (T3) nanoemulsions, alongside a control group treated with distillated water, after 21 days of storage.

6. Fruits quality parameters

The physicochemical properties of orange fruits are crucial factors for investigating since they influence fruit quality. Citrus fruits are susceptible and sensitive to many environmental stresses, including physical and chemical changes that occur following harvest (Zacarias et al., 2020).

The present study examined variations in physicochemical parameters, including weight loss, firmness, total soluble solids, titratable acidity, pH, and levels of ascorbic acid (vitamin C) of Thomson orange fruits before and after the application of *O. majorana*, *C. citratus* and *C. limon* nanoemulsions.

Concerning the physical parameters (Figure 38, A, B), the weight loss varied among the treatments; the water loss within the fruits is related with transpiration and respiration of the fruits which lead to low humidity (Zhang et al., 2022; Rokaya et al., 2016).The highest weight loss was recorded in fruits treated with *C.citratus* nanoemulsion showing a value of 4.14 %, followed by the control group and fruits treated with marjoram nanoemulsion with values of of 4.13 % and 3.74 % respectively, lesser weight loss 3.52 %, was shown in oranges fruits treated by *C. limon* nanoemulsion, however no significant difference (P=0,4555) was observed in term of weight loss within all the groups (Figure 38, A). Humidity loss in the fruits is controlled by the peel layers (Abbasi et al., 2011), the reduction in weight in fruit treated with *C. citratus* (T3) could be explained by the fact that coatings containing essential oil reduce the movement of the water from the fruits to the environment, additionally, essential oils in coatings act like barriers (Shehata et al., 2020). These results are in accordance with several researches, Khorram and Ramezanian, (2020) reported a significant difference in weight loss between treated oranges with cinnamom essential oil and the control fruits after 21 days of storage, in addition, Hassan et al. (2014) indicated that the weight loss in tangerine oranges coated with emulsion increased with storage time; however, the control fruits exhibited the highest loss in weight. In a study by Breceda-Hernandez et al. (2020) edible pectin containing lemon EO, reduced the weight loss in grapes.

Fruits firmness (Figure 38, B) decreased in all the groups after 21 days if storage. Firmness of the fruits reduces due to several factors, including water loss from the peel as seen previously, the reduction in air within the cells; destruction of the cell compounds such cellulose, pectin, proteins, which affect the cell wall rigidity (Siburian et al., 2021). There was no significant difference (P = 0,8704) between fruits treated with marjoram (T1) and those

treated with lemon T2 after 21 days of storage, while there was a significant difference between T1, T3 (P=0,0008), and T1 and the control group (P<0,0001), *C. citratus* T3 and the control group showed the lowest firmness with values of 18.01 N and 17.26 N, respectively. *C. limon* nanoemulsion T2 showed increased firmness with 20.83 N, this is porch to the results of Shehata et al. (2020) who demonstrated that the high firmness was observed in strawberries treated with lemon essential oil respectively, in contrast, Salvia-Trujilloet al. (2015) showed that coating nanoemulsion containing lemongrass essential oil maintain the constant firmness in apples fruits.

Regarding the chemical parameters (Figure 38, C, D, E, F), The total soluble solids (TSS) increased in all the fruits treated with marjoram T1, lemon T2 (P =0,3403) with no significant difference, in addition to lemongrass T3 (P = 0,6625) after 21 days of storage, except between fruits treated with *O. majorana* and the control group (P=0,0152). The total soluble solids (TSS) level is an important measure as it serves as an indicator of sweetness (Magwaza & Opara, 2015). This study involved orange fruits stored at an ambient temperature of 22-22°C, which heightened the respiration rate of the fruit and facilitated the rapid conversion of carbohydrates into soluble sugars, thereby accounting for the increase in TSS after storage (Hasbullah & Ismail, 2022; Farouk Idnan et al., 2012). Conversely, the control group exhibited a constant TSS level at the end of storage, measuring 12.33°, these findings corroborate those of Almeida et al. (2024) and Aminifard et al. (2017) who reported an increase in TSS levels in mangoes treated with lemongrass emulsion and in coated blood oranges with caraway and anise essential oils, with the control group reaching the highest level of 12.5° Brix, respectively. In contrast, Das et al. (2023) indicated that TSS decreased in the control group while remaining constant in the coated samples. Abbasi et al., (2011) indicates that the degradation of polysaccharides into soluble sugars may influence the total soluble solids (TSS) content following a storage period. Furthermore, in this study, weight loss was augmented in both treatments, which is related to the sugar content (Wijewardane, 2022).

The pH (Figure 38, D) exhibited a minimal increase in all the treatments (T1, T2, T3), with no significant difference (P > 0.05) compared to the control group, which decreased to 2.99 ± 0.13. These results align with the findings of Habibi et al. (2023) who observed an increase in pH in blood oranges coated during refrigerated storage, Radi et al. (2018) noted a negligible increase in pH readings at the end of storage, Wijewardane (2022) attributes the increase in pH level to numerous variables, including the reduction in acidity and ascorbic acid

concentration throughout storage time, as well as the higher respiration of the fruit, which may influence its acidity.

The titratable acidity (Figure 38, E) percentage decreased in all treatments (T1, T2 and T3) after 21 days of storage, with significant difference between T1 and T2 (P=0,0221) and T1 and T3 (P=0,0062), oweer tere was no significant differene between all te tree treatments and te ontrol group (P> 0.05), the highest TA level was observed in fuits treated with marjoram T1, this decline in titratable acidity is likely associated with the increased respiration of the fruit during storage (Wijewardane, 2022). Titratable acidity is influenced by various factors, including the type of fruit, its composition of organic acids, and microbial presence (Yousuf et al., 2021). In this study, the results align with Bhandari et al. (2021), where a gradual decrease in titratable acidity was observed in sweet oranges treated with wax emulsion containing various essential oils, while samples treated with wax emulsion containing 0.5 % lemongrass extract exhibited the highest titratable acidity values, Zhang et al. (2022) found a consistent drop in titratable acidity (TA) in all treatments during the storage period of mandarins treated with lemon essential oil and chitosan.

The ascorbic acid content (vitamin C) is a crucial factor in the quality of orange fruits due to its substantial health benefits. In this study, the percentage of ascorbic acid decreased in all samples (Figure 38, F) with no significant differene between T1 and T2 (P=0,4043) and between and T2 and T3 (P=0,0714), on te oter and significant difference was observed between the 3 treatments and te ontrol group (P<0,0001). The highest decline in vitamin C content was noted in fruits treated with *C. citratus* nanoemulsion T3, these results align with those of Nasirifar et al. (2018) who indicated a decrease in vitamin C across all treated orange fruits, with the highest content found in fruits coated with lipid-based formulations containing lemon essential oil.

The decrease in Vitamin C can be attributed to its thermolability; it is very susceptible to heat, light, oxygen, and oxidation and enzyme stress (NakilcioĞlu-TaŞ & ÖtleŞ, 2020). Yousuf and Srivastava, (2019) indicate that the ascorbic acid concentration (vitamin C) diminishes with the maturation of the fruit.

This study did not demonstrate a significant effect exerted by the nanoemulsions. Several studies indicate that the physicochemical parameters of fruits are primarily associated with the ripening process over time. Furthermore, these changes are connected to the low metabolic activity of these fruits post-harvest, as they no longer receive nutrients from the trees (Cháfer et al., 2012).

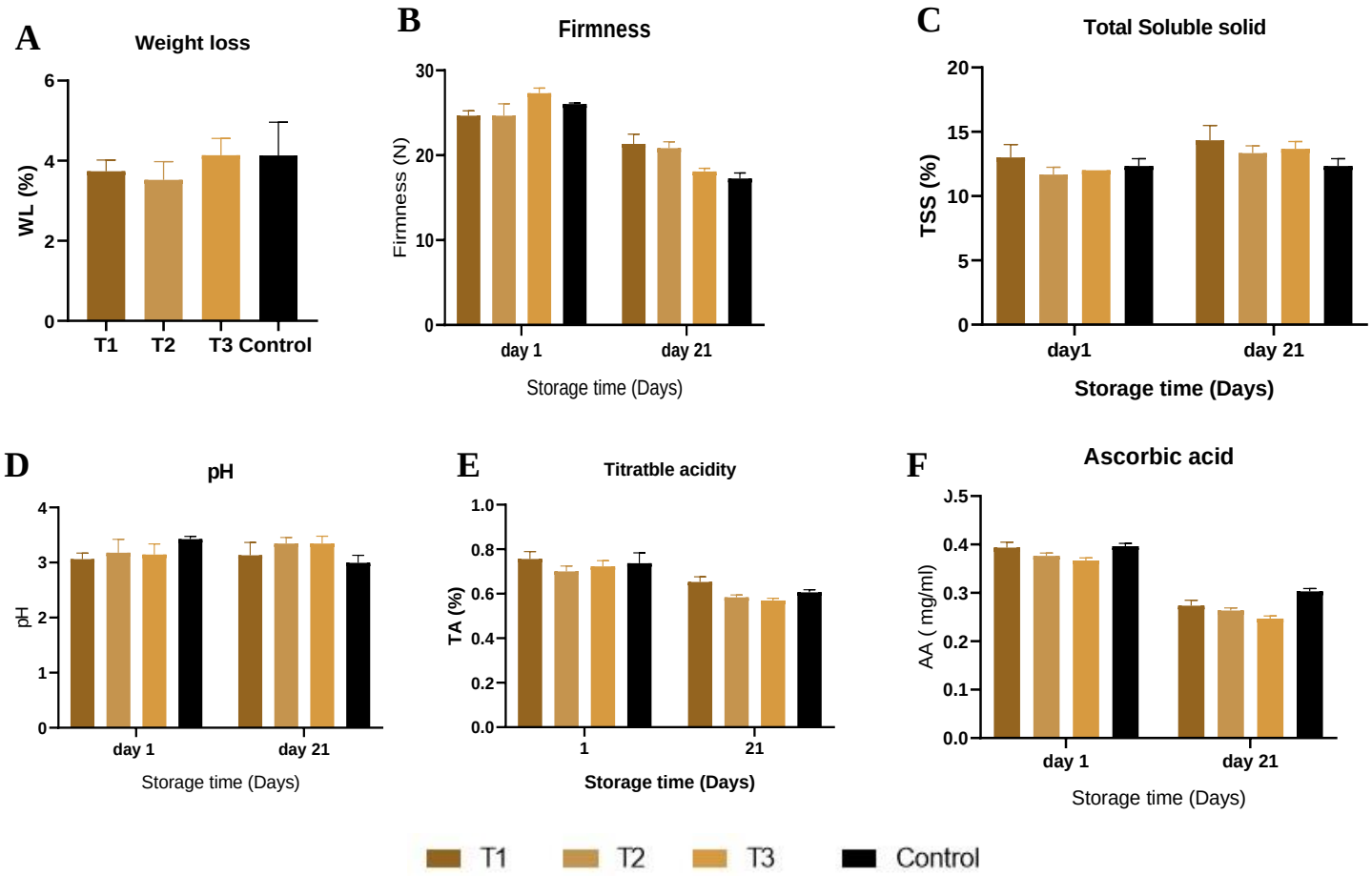


Figure 38. Effect of nanoemulsions from *O. majorana* (T1), *C. limon* (T2) and *C.citratus* (T3) on weight loss, titratable acidity, pH, total soluble solids, and ascorbic acid content of orange fruuts over 21 days of storage .

7. Sensory attributes

Essential oils have been used for many years as additives in various food products due to their potent characteristic aroma, the primary factors in the sensory analysis of essential oils include visual quality, color, odor, and taste, however, certain essential oils particularly those from citrus species, possess a potent aroma that can significantly influence the organoleptic properties of food products (Mani-López et al., 2017). This issue impacts consumer acceptance; nonetheless, it can be mitigated through the encapsulation of essential oils within nanoemulsions, which can diminish their intense aroma and flavor (Mobininejad et al., 2021).

In this study, *O. majorana*, *C. citratus* and *C. limon* nanoemulsions were applied as coatings on the surface of Thomson orange fruits, sensory evaluation was conducted on both

the first day of application and at the end of the storage period (day 21), and results were compared to a control group. The results are illustrated in the Figure 39.

On the first day (Figure 39, G) there was no significant difference among all treatments regarding all qualities (P >0,9999), except for a slightly displeasing aroma noted from *O. majorana* nanoemulsion, However, as essential oils are volatile compounds, this aroma is expected to disappear with storage time. Additionally, some panelists appreciated the aroma of *C. citratus* (T3) and *C. limon* (T2), as both nanoemulsions exhibited a citrus-like aroma that combined orange fruits well. By the end of storage, there was no significant difference between the control fruits and those treated with *C. limon* nanoemulsion. However, the aroma and taste diminished compared to the first day for all the samples, although not considerably, these findings align with the studies by Sumonsiri et al. (2020), which indicated that lemon oil did not influence the sensory properties of strawberry fruits, and Shehata et al. (2020) who found that lemon, mandarin, and orange essential oils were more favorable received concerning color, texture, flavor, and appearance attributes than untreated strawberries after 18 days of storage. Conversely to lemon and marjoram, oranges treated with *C. citratus* nanoemulsion exhibited darkening of the outer peel (P=0,0351), which was unappealing to consumers, furthermore, there was a notable decline in taste and flavor (Figure 39, H) accompanied by an alcoholic and fermented taste, this finding is consistent with numerous researches, De Bruno et al. (2023) and Siburian et al. (2021) observed that the incorporation of bergamot and cinnamon essential oils into coatings resulted in modifications in the organoleptic qualities of the product, respectively. A study by Khorram et al. (2020) indicated that Thomson oranges treated with shellac and cinnamon essential oils exhibited the highest acceptability scores compared to control fruits, conversely, fruits treated only with the essential oil were not appreciated by penalties due to surface deterioration, as previously observed in this study with *C. citratus*. Njombolwana et al. (2013) suggest that the discernible disagreeable odor and alcoholic flavor were likely a result of anaerobic respiration, which generated significant quantities of ethanol as well as acetaldehyde during storage.

In contrast, a study by Tussaadah et al. (2023) indicated that citronella essential oil preserved the sensory quality of apples after 31 days of storage compared to sesame and coconut essential oils, de Oliveira et al. (2020) also demonstrated that the application of a chitosan coating containing lemongrass essential oil yielded the highest sensory scores.

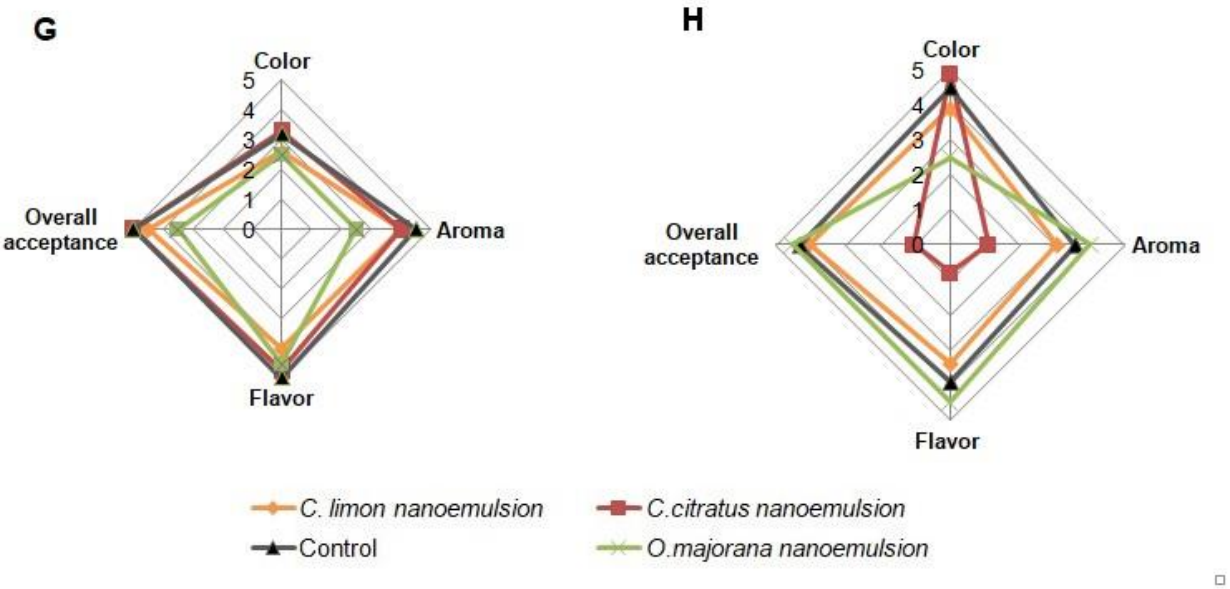
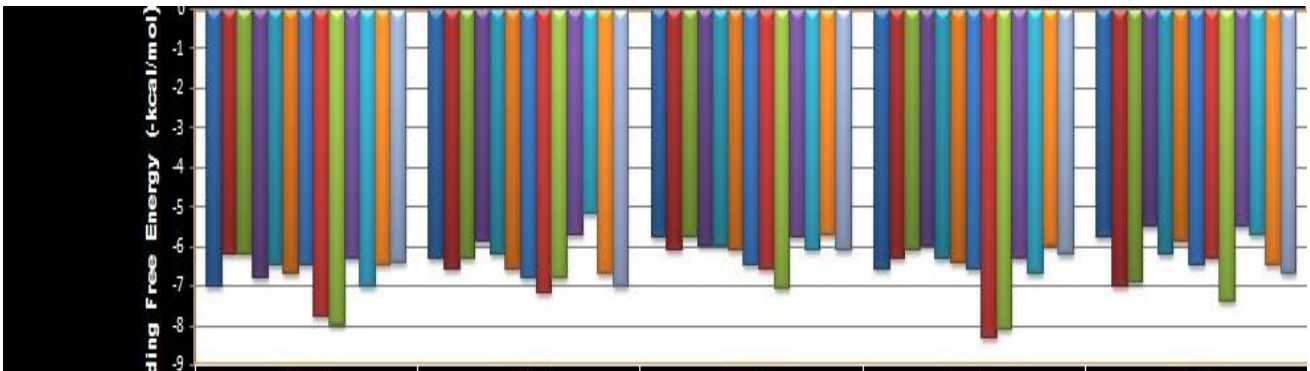


Figure 39. Radar chart illustrating sensory characterestics of orange fruits treated with *O.majorana* , *C.citratus*, and *C.limon* nanoemulsions beside a control group, at both dayb 1 (G) and day 21 (H).

8. *In silico* analysis

The interaction strength among the primary components in both oils and their nanoemulsions with fungal non-reducing polyketide synthase (8CG4), aldehyde dehydrogenase 12 (6D97), serine hydrolase (1VKH), sterol 14-α demethylase (4UYM), and patulin synthase (8BXL) was characterized by negative binding-free energy (Figure 44). Sesquiterpenes, primarily found in marjoram essential emulsion and its nanoemulsion, exhibited a strong affinity for the examined enzymes, with binding energies ranging from -6.3 to -8.3 kcal/mol for β-caryophyllene and from -6.8 to -8.1 kcal/mol for bicyclo-germacrene. This suggests that these compounds may be effective in inhibiting these fungal enzymes. The results illustrate the specific interactions of specific interactions between the chemical compounds and the enzymes with the highest scores (Figure 40, 41); these interactions are crucial to the antifungal activity observed. The major monoterpenes contained in marjoram include sabinene, α-terpinene, γ-

terpinene, sabinene hydrate, terpinene-4-ol, and α-terpineol, demonstrated docking scores ranging from -6.6 to -7.0 kcal/mol for the investigated enzymes, which are comparable to those of fennel (fenchone and anethole), varying from -6.7 to -7.0 kcal/mo. The findings indicate that sesquiterpenes in *O. majorana* have a strong affinity for the target fungal enzymes, elucidating the significant antifungal efficacy of marjoram essential oil and its nanoemulsion as an antifungal agent.



	8CG4	6D97	1VKH	4UYM	8BXL
Sabinene	-7	-6,3	-5,8	-6,6	-5,8
α-Terpinene	-6,2	-6,6	-6,1	-6,3	-7
γ-Terpinene	-6,2	-6,3	-5,8	-6,1	-6,9
Sabinene hydrate	-6,8	-5,9	-6	-6	-5,5
cis-β-Terpineol	-6,5	-6,2	-6	-6,3	-6,2
Terpinen-4-ol	-6,7	-6,6	-6,1	-6,4	-5,9
α-Terpineol	-6,5	-6,8	-6,5	-6,6	-6,5
β-Caryophyllene	-7,8	-7,2	-6,6	-8,3	-6,3
Bicyclogermacrene	-8	-6,8	-7,1	-8,1	-7,4
α-Pinene	-6,3	-5,7	-5,8	-6,3	-5,5
L-Fenchone	-7	-5,2	-6,1	-6,7	-5,7
Estragole	-6,5	-6,7	-5,7	-6	-6,5
Anethole	-6,4	-7	-6,1	-6,2	-6,7

Figure 40. The binding free energy of the major volatiles with enzymes associated with fungal biosynthesis.

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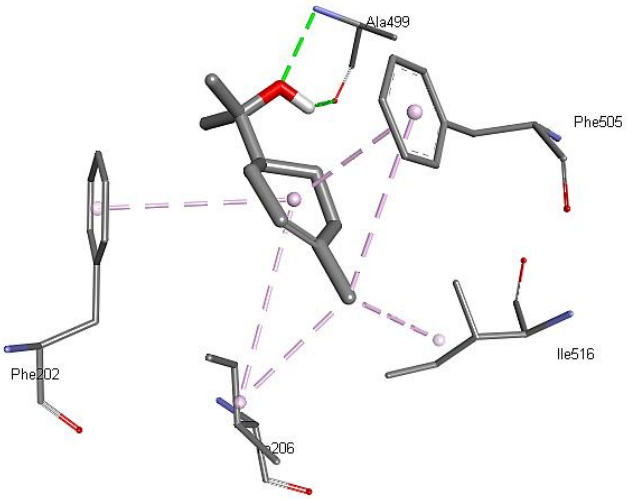
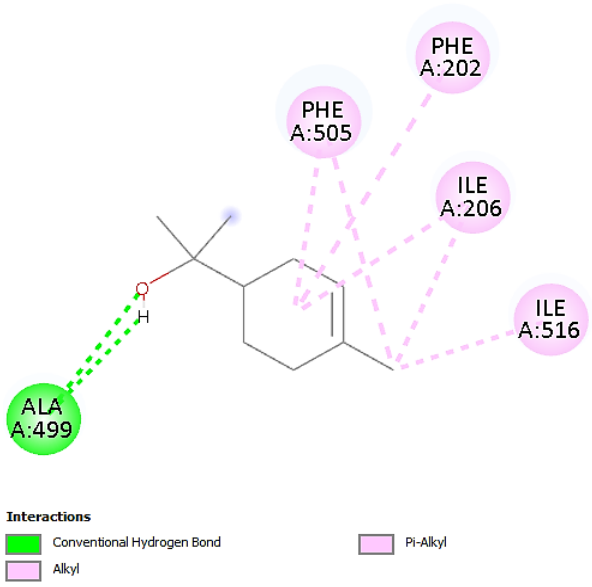
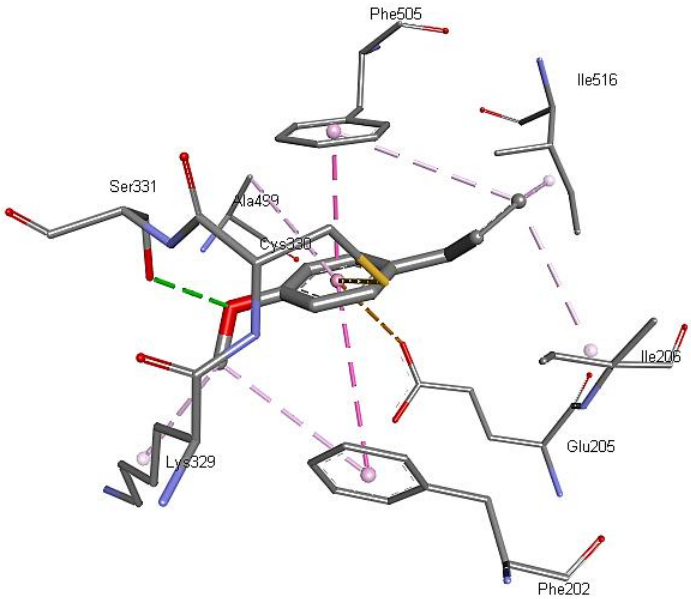
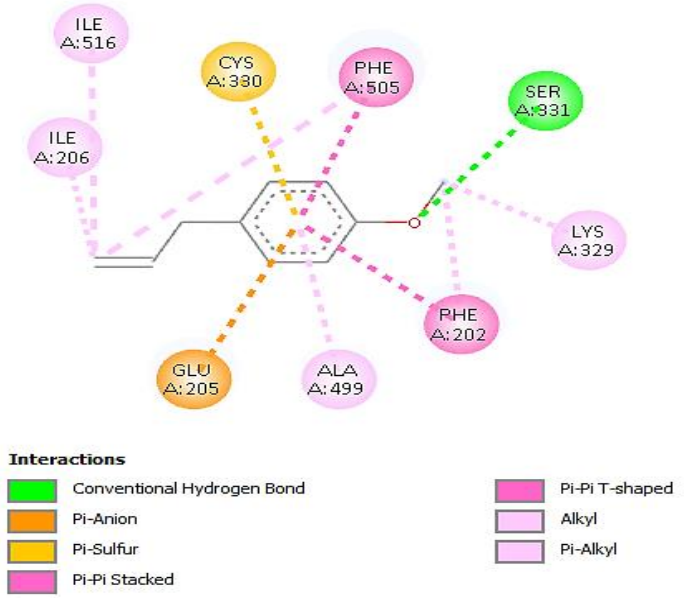
8CG4- 4-Terpinen-4-ol: -6.7 kcal/mol



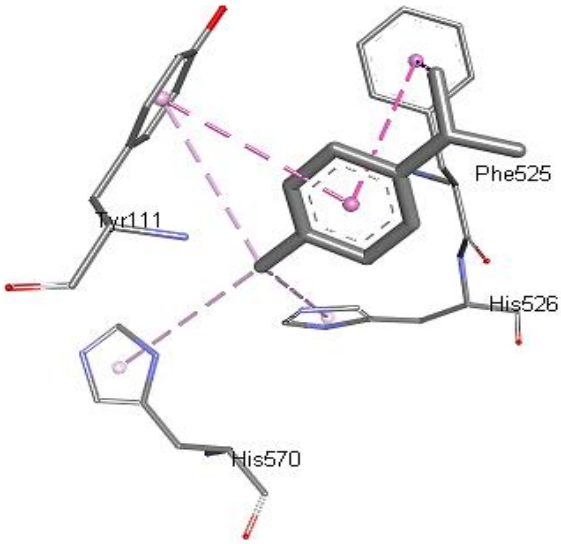


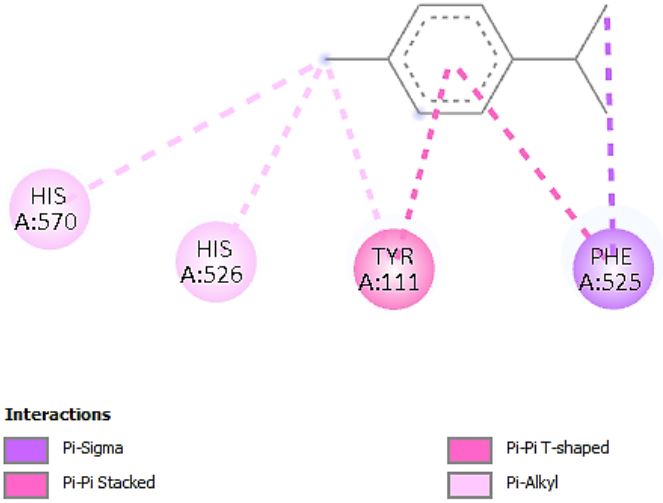
6D97-Estragole: -6.7 kcal/mol



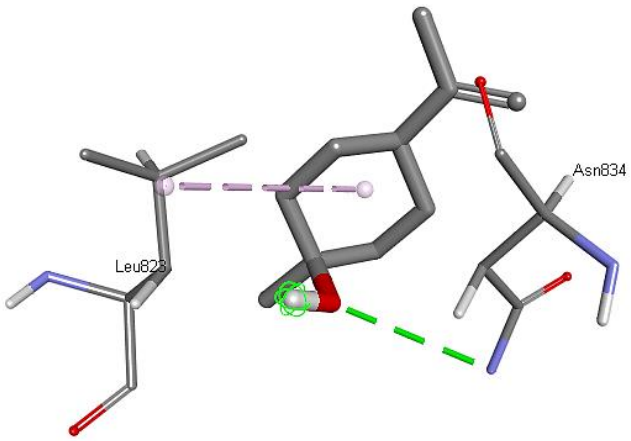


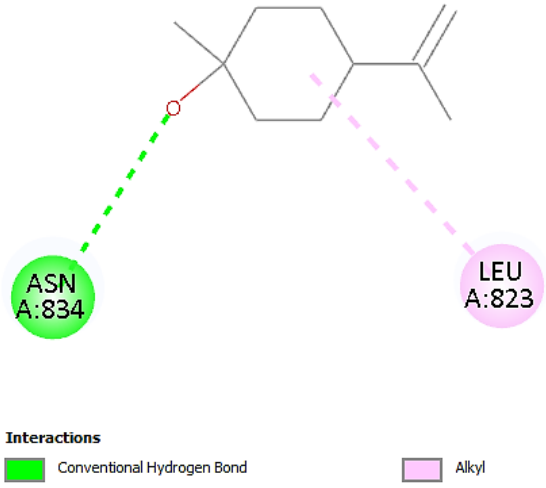
8BXL-γ-Terpinene: -6.9 kcal/mol



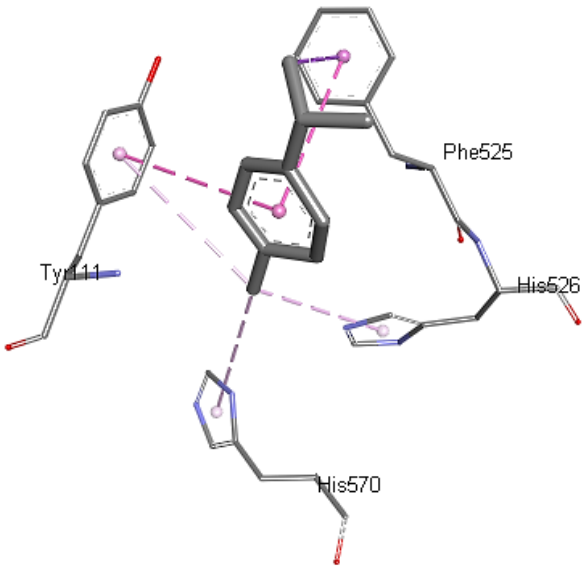


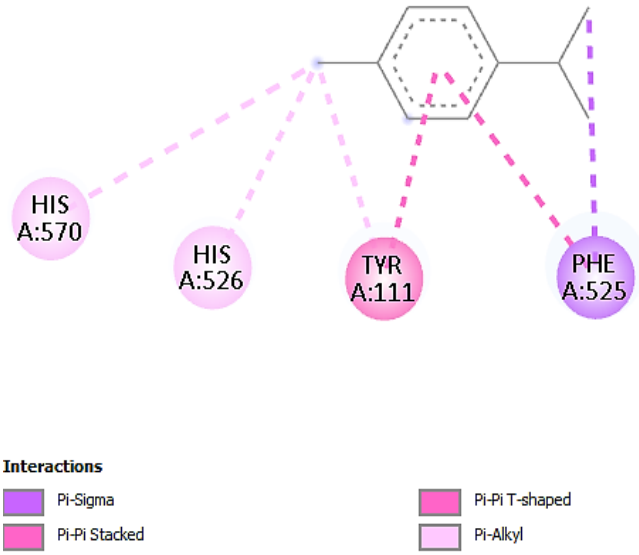
8CG4- cis-β-Terpineol: -6.5 kcal/mol



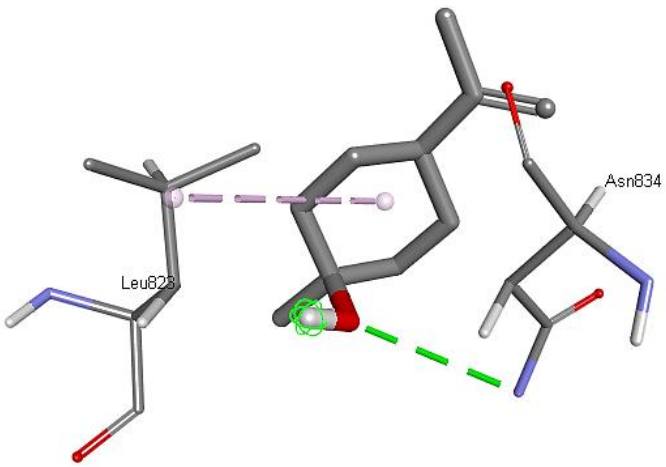


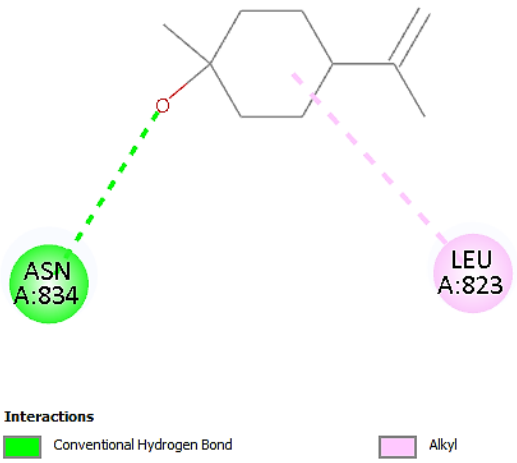
8BXL- α-Terpinene: -7.0 kcal/mol



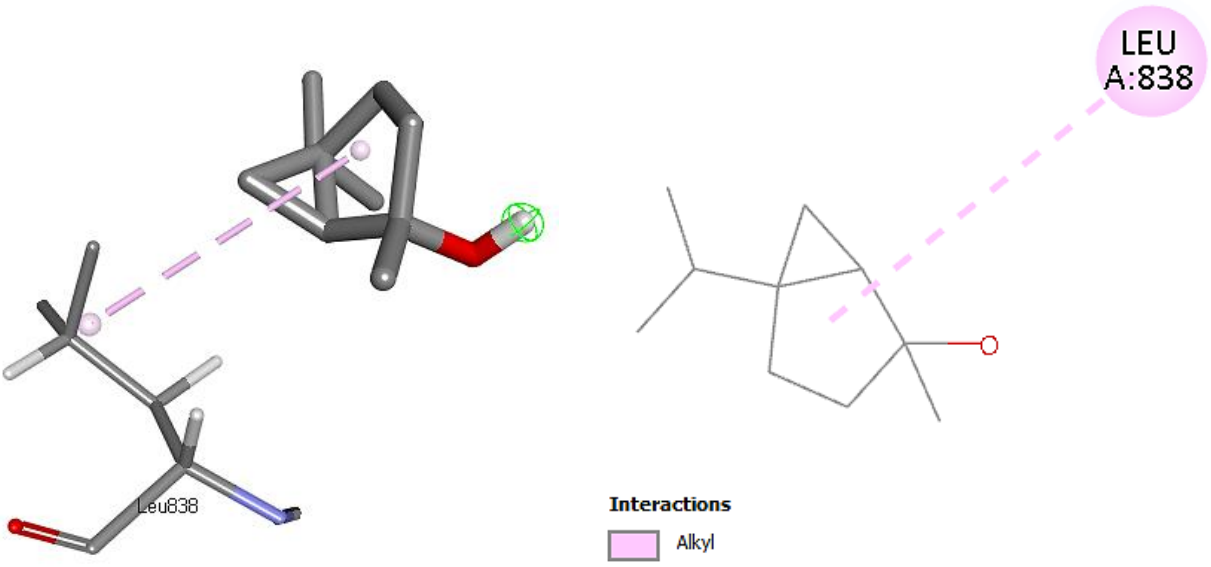


8CG4- cis-β-Terpineol: -6.5 kcal/mol

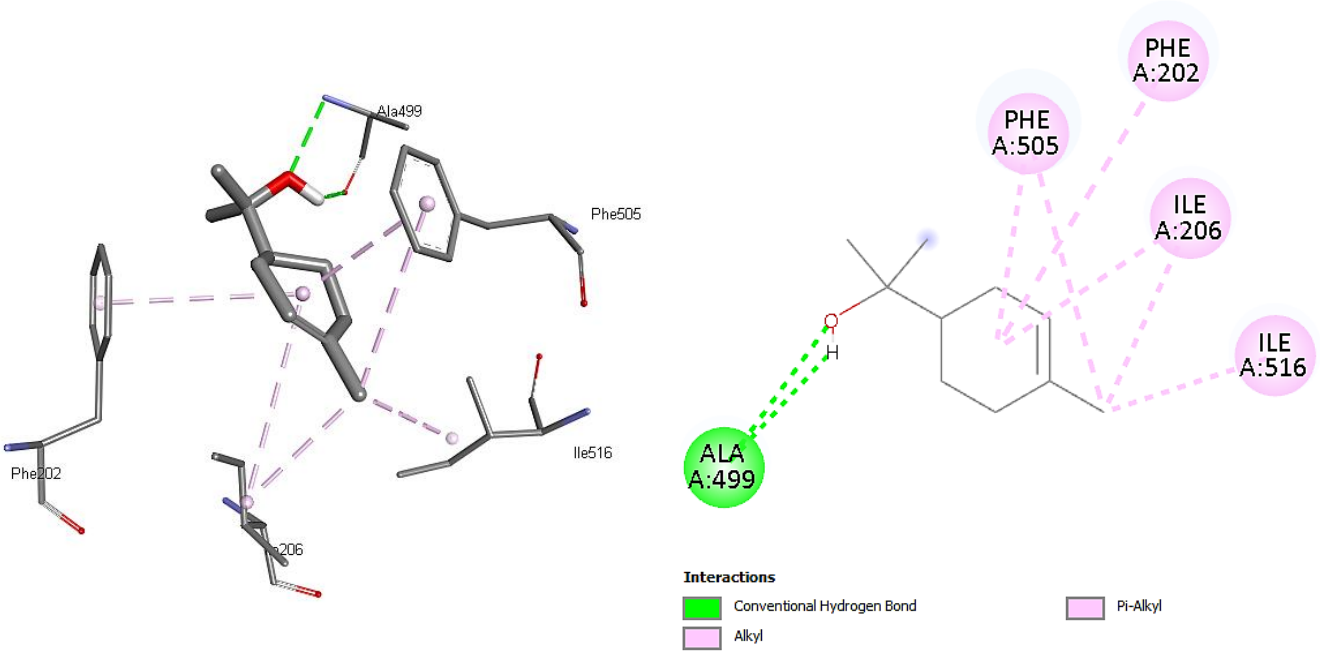




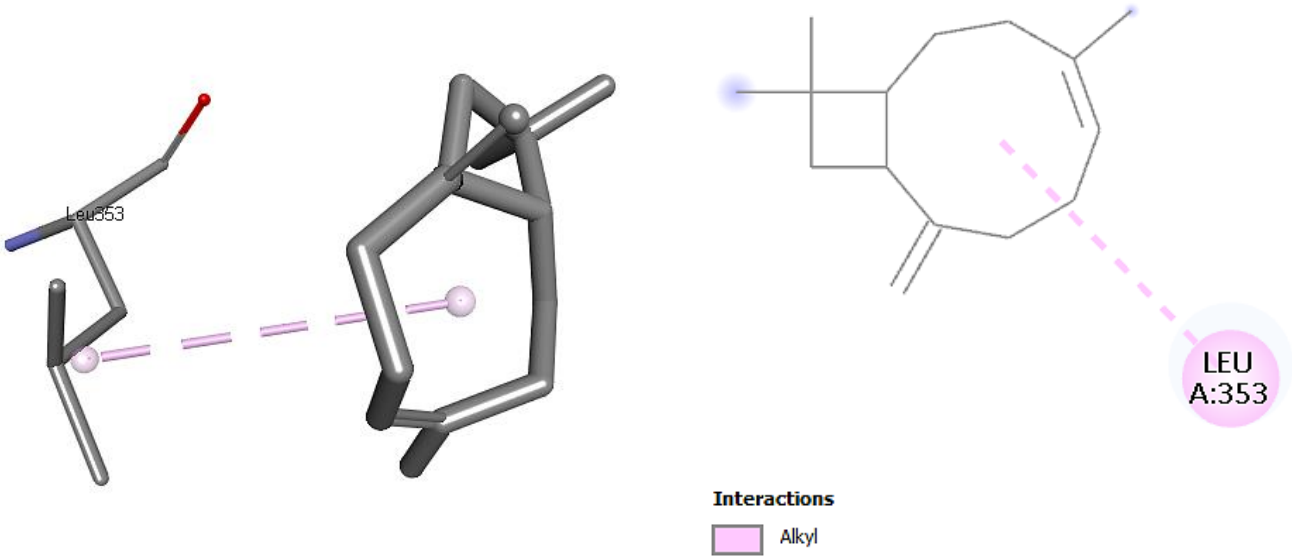
8CG4-Sabinine hydrate: -6.8 kcal/mol



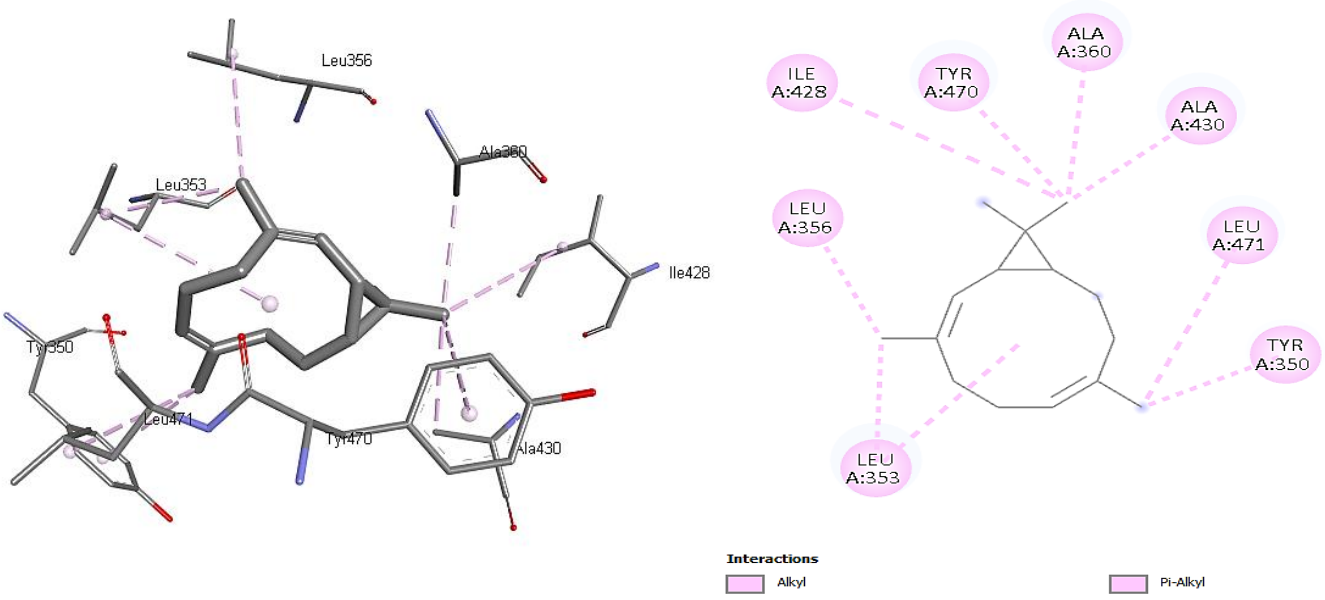
6D97-α-Terpineol: -6.8 kcal/mol



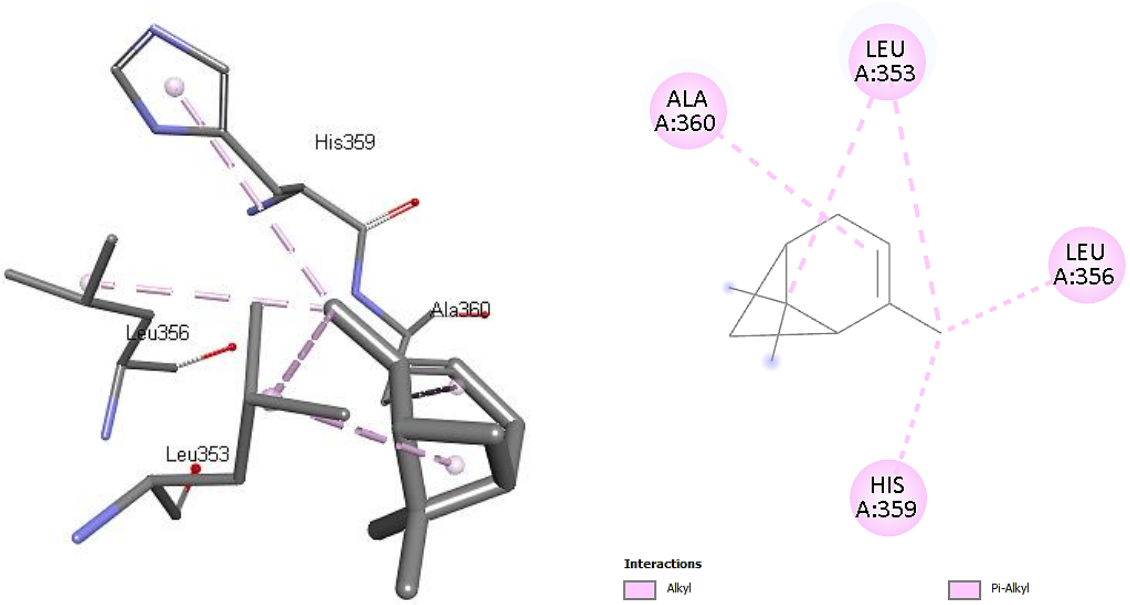
4UYM-β-Caryophyllene: -8.3 kcal/mol



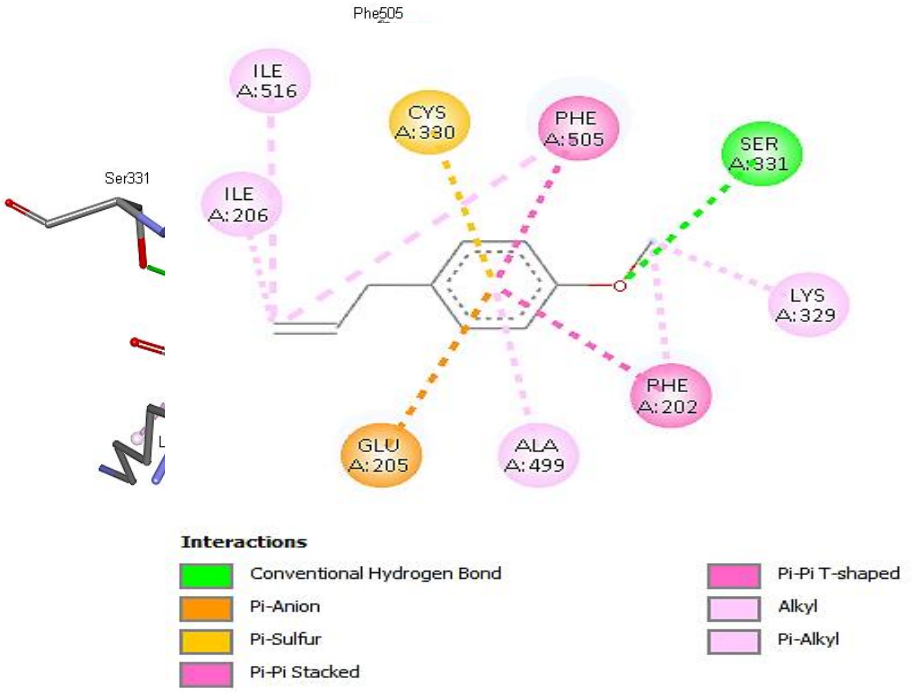
4UYM-Bicyclogermacrene: -8.1 kcal/mol



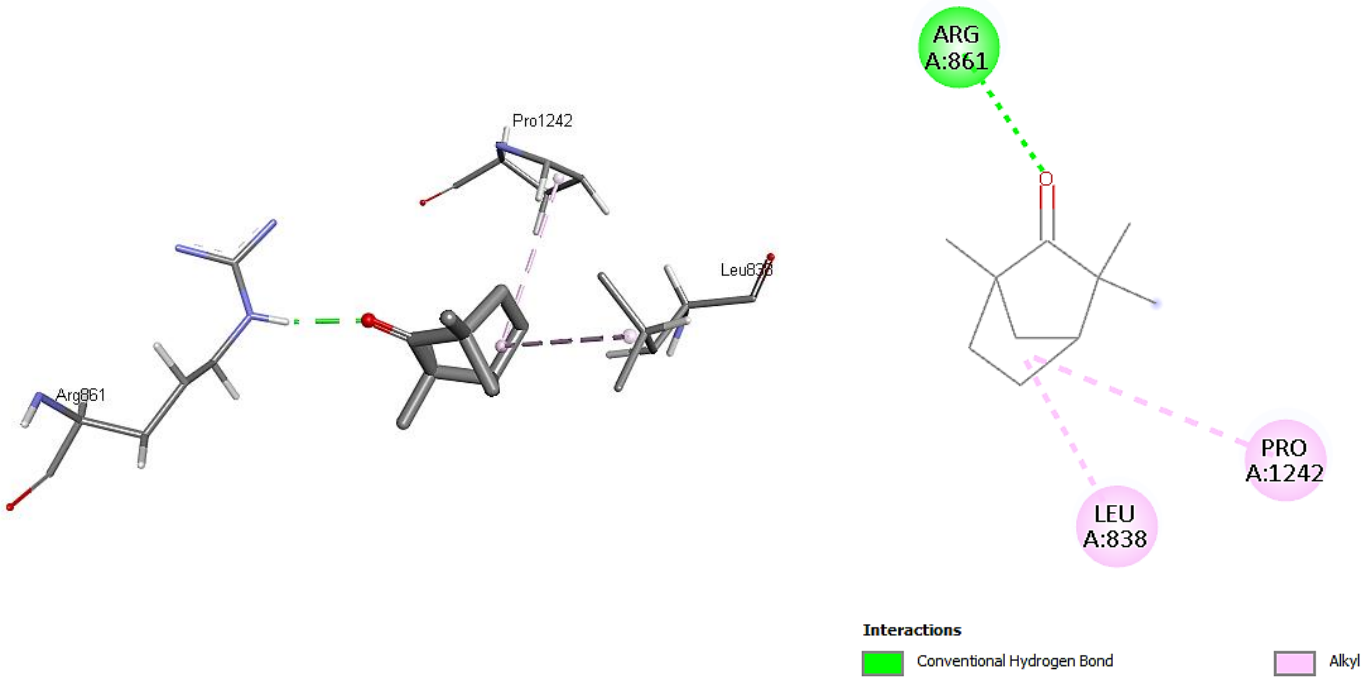
4UYM-α-Pinene: -6.3 kcal/mol



6D97-Estragole: -6.7 kcal/mol



8CG4-Fenchone: -7.0 kcal/mol



6D97-Anethole: -7.0 kcal/mol

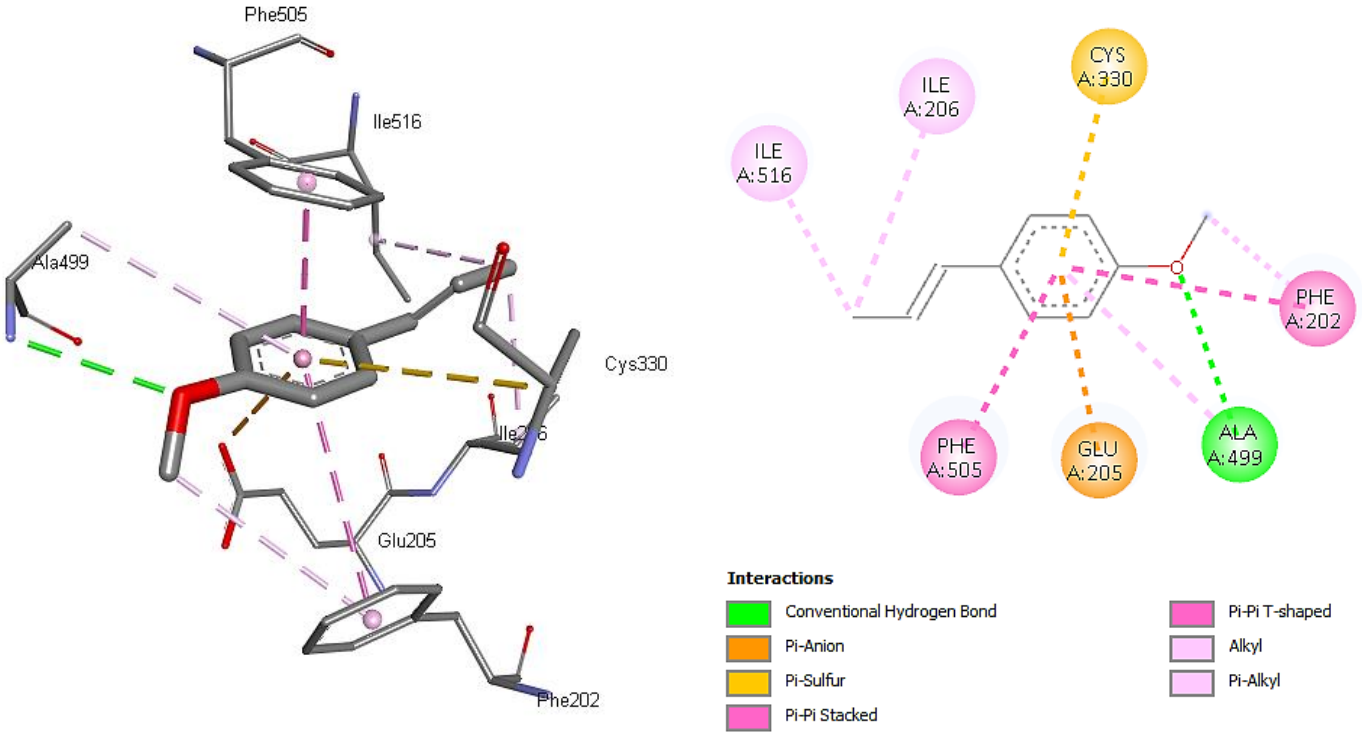


Figure 41. The interactions of chemical compounds with the tested enzymes

Our substantial findings from molecular docking assays corroborate the antifungal results derived from MFC experiments. To our knowledge, the aforementioned docking study has not been previously referenced in the literature. Enzymes were chosen based on documented pathways and docking analyses, polyketide synthases (PKS) is responsible for the manifuctaringof Aflatoxins, secreted by *Penicillium* and Aspergillus species, Aldehyde dehydrogenase is essential for fungal development and virulence (Tang et al., 2023), Polyol lipids are synthesized via cycles of chain transfer, esterification, and hydrolysis by serine hydrolase, resulting in strongly reducing polyketide intermediates (Kong et al., 2025), Sterol 14α-demethylase is essential for sterol synthesis necessary for membrane development in all species (Li et al., 2011), Patulin synthase (PatE) from *Penicillium expansum* facilitates the terminal phase of patulin production, a mycotoxin responsible for postharvest fruit deterioration (Tjallinks et al., 2023).

According to the current investigation, Li et al. (2011) demonstrated similar results between -5.06 to -7.40 kcal/mol for various commercial fungicides targeting Sterol 14α-demethylase. Tragni et al. (2021) identified seven ligands for patulin reduction based on *in vitro* findings, with binding energies ranging from -4.29 to -8.5 kcal/mol. The interactions of volatile compounds, specifically *O. majorana* (terpinene-4-ol) and *F. vulgare* (estragole), with elevated docking scores are depicted in Figure 44. The traditional hydrogen bond had the greatest interaction strength, succeeded by π-π bonds, π-alkyl interactions, and n-alkyl interactions (Ritika et al., 2021). The current investigation confirms that marjoram has greater affinity for fungal enzymes than fennel, indicating its high potency

Conclusion & Perspectives

Conclusion and Perspectives

Thomson orange (*Citrus sinensis*) fruits are among the most important economic crops in Algeria; since these fruits are widely produced and consumed especially in winter. However, both consumers and fruits merchants are complaining about the fungal occurrence in orange fruit even after conservation in refrigerated fruits at low temperatures, the orange fruits are prone to fungal attack in different stages from harvesting to consumption, *P. digitatum* beside *P. italicum* are among the most devastating molds that completely deteriorate citrus fruits.

The current study aimed to evaluate the antimicrobial abilities of marjoram, lemongrass, wild fennel and lemon essential oils and their nanoformulations in reducing and inhibiting the growth of the pathogenic microorganisms with a special attention to *P. digitatum* mold, in addition evaluating their effect on prolonging the shelf life of sweet oranges and maintaining their physicochemical characteristics after the application of the nanoemulsions at room temperature. Results indicate the effect of the nanoemulsification process via high-energy method (Ultrasonication) on the chemical composition of crude essential oils, wherein certain compounds increased while others were decreased, alongside the absence of some compounds in the nanoemulsion; the tested nanoemulsions exhibited stability. This investigation demonstrated the potent both *in vitro* and *in vivo* antifungal activity of the essential oils and their nanoemulsions, in addition to *in silico* antifungal activity of marjoram and fennel.

The current study offers new natural alternatives such nanoemulsions obtained from marjoram, lemongrass and lemon in extending the shelf life of orange fruits and inhibiting the growth of pathogenic fungi, in addition *P. expansum* was reported for the first time in this research as an isolate from orange fruits, knowing that it is found in apples fruits in particular. Despite the great antifungal activity shown by the tested essential oils obtained from *O. majorana*, *C. citratus* and *C. limon* and their nanoemulsions, the antibacterial activity was reduced, especially for the case of *F. vulgare* and *C. limon*. In addition, lemongrass (*C. citratus*) affected negatively the sensory qualities of the fruits at the end of the storage period.

This work offer new ways for innovates solutions for the bio-preservation of fruits using natural compounds meeting consumers demand, and at the same time reducing agricultural and economic losses of the fruits.

- Future studies in Algeria should concentrate on replacing synthetic preservation chemicals, such as conventional pesticides, with safer natural alternatives, given their widespread use and numerous negative effects. Moreover, integrating these nanotechnologies with other natural bioactive compounds could enhance biological activity while maintaining fruit quality for extended storage periods.
- Further research should focus on the use of natural surfactants and co-surfactants in nanoemulsions to improve physical stability while relying on non-toxic compounds.
- The valorization of by-products, particularly through the use of agro-industrial residues as sources of natural bioactive molecules, should also be encouraged.
- In addition, there is a need to develop preservation methods that are not only effective, but also simple and low-cost, so that they can be realistically implemented by farmers, storage facilities, and the food industry in local contexts.
- It should be emphasized that the present study is preliminary and represents only a first step in this field. Further optimization, larger-scale experiments such industrial-scale evaluations are needed to confirm these findings and to improve the proposed formulations.
- Finally, highlighting *P. expansum* as a significant postharvest pathogen will remain important for better understanding and managing fungal spoilage in stored fruits.

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❖ Webography

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Appendices

Appendix 1: Material and Methods

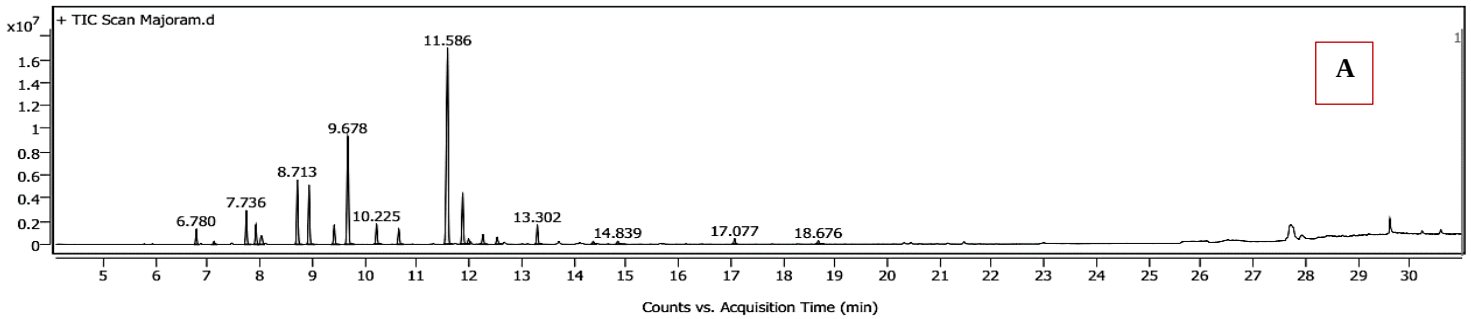
Figure A1.1. Clevenger apparatus

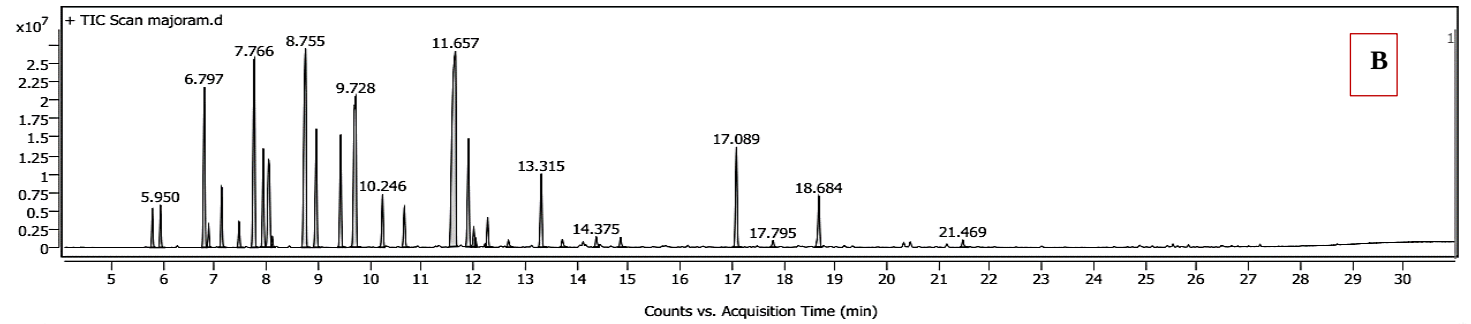
Figure A1.2. Probe sonicator

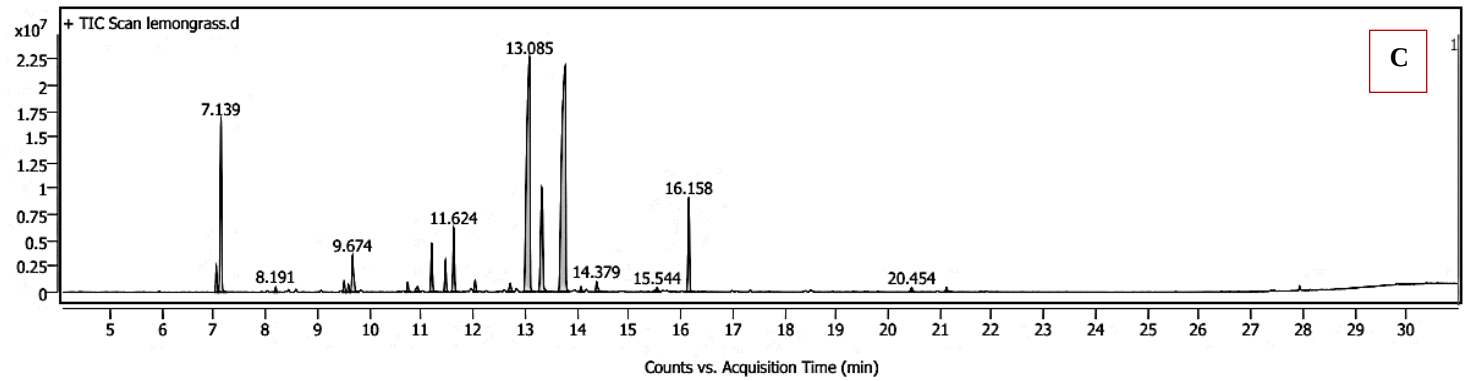
Figure A1.3. GC (Agilent 8890 System) coupled with MS (Agilent 5977B GC/MSD) NRC, Cairo, Egypt.

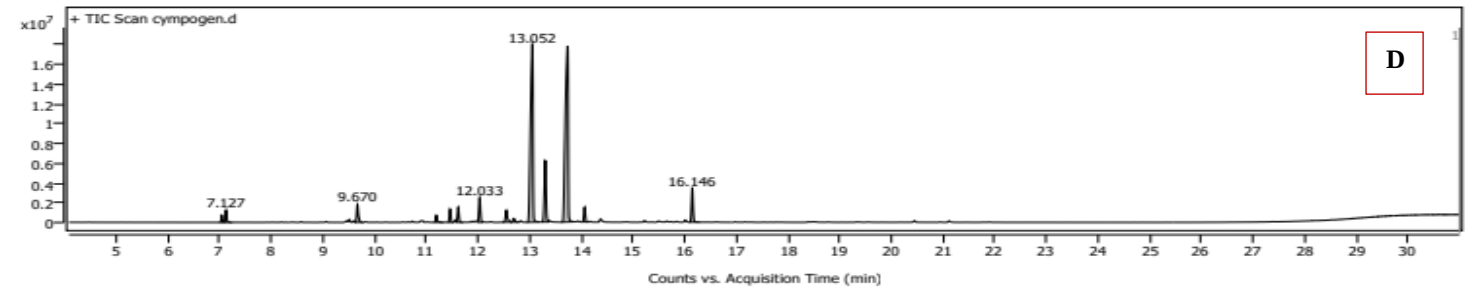
Figure A1.4. Dipping method used.

Appendix 2: Results









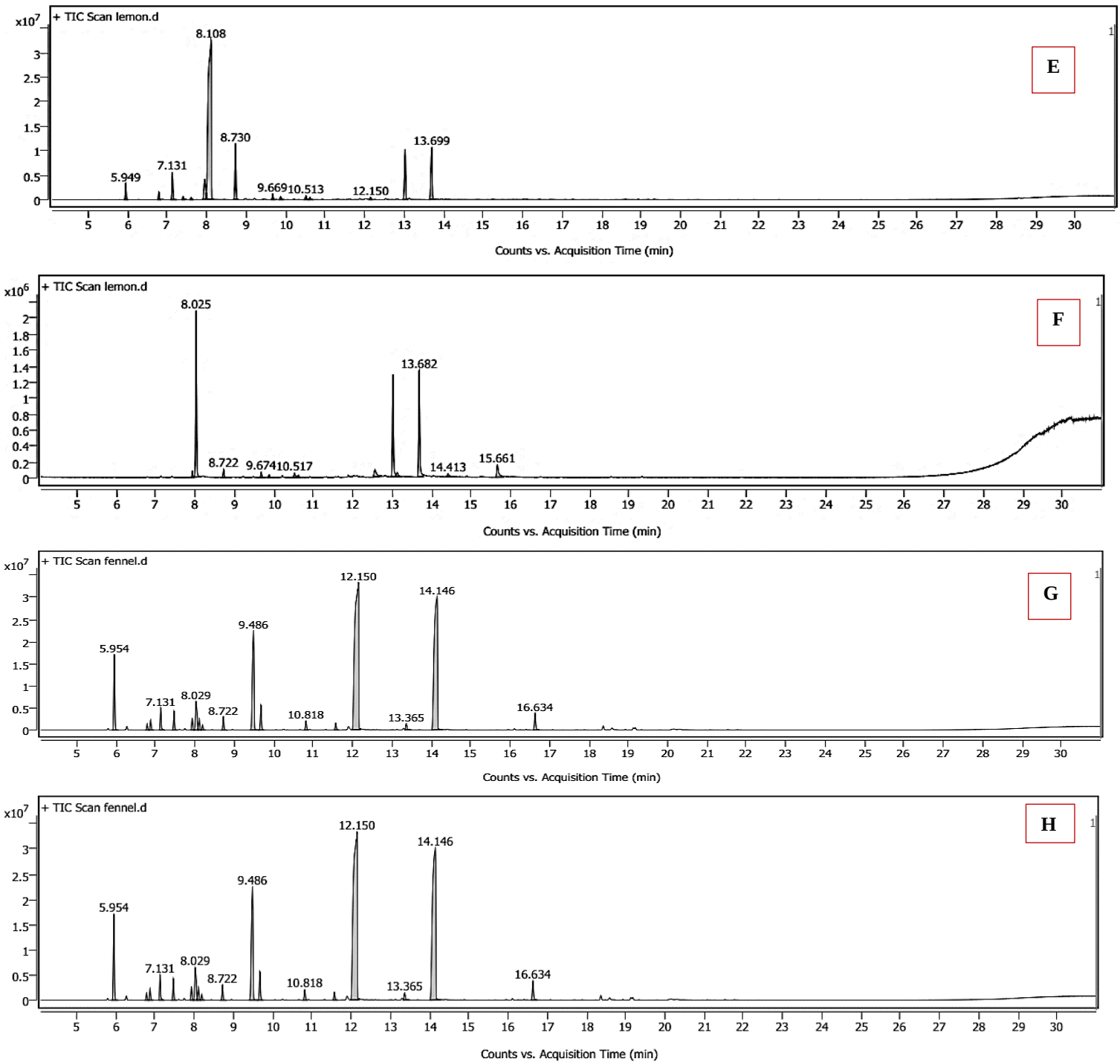


Figure A2.1. Chromatograms of GC-MS analysis of *O. majorana* (A), *C. citratus* (C), *C. limon* (E), *F. vulgare* (G) EOs and theit nanoemulsions (B, D, F, H), respectively.

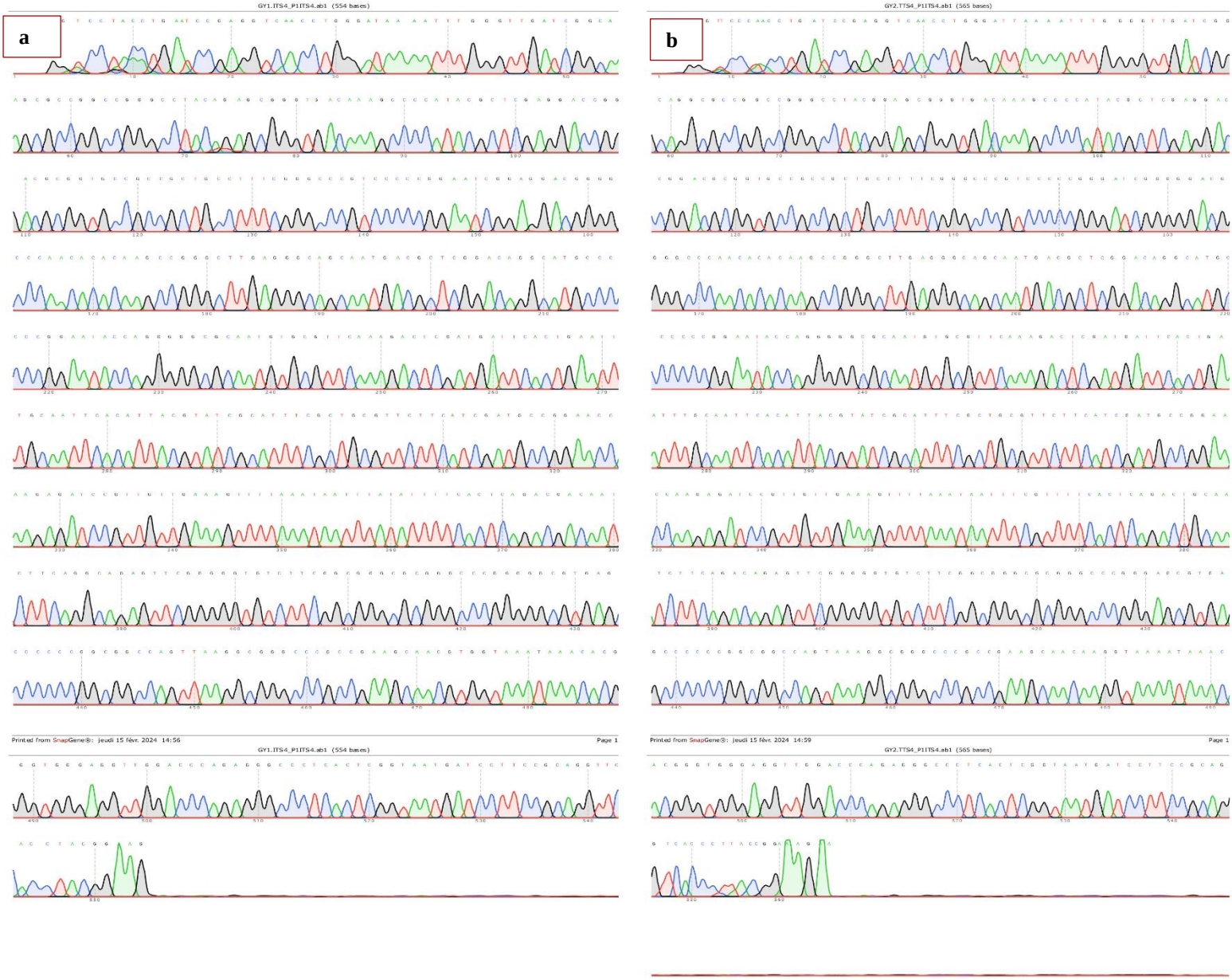


Figure A2.2. Electrophoretic Sequences of GY1 (a) and GY2 (b) Isolates from the ITS Region

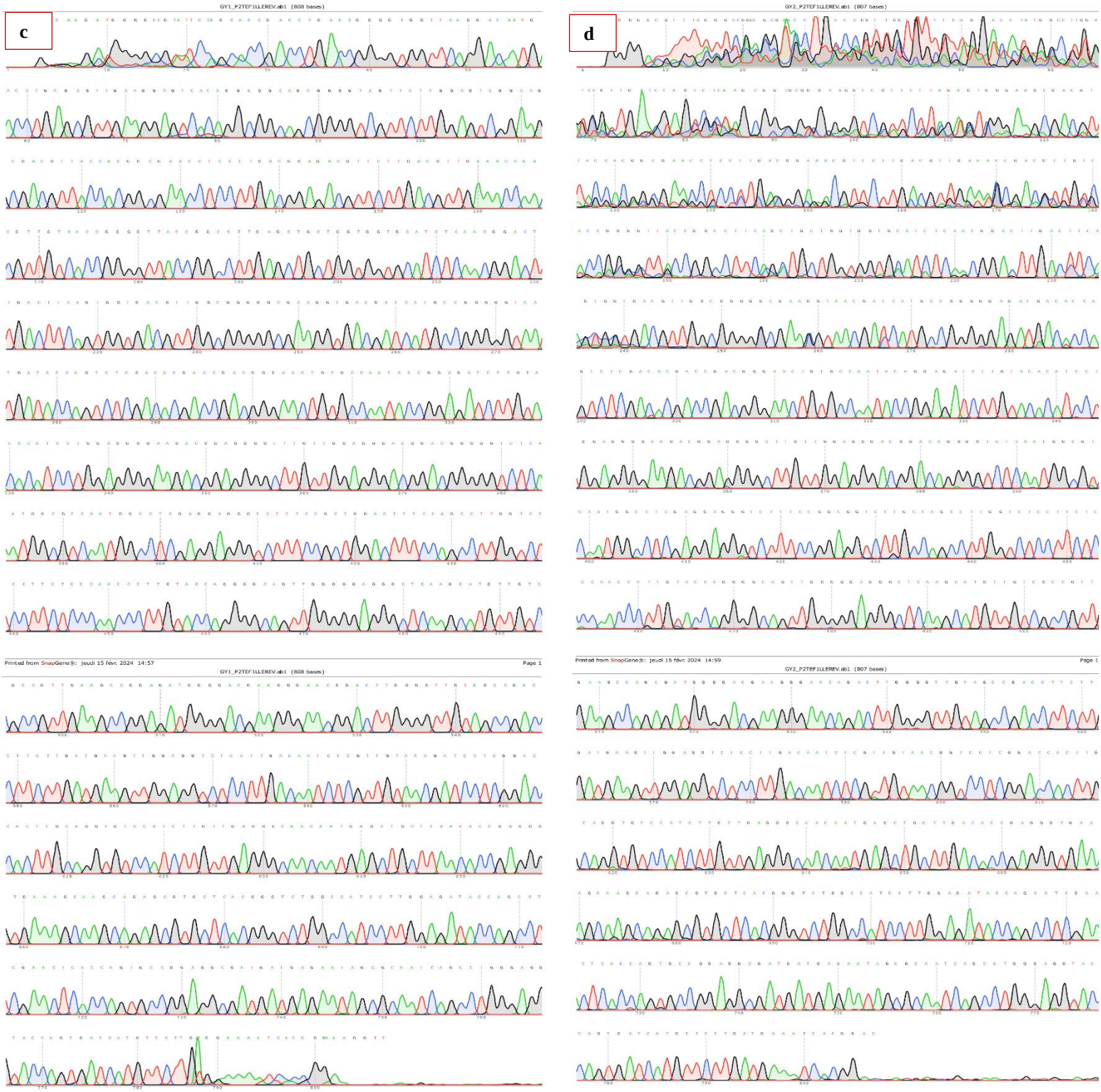
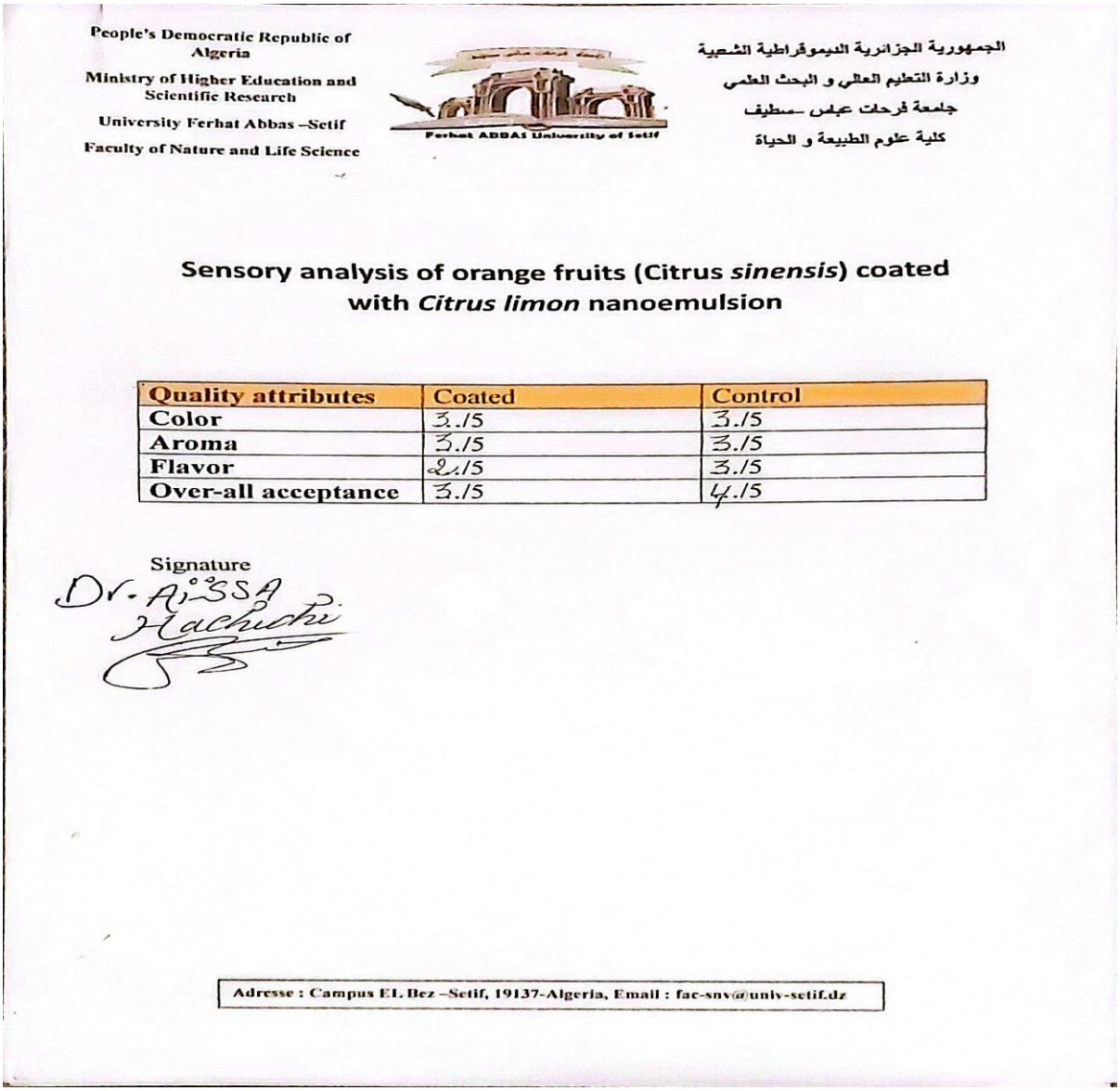


Figure A2.3. Electrophoretic Sequences of GY1 (c) and GY2 (d) Isolates from the EF1 Region

Figure A2.4. An example of the scale used for color in sensory analysis (Tarancón et al., 2022).



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الجمهورية الجزائرية الديموقراطية الشعبية
وزارة التعليم العالي و البحث العلمي
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Sensory analysis of orange fruits (Citrus *sinensis*) coated with *Citrus limon* nanoemulsion

Quality attributes	Coated	Control
Color	3./5	3./5
Aroma	3./5	3./5
Flavor	2./5	3./5
Over-all acceptance	3./5	4./5

Signature
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Figure A2.5. An example of sensory evaluation of Thomson orange fruits.

Publications:

Gharzouli, M., Aouf, A., Mahmoud, E., Ali, H., Alsulami, T., Badr, A. N., ... & Farouk, A. (2024). Antifungal effect of Algerian essential oil nanoemulsions to control Penicillium digitatum and Penicillium expansum in Thomson Navel oranges (Citrus sinensis L. Osbeck). *Frontiers in Plant Science*, *15*, 1491491. https://doi.org/10.3389/fpls.2024.1491491

Gharzouli, M., Aouf, A. H., Moawad, S., Ali, H., Alsulami, T., Farouk, A., ... & Badr, A. N. (2025). Bio-preservative potential of marjoram and fennel essential oil nano-emulsions against toxigenic fungi in citrus: integrating in-vitro, in-vivo, and in-silico approaches. *Food Additives & Contaminants: Part A*, *42*(5), 632-650. https://doi.org/10.1080/19440049.2025.2473551

الملخص

يمثل تلوث المحاصيل الحمضية بالفطريات مشكلة كبيرة في الجزائر. الهدف الرئيسي من هذه الدراسة هو الحد من نمو الفطريات في ثمار البرتقال باستخدام المستحلبات النانوية المستخلصة من نباتات *Origanum majorana*، *Citrus limon*، *Cymbopogon citratus* و *Foeniculum vulgare*. تم استخراج الزيوت الأساسية من هذه النباتات بطريقة التقطير المائي، كما تم إنتاج مستحلباتها النانوية باستخدام الموجات فوق الصوتية. تم تحليل التركيب الكيميائي باستخدام جهاز الكروماتوغرافية الغازية والمطيافية الكتلية، ثم تم تقييم الخصائص الفيزيائية والكيميائية للمستحلبات النانوية. تم تقييم الفعالية المضادة للميكروبات مخبريا. اضافة الى ذلك، تم تقييم الإمكانات المضادة للفطريات للبردقوش والشمر حاسوبيا. أظهر تحليل الكروماتوغرافية الغازية والمطيافية الكتلية أن الاستحلاب النانوي يزيد من تراكيز التربينين-4-أول (terpinen-4-ol) و سيس بيتا تربينيول (cis-β-terpineol) في البردقوش، وكذلك الإستراجول (estragole) والأنيثول (anethole) في الشمر، بالإضافة الى النيرال (neral) والجرانيال (geranial) في كل من زيت الليمون وزيت عشبة الليمون، مع انخفاض مستويات الفا تربينين (α-Terpinene)، الفينكون (fenchone)، الليمونين (limonene)، قاماتربينين (γ-terpinene)، وبيتا ميرسين (β-myrcene) .أظهرت صور المجهر الا لكتروني النافذ للمستحلبات النانوية مورفولوجية كروية بأبعاد نانوية متنوعة. أظهر التحليل الجزيئي والتحليل التطوري العرقي للفطريات المعزولة من ثمار البرتقال تطابقًا بنسبة 100% مع *P. expansum* و *P. digitatum*. أظهرت الزيوت الأساسية فعالية أكبر مقارنةً مع مستحلباتها النانوية، حيث تراوحت قيم الحد الأدنى من التركيز المثبط (MIC) بين 0.12% و2% حجم/ حجم ضد البكتيريا ومن 0.03% إلى1 % حجم/ حجم ضد الفطريات. علاوة على ذلك، تم تثبيط الغالبية العظمى من الفطريات المختبرة تمامًا بواسطة عشبة الليمون، من ناحية أخرى، أظهرت *F.vulgare* أضعف نشاطية مضادة للميكروبات. تمت ملاحظة قيم قوية للطاقة الحرة للتفاعل في التفاعلات بين الزيوت الأساسية أو المستحلبات النانوية وإنزيمات الفطريات، مع درجات ربط ملحوظة (−6.6 إلى−7.0 كيلوكالوري/مول) للمكونات العطرية. تطبيق المستحلبات النانوية للبردقوش، الليمون وعشبة الليمون كغلاف على ثمار البرتقال منع بشكل كبير نمو فطريات *P. expansum* و *P. digitatum* مقارنة بالعينات غير المغلفة. استخدام المستحلبات النانوية يقلل من التغيرات السلبية في معايير جودة ثمار البرتقال طوال فترة التخزين، بما في ذلك الحموضة القابلة للمعايرة (TA)، الرقم الهيدروجيني(pH) ، المواد الصلبة الذائبة الكلية (TSS)، تركيز حمض الأسكوربيك، فقدان الوزن والصلابة. لم تؤثر المستحلبات النانوية ل*O.majorana* و*C. limon* على الخصائص الحسية للفواكه المغلفة مقارنةً بالمستحلب النانوي ل*C. citratus*.بشكل عام، أظهرت المستحلبات النانوية التي تم اختبارها فعاليتها العالية في إدارة مسببات الأمراض التي تؤثر على ثمار البرتقال.

الكلمات المفتاحية: *Origanum majorana*، *Cymbopogon citratus*، *Citrus limon*، *Penicillium digitatum*، *Penicillium expansum*، الزيوت الاساسية، المستحلبات النانوية، برتقال تومسون.

Abstract

Fungal contamination of citrus crops represents a significant issue in Algeria. The primary aim of this study is to limit fungal growth on orange fruits using the nanoemulsions obtained from plants of *Origanum majorana*, *Citrus limon*, *Cymbopogon citratus and Foeniculum vulgare.* The essential oils from these plants were extracted using hydrodistillation method, and their nanoemulsions were produced using ultrasonication method. The chemical composition was analyzed using a GC-MS system, and the physicochemical properties of the nanoemulsions were assessed. The antimicrobial efficacy was evaluated *in vitro*. Furthermore, the antifungal potential of marjoram and fennel was assessed *in silico*. The GC-MS analysis demonstrated that the nanoemulsification increases the concentrations of terpinen-4-ol and cis-β-terpineol in marjoram, as well as estragole and anethole in fennel, in addition to neral and geranial in both lemon and lemongrass oils, while reducing the levels of *α*-terpinene, fenchone, limonene, γ-terpinene, and β-myrcene. The nanoparticles exhibited a narrow particle size distribution, indicating high stability and monodispersity of the nanoemulsions. The TEM imaging of the nanoemulsions showed a spherical morphology with diverse nanometer-scale dimensions. The molecular and the phylogenetic analysis of the isolated fungi from orange fruits showed 100% homology to *P. expansum* and *P. digitatum*. the essential oils exhibited greater potency compared to their nanoemulsions, with MIC values spanning from 2% to 0.12% against bacteria and from 1% to 0.03% against fungus. Furthermore, the majority of the tested fungi were completely inhibited by lemongrass. On the other hand, *F. vulgare* showed the weakest antimicrobial activity. Strong binding-free energy values were observed in the interactions between essential oil or nanoemulsions and fungal enzymes, with notable docking scores (−6.6 to −7.0 kcal/mol) for aromatic components. The application of nanoemulsified marjoram, lemon and lemongrass as a coating on orange fruits significantly inhibit the growth of *P. expansum* and *P. digitatum* molds compared to uncoated samples. The use of nanoemulsions minimize negative changes in quality parameters of orange fruits throughout storage, encompassing titratable acidity (TA), pH, total soluble solids (TSS), ascorbic acid concentration, weight loss and firmness. The nanoemulsified *O. majorana* and *C. limon* did not influence the sensory attributes of the coated fruits as compared to the *C. citratus* nanoemulsion. Overall, the tested nanoemulsions demonstrated their high efficacy in managing pathogens affecting orange fruits.

Key words: *Origanum majorana*, *Foeniculum vulgare*, *Cymbopogon citratus*, *Citrus limon*, *Penicillium digitatum*, *Penicillium expansum*, nanoemulsion, Thomson orange.

Résumé

La contamination fongique des cultures d'agrumes représente un problème important en Algérie. L'objectif principal de cette étude est de limiter la croissance fongique sur les fruits d'orange en utilisant les nanoemulsions obtenues à partir de plantes d'*Origanum majorana*, *Citrus limon*, *Cymbopogon citratus* et *Foeniculum vulgare*. Les huiles essentielles de ces plantes ont été extraites par hydrodistillation et leurs nanoémulsions ont été obtenues par ultrasonication. La composition chimique a été analysée à l'aide d'un appareil GC-MS, puis les propriétés physico-chimiques des nanoémulsions ont été évaluées. L'efficacité antimicrobienne a été évaluée *in vitro*. De plus, le potentiel antifongique de la marjolaine et du fenouil a été évalué *in silico*. L'analyse GC-MS a démontré que la nanoémulsification augmente les concentrations de terpinen-4-ol et de cis-β-terpinéol dans la marjolaine, ainsi que d'estragole et d'anéthole dans le fenouil, en plus de néral et de géranial dans les huiles de citron et de citronnelle, tout en réduisant les niveaux de α-terpinène, de fenchone, de limonène, de γ-terpinène et de β-myrcène. Les nanoparticules ont montré une distribution étroite des tailles de particules, indiquant une grande stabilité et une monodispersité des nanoémulsions. L'imagerie MET des nanoémulsions a montré une morphologie sphérique avec des dimensions variées à l'échelle nanométrique. L'analyse moléculaire et phylogenetique des moisissures isolées des fruits d'orange a montré une homologie de 100 % avec *P. expansum* et *P. digitatum*. Les huiles essentielles ont montré une plus grande efficacité par rapport à leurs nanoémulsions, avec des valeurs de CMI allant de 2 % à 0,12 % contre les bactéries et de 1 % à 0,03 % contre les moisissures. De plus, la majorité des moisissures testées ont été complètement inhibés par la citronelle. D'autre part, *F. vulgare* a montré la plus faible activité antimicrobienne. Des valeurs élevées d'énergie libre de liaison ont été observées dans les interactions entre les huiles essentielles ou les nanoémulsions et les enzymes fongiques, avec des scores de docking notables (−6,6 à −7,0 kcal/mol) pour les composants aromatiques. L'application de nanoémulsions de la marjolaine, le citron et la citronelle comme revêtement sur les fruits d'orange inhibe significativement la croissance des moisissures *P. expansum* et *P. digitatum* par rapport aux échantillons non revêtus. L'utilisation de nanoémulsions minimise les changements négatifs dans les paramètres de qualité des fruits d'orange tout au long du stockage, englobant l'acidité titrable (AT), le pH, les solides solubles totaux (SST), la concentration en acide ascorbique, la perte de poids et la fermeté. Les nanoémulsions d'*O.majorana* et de *C. limon* n'ont pas influencé les attributs sensoriels des fruits enrobés par rapport à la nanoémulsion de *C. citratus*. Dans l'ensemble, les nanoémulsions testées ont démontré leur haute efficacité dans le control des pathogènes affectant les fruits d'orange.

Mots-clés : *Origanum majorana*, *Foeniculum vulgare*, *Cymbopogon citratus*, *Citrus limon*, *Penicillium digitatum*, *Penicillium expansum*, nanoémulsion, orange Thomson.