Preparation of Essential Oils Loaded Sodium Alginate/Gelatin Blended Films: Their Antibacterial and Antioxidant Activities

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ABSTRACT

Antibacterial resistance has become a major health care problem around the world. So, need for new novel drugs that can act as antibacterial agents became essential to overcome the bacterial resistance in the last decade. Essential oils (EOs) are known as aromatic oily materials that found as liquid with a strong odor, and produced from different parts of plants. EOs provide a strong antibacterial activity that leads to their role in the protection of plants against different species of bacteria.

Within this study, complexes of sodium alginate and gelatin polymers were prepared. Two different EOs which were extracted from two plants from *Citrus* species which are orange (*Citrus sinensis*), and lemon (*Citrus limon*) were loaded on the polymer complexes. The final products were diluted in order to prepare different concentrations and examined for antibacterial activity and antioxidant activity. For antibacterial activity, Broth Microdilution assay was conducted in order to determine MICs for the products. While for the antioxidant activity, four different assays were conducted including; Total phenol and flavonoid contents, FRAP, and Ferrous ion-chelating effect. The results obtained from this study indicate that the final products exhibit antioxidant activity that is correlated with the concentration of essential oils within the polymer complexes. While for that antibacterial activity, the resulted showed that the lemon EO polymer complex exhibited higher activity than orange EO polymer complex.

Keywords: EOs, FRAP, Polymer, Medicinal Chemistry, MIC, Phenol, Flavonoid.

ÖZ

Antibakteriyel direnç, dünya çapında önemli bir sağlık sorunu haline gelmiştir. Bu nedenle, son on yılda bakteriyel direncin üstesinden gelmek için antibakteriyel ajan olarak davranabilen yeni ilaçlara ihtiyaç duyulmuştur. Uçucu yağlar, güçlü bir kokuya sahip sıvı halde bulunan ve bitkilerin farklı kısımlarından üretilen aromatik yağlı maddeler olarak bilinir. Uçucu yağlar sahip oldukları güçlü antibakteriyel aktiviteden dolayı bitkileri farklı bakteri türlerine karşı korunma rollünü sağlar.

Bu çalışma kapsamında sodyum aljinat ve jelatin polimerlerinin kompleksleri hazırlanmıştır. Polimer komplekslerine Citrus türlerinden portakal (*Citrus sinensis*) ve limon (*Citrus limon*) olmak üzere iki bitkiden ekstrakte edilen iki farklı Uçucu yağ yüklenmiştir. Elde edilen ürünler, farklı konsantrasyonlar hazırlamak için seyreltilmiş, antibakteriyel ve antioksidan aktiviteleri incelenmiştir. Antibakteriyel aktivite için ürünlerin minimal inhibisyon konsantrasyonu (MİK) değerleri belirlenmek amacıyla Broth Mikrodilüsyon testi kullanılmıştır. Antioksidan aktivite için ise dört farklı test uygulanmıştır, bu testler; toplam fenol ve toplam flavonoit içeriklerinin belirlenmesi, ferrik iyon indirgeme antioksidan gücünün tayini (FRAP) ve demir iyonu şelasyon etkinin tayinidir etkisidir. Bu çalışmada bulunan sonuçlar, elde edilen ürünlerin antioksidan aktivitelerinin, polimer komplekslerinin içindeki uçucu yağ konsantrasyonu ile ilişkili olduğunu göstermiştir. Antibakteriyel aktivite testinden elde edilen sonuçlar limon uçucu yağ polimer kompleksinin portakal uçucu yağ polimer kompleksinden daha yüksek aktivite sergilediğini göstermiştir.

Anahtar Kelimeler: Uçucu yağlar, FRAP, Polimer, Tıbbi Kimya, MIK, Flavonoid.

DEDICATION

To My Family

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LIST OF ABBREVIATIONS

^{1}H-NMR	Proton Nuclear Magnetic Resonance
^{13}C-NMR	13-Carbon Nuclear Magnetic Resonance
AES	Atomic Emission Spectroscopy
CCC	Countercurrent Chromatography
CFU	Colony-Forming Unit
EDTA	Ethylene Diamine Tetra Acetic Acid
EOs	Essential Oils
FRAP	Ferric-reducing Antioxidant Power Assay
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
HPLC	High-Pressure Liquid Chromatography
HSV	Herpes Simplex Virus
IR	Infrared Spectroscopy
IRMS	Isotope-Ratio Mass Spectrometry
LC	Liquid Chromatography
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
MS	Mass Spectroscopy
NA	No Activity
NIR	Near-Infrared Spectroscopy
SFC	Supercritical Fluid Chromatography
UV	Ultraviolet–visible Spectroscopy

Chapter 1

INTRODUCTION

In the past decades, the discovery of antibiotics lead to a successful treatment for different species of bacterial infections (Dengler Haunreiter et al., 2019). However, the bacterial resistance became a significant health care problem around the world in the last decade (Namivandi-Zangeneh et al., 2020). So, the need for new novel drugs that can act as antibacterial agents became essential to overcome the bacterial resistance (Rossiter et al., 2017).

Plant kingdom is considered as the main source for different important materials for humans. However, the plants are considered as a source of secondary metabolites that do not have a role in either growth or development of plants (Figueiredo et al., 2008). Essential oils (EOs) are known as aromatic oily material that is found as liquid with a strong odor, and produced from different parts of plants including; fruits, roots, herbs, and flowers. Due to the oily and aromatic characteristics of essential oils, they are also called the volatile or ethereal oils (Palazzolo et al., 2013). Naturally, essential oils provide a strong antibacterial activity that leads to their role in the protection of plants against different species of bacteria (Carson & Riley, 2003).

The *Citrus* peel EOs shows different quantity and quality depending on the climate, soil, genotype, nature of the fruits, and the extraction method (Dugo et al., 2000). The range of 0.5-5.0% is considered as the main content of EOs within the *Citrus* peel

(Palazzolo et al., 2013). The EOs found within the *Citrus* peel show a very complex matrix which contains various classes of numerous chemicals that produce different biological activities (Flamini et al., 2007). The limonene is considered as the major component that is found in *Citrus* peel EOs with a content of 32-98% (Moufida & Marzouk, 2003).

For the biological activity of *Citrus* species, it is known that *C. aurantium* L. is considered as the most usable species for biological activities (Palazzolo et al., 2013). *Citrus* EOs show the ability to produce antitumor activity due to the presence of monoterpenes. The antitumor effect was successfully studied against cancers in rodents in different organs including; forestomach, liver, mammary, skin, and lung (Crowell, 1999). Also, the presence of terpenes within the *Citrus* EOs leads to the production of anti-ulcer activity of EOs against stomach and intestinal ulcers (Lewis & Hanson, 1991). Additionally, *Citrus* EOs has been recorded to exhibit antiinflammatory activity (Moraes et al., 2009), antidepressant activity (Komori et al., 1995), antifungal activity (Sanguinetti et al., 2007), and sleep aiding activity (Tsuchiya et al., 1991).

Recently, the combination of different natural biomolecules is commonly applied in different industries in order to improve the characteristics and properties of the final product (Shahidi & Hossain, 2020). The different polymers that have been used for coating of EOs can be chitosan, gelatin (Y. Li et al., 2021), and alginate (Shankar et al., 2019).

Chapter 2

LITERATURE REVIEW

2.1 Phytochemicals

Pharmacognosy is known as one of the oldest pharmaceutical sciences which is interesting in the study of naturally originated drugs by investigating their chemical, physical, biological, and biochemical characteristics. Also, it is obvious that the subject of pharmacognosy has the power to incorporate in many aspects including; biochemistry, medicinal chemistry, organic chemistry, and biology (Lemke et al., 2012). Medicinal plant term is used to describe various species of plants which have the ability to exhibit different pharmaceutical activities. These species contain different types of phytochemicals which is the main reason for their pharmaceutical activities. Among the 17,000 species of higher plants, there are approximately 8,000 species of plants that can be considered as medicinal plants. These species were used in different traditional medicine systems such as; Traditional Chinese Medicine (TCM), and Ayurveda (R. Singh, 2015).

Phytochemicals or the isolated pharmacologically active substances from the plants are considered as one of the greatest approaches related to the drug discovery process (Sneader, 2005). Phytochemicals are chemicals isolated from plants that are biologically active as they have the ability to provide health benefits for humans. They are known as the secondary metabolites of the plants with their ability to exhibit different biological activities including; antioxidant activity, immune system

modulation, anticancer activity, detoxification activity, and anticancer activity. The plants that contain these substances are called herbal plants or medicinal plants, and had been used since the beginning of history (Saxena et al., 2013).

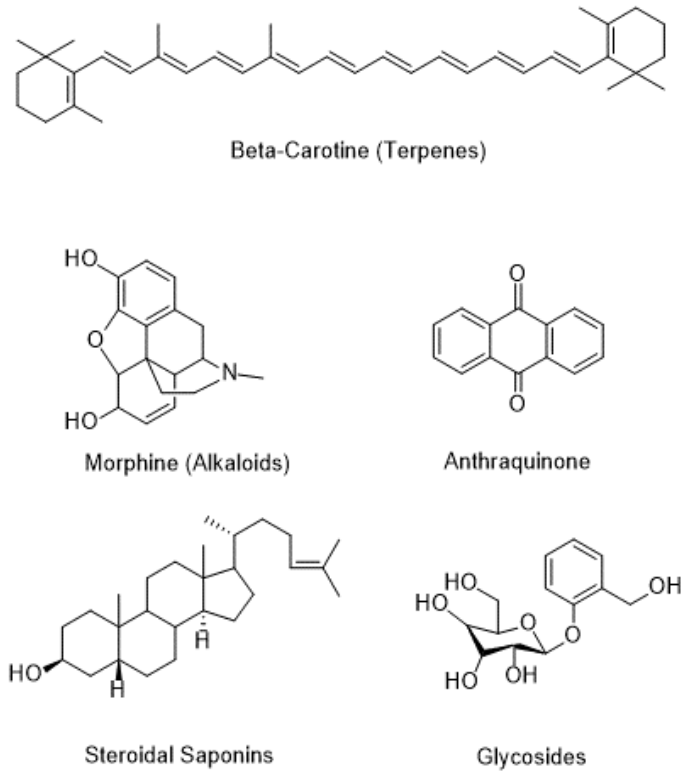

Figure 1: Some examples of phytochemicals (Saxena et al., 2013)

Phytochemicals play an essential role within the plants especially a protection function against different types of pests in addition to their therapeutic activities. According to their chemical composition, they can be divided into; alkaloids, glycosides, polyphenols including flavonoids, and phenolics tannins, saponins, terpenes including carotenoids, and steroids, as well as anthraquinones (Arvind Kumar Shakya, 2016). Recently, the interest in the phytochemicals increased due to the diversity in their chemical structure, in addition to their biological activity. Additionally, it was estimated that there are over 250,000 flower plant species that can be used as a source for novel drug molecules (Hosseinzadeh et al., 2015).

2.2 Essential Oils as Phytochemicals

Essential oils (EOs) are volatile oils which are highly concentrated natural products with a high content of high volatile compounds. These volatile compounds are found as a mixture of monoterpenoids, sesquiterpenoids, diterpenoids, phenylpropanoids, and benzenoids. Also, these compounds have the ability to exhibit various biological activities including many pharmaceutical activities such as antibacterial, antioxidant, and anticancer activities (Adorjan & Buchbauer, 2010). The first systematic investigation of the essential oil components was conducted by the French chemist M.J. Dumas, and published in 1833. Within this investigation, various types of hydrocarbons, oxygen-containing compounds, and sulphur-containing compounds were analyzed. While in 1859, the French researcher M. Berthelot was able to use optical rotation in order to characterize different kinds of these natural products (Kubeczka, 2020).

2.2.1 Biosynthesis of Essential Oils

Through photosynthesis, the green plants are able to convert both carbon dioxide and water into glucose, which is then producing phosphoenolpyruvate by cleavage. Phosphoenolpyruvate is considered as the main precursor for the natural products derived from shikimic acid pathway such as lignans, coumarins, and flavonoids. After that, phosphoenolpyruvate is decarboxylated giving coenzyme-A which is esterified giving acetyl Co-A. Acetyl Co-A has two different pathways including either self-condensation producing lipids and polyketides, or conversion to mevalonic acid which is the precursor for terpenoids (Sell, 2020).

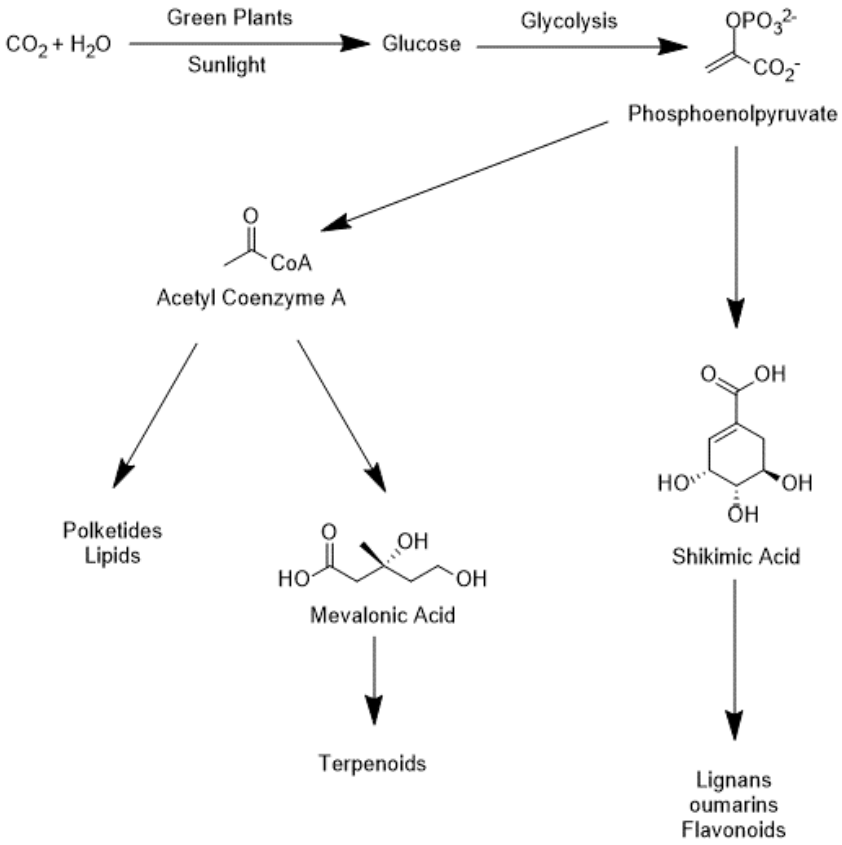

Figure 2: General scheme for secondary metabolite biosynthesis (Sell, 2020)

2.2.2 Sources of Essential Oils

EOs can be found in different genera among the plant kingdom where these genera are distributed to approximately 60 different families. However, there are seven families that are mostly used to produce EOs for either medicinal or industrial purposes which are Poaceae, Myrtaceae, Asteraceae, Alliaceae, Lamiaceae, Apiaceae, and Rutaceae (Hammer & Carson, 2010). Terpenoids are considered as the major constituents in the EO producing families. However, phenylpropanoids are more frequent in Apiaceae, Myrtaceae, Lamiaceae, and Rutaceae (Raut & Karuppayil, 2014).

Table 1: Some important EOs from some famous plant families (Raut & Karuppayil, 2014)

Family	Essential Oil	Therapeutic Activity
Asteraceae	✓ *Artemisia judaica*	• Antifungal • Antiviral • Anticancer
Rutaceae	✓ *Citrus* sp. (Lemon) ✓ *C. paradisi* (Grape fruit)	• Antifungal • Antibacterial • Anticancer
Lamiaceae	✓ *Melissa officinalis* (Lemon balm) ✓ *Mentha longifolia* (Wild Mint) ✓ *M. piperita* (Peppermint) ✓ *Salvia officinalis* (Sage) ✓ *Rosmarinus officinalis* (Rosemary) ✓ *Lavandula officinalis* (Lavender) ✓ *Origanum vulgare* (Oregano)	• Antibacterial • Antifungal • Anticancer • Antiviral • Antidiabetic • Antimutagenic • Antiprotozoal • Anti-inflammatory • Antioxidant
Apiaceae	✓ *Carum nigrum* (Black caraway) ✓ *Cuminum cyminum* (Cumin) ✓ *Foeniculum vulgare* (Fennel)	• Anti-diabetic • Antimicrobial • Anticancer
Rosaceae	✓ *Rosa* sp.	• Antifungal
Myrtaceae	✓ *Thymus vulgaris* (Thyme) ✓ *Melaleuca alternifolia* (Tea tree) ✓ *Eucalyptus globulus* (Blue gum)	• Antimutagenic • Anti-inflammatory • Antimicrobial
Liliaceae	✓ *Allium cepa* (onion) ✓ *Allium sativum* (Garlic)	• Antimicrobial
Zingiberaceae	✓ *Zingiber officinale* (Ginger) ✓ *Curcuma longa* (Turmeric)	• Anticancer • Antioxidant • Antimutagenic
Lauraceae	✓ *Cinnamomum* sp. (Cinnamon)	• Antimicrobial • Antimutagenic • Anti-inflammatory

The Poaceae family is a good source for many types of EOs including citronella oil from *Cymbopogon nardus*, lemongrass oil from *Cymbopogon citratus*, and palmarosa oil from *Cymbopogon martini*. These EOs are rich in geraniol, geranyl acetate, and citral which exhibit anticancer and antimicrobial activities. While Citrus oils are

derived from the fruit peel of plants from Rutaceae family and rich in linalool, and limonene that exhibit antimicrobial activity (Bedi, 2010). On the other hand, cinnamon oil which is obtained from *Cinnamomum verum* from Lauraceae family is rich in eugenol that exhibit both antimicrobial, and anticancer activities (Hammer & Carson, 2010).

2.2.3 Chemistry of Essential Oils

After the analysis of pure EOs, there are approximately 200 different components can be identified with the presence of either terpenes or phenylpropanoid derivatives (Rao & Pandey, 2006). The mixtures of EOs can be categorized into two different fractions; volatile fraction, and nonvolatile fraction. The volatile fraction represents 90-95% of the total weight of oil, and composed of different categories of terpenes, alcohols, aldehydes and esters. On the other hand, the nonvolatile fraction represents 1-10% of the total weight of oil, and composed of hydrocarbons, flavonoids, waxes, carotenoids, sterols, and fatty acids (Hanif et al., 2019).

Terpenes are considered as the main structural block for the components of EOs. They are the results of condensation of a pentacarbonate unit that carry two unsaturated bonds and called isoprene. Terpenes can be categorized according to the number of isoprene units within the structure into hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, and so on. However, the most frequent terpenes found in EOs are monoterpenes, and sesquiterpenes (Zuzarte & Salgueiro, 2015).

Monoterpenes have a formula of $C_{10}H_{16}$, and can be identified from mostly 90% of EOs (Bakkali et al., 2008). Linear hydrocarbons of this category have a 2,6-dimethyloctane structure carrying three double bonds, while the bicyclic constituents have a second ring with three, four, or five carbons attached to the hexane ring (Hunter,

2009). *Limonene, phellandrene, β-myrcene, pinene, sabinene,* and *terpinene* are some examples of monoterpenes hydrocarbons (Zuzarte & Salgueiro, 2015).

On the other hand monoterpene oxygenated derivatives can be a result of biochemical modifications such as oxidation (Hunter, 2009). This category includes *citral, citronellal, citronellol, geraniol, linalool, menthol,* and *thymol.* Going to sesquiterpenes, they have a formula of $C_{15}H_{24}$, and have lower volatilities with higher boiling point than monoterpenes with high structural diversity (Sell, 2020). *Cadinene, caryophyllene, elemene, farnesene,* and *zingiberene* are the main examples for sesquiterpene hydrocarbons, while *carotol, bisabolol, caryophyllene oxide, farnesol,* and *turmerones* are the main example for sesquiterpene oxygenated derivatives (Zuzarte & Salgueiro, 2015).

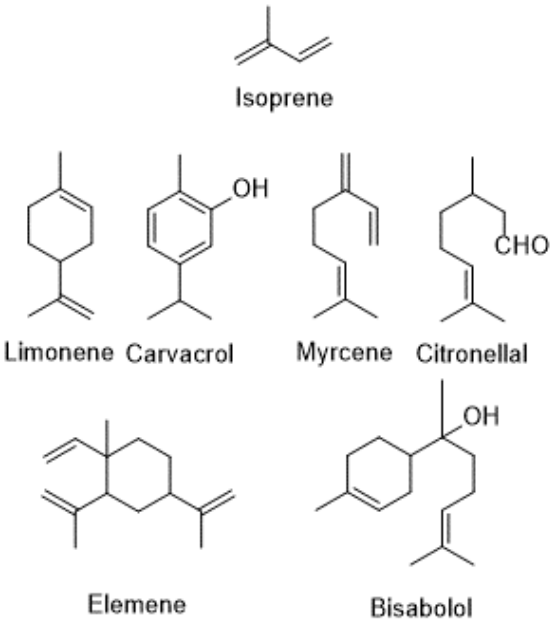

Figure 3: Isoprene structure and some examples of Mono- and Sesquiterpenes (Zuzarte & Salgueiro, 2015)

2.2.4 Analysis of Essential Oils

Before the analysis of EOs, sample preparation process is an essential procedure where there are many conventional techniques can be used for this purpose. The sample is prepared by extraction from the plant part that is known to be rich in EOs (Rubiolo et al., 2010). Some of these techniques are simple such as maceration, enfleurage, cold pressing, solvent extraction, and distillation which is categorized into hydro-distillation, steam distillation, turbo distillation extraction, simultaneous distillation-extraction (Hanif et al., 2019). However, there are some complicated techniques which show better extraction for the EOs. These techniques include supercritical fluid extraction especially supercritical CO_2 extraction, microwave-assisted extraction and hydro-distillation (MAE and MA-HD), and dynamic (D-HS), static (S-HS), and high concentration capacity headspace (HCC-HS) sampling (Rubiolo et al., 2010).

Going to the analysis, the procedure that can be used in the analysis of EOs can be classified into two main categories; classical analytical techniques, and modern analytical techniques (Hanif et al., 2019). Going to the classical analytical techniques, gravimetric methods and titrimetric methods are useful tools in the analysis of EOs. However, specific gravity method is considered as the most frequently used methods in the analysis of physiochemical characteristics of EOs (Guenther, 2013). On the other hand, the modern analytical techniques show higher effectivity due to their ability to give either qualitative or quantitative information with any kind of EOs samples especially chromatography (Arigò et al., 2020). Chromatographic techniques are the most frequently used techniques in the modern analytical methods. However, there is a need for another technique for the conformation and getting a reliable identification (Hanif et al., 2019).

Table 2: Different techniques used in the analysis of EOs (Kubeczka, 2020)

Chromatographic Techniques	Spectroscopic Techniques	Complex Techniques
TLC	MS	GC-UV
GC	IR	SFE-GC
LC	NIR	GC-MS
HPLC	UV	HPLC-GC
CCC	^{1}H-NMR	GC-AES
SFC	^{13}C-NMR	GC-FTIR
	Raman	SFC-GC
		GC-IRMS
		GC-FTIR-MS
		HPLC-NMR
		HPLC-MS

2.2.5 Therapeutic Activities of EOs

Despite the importance of EOs for the plants, they have the ability to exhibit biological and therapeutic activities (Gershenzon & Dudareva, 2007). These biological activities can be summarized as antithrombotic, immunomodulatory, antiplatelet, antioxidant, analgesic, antiinflammatory, anticancer, and antimicrobial activities (Carson & Hammer, 2011). Going to antibacterial activity, different EOs from different plants show the ability to produce antibacterial activity against wide range of either gram positive and gram negative bacteria (Raut & Karuppayil, 2014). However, their prolonged use of high concentration is limited due to their toxicity because of their serious side effects (Galvão et al., 2012). The efficacy of EOs as antibacterial agents can be a result of their high lipophilicity property which allows them to easily penetrate cell wall and cell membrane. Also, they show some interactions with different components of cell membrane such as polysaccharides, phospholipids, and fatty acids

leading to an increase in bacterial membrane permeability. That increase in permeability can lead to the loss of cell contents and ions resulting in the cell death (Saad et al., 2013).

Antimutagenic activity was another reported activity for EOs from some species due to their components (Jeena et al., 2013; Varona et al., 2013). EO extracted from *Matricaria chamomilla* shows the ability to some induced mutagenic errors in mouse bone marrow, while the EOs extracted from *Cinnamomum camphora*, *Helichrysum italicum, Origanum compactum,* and *Ledum groenlandicum* show the ability to inhibit the induced mutations in *Drosophila melanogaster* (Raut & Karuppayil, 2014). Also, EOs extracted from *Abies balsamea* (L.) Mill., *Croton regelianus* Muell. Arg., *Cymbopogon flexuosus*, *Eucalyptus benthamii*, *Guatteria friesiana*, *Guatteria pogonopus*, and *Lindera umbellate* show their ability to exhibit antitumor activity against different types of cancer cells (Magalhães & Sousa, 2015).

Another reported activity was their antiviral activity, as it is believed that the presence of terpenoid, and phenylpropanoid constituents are responsible for this activity (Astani et al., 2011). EO extracted from *Thymus* species is reported to have the ability to inhibit the replication of Epstein–Barr virus (EBV) (Hamid, A.A., Aiyelaagbe, O.O. and Usman, 2011). While *Melissa officinalis* L. EO shows the ability to prevent the replication of HSV-2 (Raut & Karuppayil, 2014), and *Melaleuca alternifolia* EO has antiviral activity against influenza virus & HSV-1 (Garozzo et al., 2011).

Another important therapeutic activity of EOs is the antioxidant activity via the maintenance of cellular balance of free radicals. EOs have the ability to exhibit the antioxidant activity due to the presence of terpenoids, flavonoids, and phenolic

compounds (Sánchez-Vioque et al., 2013). Rutaceae family is considered as one of the family that is rich in EOs with antioxidant activity especially *Citrus limon* L. (lemon), and *Citrus paradisi* Macfad. (grapefruit). Also, EOs extracted from *Caryophyllus aromaticum* (clove) from Myrtaceae family, and *Coriandrum sativum* L. (coriander) from Apiaceae family show an antioxidant activity (Adorjan & Buchbauer, 2010). Going to the antiinflammatory activity, EOs from Aloe-vera (*Aloe barbadensis*), bergamot (*Citrus aurantium*), ylang-ylang (*Cananga odorata*), anise star (*Illicium verum*), and lavender (*Lavandula officinalis*) show their ability to inhibit lipoxygenase, and cyclooxygenase enzymes, in addition to prevent the leukotriene synthesis resulting in a potent antiinflammatory effect (Miguel, 2010).

2.3 *Citrus* Peel Essential Oil

One of the most popular and traded crops across the world is the genus *Citrus* from the family Rutaceae. Also, this genus is considered as one of the ancient crops as there is an earliest record related to its cultivation dating back to 2100 BC (Moore, 2001). It is known that the *Citrus* fruits are highly consumed freshly or manufactured in order to produce either jam or juice. The fruits are considered as an excellent source of vitamin C which can be a good explanation for the high consumption. However, the use of *Citrus* fruit results in a high amount of waste including; pulps, seeds, and peels which resemble almost 50% of the fruit itself (Anwar et al., 2008).

There are numerous species combined under the *Citrus* genus including; orange (*Citrus sinensis*), lemon (*Citrus limon*), mandarin (*Citrus reticulata*), kumquat (*Citrus japonica*), limes (*Citrus aurantifolia*), bergamot (*Citrus bergamia*), grapefruit (*Citrus paradisi*), and yuzu (*Citrus junos*) (Dosoky & Setzer, 2018).

2.3.1 Chemical Composition of *Citrus* Peel Essential Oil

The *Citrus* essential oils can be considered as a mixture of different chemical constituents including; different kinds of hydrocarbons, and oxygenated derivatives of either terpenoids or non-terpenoids origins. These oxygenated derivatives exhibit different functional groups such as; ketones, alcohols, esters, aldehydes, and organic acids (Bora et al., 2020; Bustamante et al., 2016). The main chemical classes of essential oil components that could be identified in *Citrus* peel are hydrocarbons which can be either monoterpenes or sesquiterpenes, and oxygenated derivatives of either monoterpenes or sesquiterpenes (J. Li et al., 2020).

The composition of EOs in *Citrus* peel is changed according to the maturation of the fruits. As in a study conducted on sour orange from southwest of Iran, it was reported that the hydrocarbon constituents were found to be higher in the ripe peel, while the oxygenated derivatives were found to be higher in unripe peel (Azhdarzadeh & Hojjati, 2016). Also, the plant origin plays an important role in the composition of the EOs, as it was found that the composition of EOs is different depending on the country where the *Citrus* species are cultivated (B. Singh et al., 2021). In a study conducted on different *Citrus* species in Egypt, it was reported that the percentage of monoterpenes hydrocarbons was 93.1% in mandarin, 90.6% in orange, 88.5% in lime, and 91.8% in grapefruit. While the oxygenated derivatives represent 3.7, 4.7, 8.3 and 3.0%, respectively of the same species (Abd-Elwahab et al., 2016). While in a Turkish study conducted on 3 different *Citrus* species, it was reported that the percentage of monoterpenes hydrocarbons was 89.9% in lemon, 96.4% in grapefruit, and 97.3% in bitter orange. While the oxygenated derivatives represent 5.1, 1.2 and 2.5%, respectively, and the

sesquiterpenes hydrocarbons represent 3.3, 0.8 and 0.1%, respectively (Kirbaşlar & Boz, 2011).

Table 3: Classification of chemical constituents obtained from Citrus peel EOs (Espina et al., 2011; B. Singh et al., 2021)

Class	Compound	Molecular Formula	IUPAC name
Monoterpene hydrocarbons	α-Pinene	$C_{10}H_{16}$	2,6,6-trimethylbicyclo [3.1.1] hept-2-ene
	Limonene	$C_{10}H_{16}$	1-methyl-4-prop-1-en-2-ylcyclohexene
	β-Phellandrene	$C_{10}H_{16}$	3-methylidene-6-propan-2-ylcyclohexene
	β-Pinene	$C_{10}H_{16}$	6,6-dimethyl-2-methylidene bicyclo [3.1.1] heptane
	β-Myrcene	$C_{10}H_{16}$	7-methyl-3-methylideneocta-1,6-diene
	γ-Terpinene	$C_{10}H_{16}$	1-methyl-4-propan-2-ylcyclohexa-1,4-diene
	Sabinene	$C_{10}H_{16}$	4-methylidene-1-propan-2-ylbicyclo [3.1.0] hexane
	α-Terpinolene	$C_{10}H_{16}$	1-methyl-4-propan-2-ylidenecyclohexene
	(E)-β-Ocimene	$C_{10}H_{16}$	(3~{E})-3,7-dimethylocta-1,3,6-triene
	Δ-Carene	$C_{10}H_{16}$	(1~{R},6~{S})-3,7,7-trimethylbicyclo [4.1.0] hept-3-ene
Sesquiterpene hydrocarbons	β-Elemene	$C_{15}H_{24}$	(1~{S},2~{S},4~{R})-1-ethenyl-1-methyl-2,4- bis(prop-1-en-2-yl) cyclohexane
	α-Humulene	$C_{15}H_{24}$	(1E,4E,8E)-2,6,6,9-tetramethylcycloundeca-1,4,8-triene
	Thujopsen	$C_{15}H_{24}$	1aS,4aS,8aS)-2,4a,8,8-tetramethyl-1,1a,4,5,6,7-hexahydrocyclopropa[j]naphthalene
	β-Caryophyllene	$C_{15}H_{24}$	(1~{R},4~{E},9~{S})-4,11,11-trimethyl-8- methylidenebicyclo [7.2.0] undec-4-ene
	Copaene	$C_{15}H_{24}$	1,3-dimethyl-8-propan-2-yltricyclo [4.4.0.0^ {2,7}] dec-3-ene

	δ-Cadinene	$C_{15}H_{24}$	(1S,8aR)-4,7-dimethyl-1-propan-2-yl-1,2,3,5,6,8a-hexahydronaphthalene
	Valencene	$C_{15}H_{24}$	(3~{R},4~{a}~{S},5~{R})-4~{a},5-dimethyl-3-prop-1-en-2-yl-2,3,4,5,6,7-hexahydro-1~{H}- naphthalene
	α-Cedrene	$C_{15}H_{24}$	(1S,2R,5S,7S)-2,6,6,8-tetramethyltricyclo [5.3.1.01,5] undec-8-ene
	β-Bisabolene	$C_{15}H_{24}$	(4~{S})-1-methyl-4-(6-methylhepta-1,5-dien-2- yl) cyclohexene
Oxygenated Monoterpenes	Citronellal	$C_{10}H_{18}O$	3,7-dimethyloct-6-enal
	Linalool	$C_{10}H_{18}O$	3,7-dimethylocta-1,6-dien-3-ol
	Decanone	$C_{10}H_{20}O$	Decan-2-one
	Decanal	$C_{10}H_{20}O$	Decanal
	Verbenone	$C_{10}H_{14}O$	2,6,6-trimethylbicyclo [3.1.1] hept-2-en-4-one
	Geraniol	$C_{10}H_{18}O$	(2~{E})-3,7-dimethylocta-2,6-dien-1-ol
	α-Terpineol	$C_{10}H_{18}O$	2-(4-methylcyclohex-3-en-1-yl) propan-2-ol
	Nerol	$C_{10}H_{18}O$	(2Z)-3,7-dimethylocta-2,6-dien-1-ol
	Terpinen-4-ol	$C_{10}H_{18}O$	4-methyl-1-propan-2-ylcyclohex-3-en-1-ol
	Limonene oxide	$C_{10}H_{16}O$	1-methyl-4-prop-1-en-2-yl-7-oxabicyclo [4.1.0] heptane
	Octanal	$C_8H_{16}O$	Octanal
	trans-Carveol	$C_{10}H_{16}O$	(1S,5R)-2-methyl-5-prop-1-en-2-ylcyclohex-2-en-1-ol
	Citral	$C_{10}H_{16}O$	(2~{E})-3,7-dimethylocta-2,6-dienal
	d-Carvone	$C_{10}H_{14}O$	(5S)-2-methyl-5-prop-1-en-2-ylcyclohex-2-en-1- one
	Decadienal	$C_{10}H_{16}O$	deca-2,4-dienal
	Geranyl acetate	$C_{12}H_{20}O_2$	[(2~{E})-3,7-dimethylocta-2,6-dienyl] acetate [(2Z)-3,7-dimethylocta-2,6-dienyl] acetate
	4′-Methoxyacetophenone	$C_9H_{10}O_2$	1-(4-methoxyphenyl) ethenone

	Isopulegol acetate	$C_{12}H_{20}O_2$	[(1R,2S,5R)-5-methyl-2-prop-1-en-2-ylcyclohexyl] acetate
	Linalool acetate	$C_{12}H_{20}O_2$	3,7-dimethylocta-1,6-dien-3-yl acetate
	Neryl propionate	$C_{13}H_{22}O_2$	[(2Z)-3,7-dimethylocta-2,6-dienyl] propanoate
	Citronellyl acetate	$C_{12}H_{22}O_2$	3,7-dimethyloct-6-enyl acetate
Oxygenated sesquiterpenes	Nootkatone	$C_{15}H_{22}O$	(4~{R},4~{a}~{S},6~{R})-4,4~{a}-dimethyl6--prop-1-en-2-yl-3,4,5,6,7,8-hexahydronaphthalen-2-one
	Farnesol	$C_{15}H_{26}O$	(2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-ol
	Nerolidol	$C_{15}H_{26}O$	(6~{E})-3,7,11-trimethyldodeca-1,6,10-trien-3-ol
	α-Sinensal	$C_{15}H_{22}O$	(2~{E},6~{E},9~{E})-2,6,10-trimethyldodeca-2,6,9,11-tetraenal

By making a comparison between orange and lemon, it can be obvious that their composition of EOs in their peels is showing a vast difference. According to Kirbaşlar & Boz, 2011, limonene content in *Citrus sinensis* which is obtained from Turkey was 91.6% of total EOs content, while in *Citrus limon*, it was 61.8% from the total EOs content. Also, β-pinene was 8.1% in lemon, but it was not obtained from orange (Kirbaşlar & Boz, 2011).

Table 4: Comparison between Turkish sweet orange and lemon EOs content in fruit's peel (Kirbaşlar & Boz, 2011)

Chemical Compound	Citrus sinensis (Sweet Orange)	Citrus limon (Lemon)
Limonene	91.6%	61.8%
Myrcene	1.3%	----
sabinene	1.0%	----
α-pinene	0.9%	----
β-pinene	----	8.1%
β-caryophyllene	0.1%	----
α-copaene	0.1%	----
Octanal	1.4%	0.1%
Decanal	0.2%	0.1%
Geranial	0.2%	1.3%
Linalool	0.4%	----
α-terpineol	0.1%	----
Geraniol	0.1%	----
Geranyl acetate	0.1%	0.6%
Neryl acetate	0.1%	1.2%
γ-terpinene	----	10.6%
β-bisabolene	----	1.6%
β-caryophyllene	----	0.7%
α-bergamotene	----	1.0%
Neral	----	0.7%

2.4 Alginate

Alginate is considered as anionic polymer which is naturally occurring and obtained from brown seaweed (Lee & Mooney, 2012). The main source for the commercial alginate is its extraction from the *Phaeophyceae* which commonly known as brown algae. There are mainly five members of this family where the alginate is extracted

including; *Macrocystis pyrifera, Laminaria digitata, Ascophyllum nodosum, Laminaria hyperborea,* and *Laminaria japonica* (Smidsrød & Skjåk-Bræk, 1990). The extraction process is mostly conducted via the treatment of these algae with aqueous alkali solutions especially sodium hydroxide (NaOH) (Lee & Mooney, 2012). Commercially, the alginate contents for *Laminaria digitata* are 25-44%, while the alginate contents for *Laminaria digitata* are 22-30% based on the dry weight (Qin, 2008).

Alginate is known as an entire stock of linear copolymers which are composed of blocks that are linked via (1,4) linkages. These linkages are connected two different residues which are α-L-guluronate (G), and β-D-mannuronate (M). These blocks show three main different connections as they can be consecutive M residues, consecutive G residues, or alternating M and G residues. The length of each block in addition to M and G residues contents are mainly depending on the source of alginate leading to manufacture of more than 200 different commercial alginates currently (Tønnesen & Karlsen, 2002).

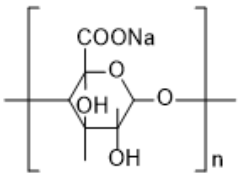

Figure 4: Alginate structure

2.4.1 Pharmaceutical Applications of Alginate

The alginate is traditionally used in the pharmaceutical industry as stabilizing agent, gel forming agent, or thickening agent. Since it shows these abilities, it is used on order

to control the release of drugs especially for the oral dosage forms. But the introduction of alginate in the localized drug delivery is increasing recently (Lee & Mooney, 2012).

The first main use for alginate is the small molecular drugs delivery based on the alginate gels. The nanoporous structure of alginate gels due to the pore sizes of almost 5 nm plays an important role in the rapid diffusion of small molecules (Boontheekul et al., 2005). Also, the antineoplastic agents were successfully controlled and delivered locally based on the use of partially oxidized alginate gels (Bouhadir et al., 2001). Protein delivery is considered as the second main application of alginate in the pharmaceutical industry as the protein-based drugs market is growing rapidly. The use of alginate gels is helpful for the reduction of denaturation process of proteins in addition to their protection from degradation (Lee & Mooney, 2012). Generally, the porosity of gels in addition to their hydrophilic nature led to a rapid release rate (Lee et al., 2003). However, some protein-based drugs can exhibit reversible binding interactions with alginate hydrogels such as heparin binding growth factors. These reversible interactions enable sustained release of the drug in addition to a localized delivery (Silva & Mooney, 2010).

2.5 Gelatin

Gelatin is known as a heterogeneous mixture of peptides which obtained via the hydrolysis of parent protein that is known as collagen. The hydrolysis process is conducted through the destruction of the polypeptide bonds (Liu et al., 2015). However, for the production of gelatin hydrolysates, an extensive enzymatic degradation can further be applied on the gelatin (Zhou et al., 2006). The production of gelatin from collagen process is mainly based on the denaturation of collagen suggests a partial destruction of either secondary and tertiary structure. Also, it was

suggested that some primary structure can be destructed in order to produce a mixture of polypeptides and proteins (See et al., 2015).

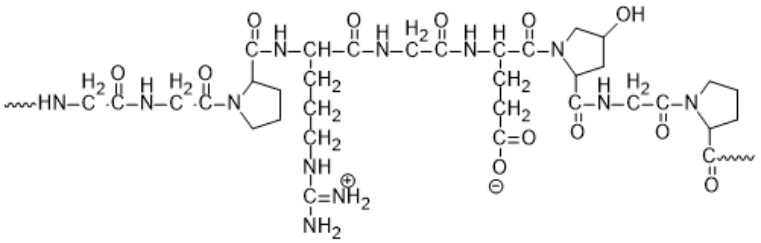

Figure 5: Gelatin structure

The commercial gelatin is obtained from the mammalian tissues including bones and skin. The main sources for these mammalian tissues are caprine, porcine, and bovine species (Gaspar-Pintiliescu et al., 2019). However, the interest in finding alternative sources especially the marine organisms for gelatin has been increased in order to avoid zoonotic diseases such as; swine flu, in addition to some religious reasons (Karim & Bhat, 2009). Additionally, the byproducts that are produced from different fish species can be used for the extraction of marine-based gelatin such as; squids (Chan-Higuera et al., 2016), sponges (Tziveleka et al., 2017), jellyfishes (Chancharern et al., 2016), and snails (Nazeer & Sri Suganya, 2014). There are different species of fishes that are used as source for fish gelatin including; megrim (*Lepidorhombus boscii*), black tilapia (*Oreochromis mossambicus*), red tilapia (*Oreochromis nilotica*), Nile perch (*Lates niloticus*), pollock skins (*Alaskan pollock*), yellowfin tuna (*Thunnus albacares*), dover sole (*Solea vulgaris*), bigeye snapper (*Priacanthus macracanthus*), Atlantic salmon (*Salmo salar*), and Nile tilapia (*Oreochromis niloticus*) (Karim & Bhat, 2009).

Gelatin is used as a natural biomaterial extensively due to its physiochemical properties (Gaspar-Pintiliescu et al., 2019). It is considered as the kind of protein that plays a major role in the enhancement of viscosity for the continuous phase of the two-phases systems or emulsions. Also, it shows the ability to delay coalescence and flocculation formation within the emulsions leading to an enhancement in the stability of emulsions specially the oil-in-water emulsions (Djagny et al., 2001). The use of gelatin as a natural biomaterial in tissue engineering is related to it characteristics including; its low antigenicity, high biodegradability, and high biocompatibility. Also, it shows high ability in stimulation of either cellular growth or attachment for wound healing (Nikkhah et al., 2016). On the other hand, it was reported that the gelatin which is isolated from *Rapana venosa* snail shows the ability to promote cell adhesion and cell proliferation (Gaspar-Pintiliescu et al., 2019). Additionally, protein hydrolysates that was isolated from *Rapana venosa* snail show the ability to exhibit a significant antioxidant activity (Luo et al., 2018).

Chapter 3

EXPERIMENTAL

3.1 Essential Oil Extraction and Preparation

In March 2021, mature orange (*Citrus sinensis* L.), and lemon (*Citrus limon* L.) fruits were collected from Guzelyurt, TRNC. The peels of both fruits were peeled off in order to be used fresh for the essential oil extraction process. Then, 100 g of peels from orange and lemon were preheaed at 45 ºC and kept for two hours. Finally, 200 mL of distilled water were added to both collected peels in order to start the hydrodistillation process for 120 minutes using Clevenger-type apparatus. The yield percentage was calculated according to the following formula;

$$\%Yield = \frac{Amount\ of\ EO\ extracted}{Amount\ of\ peels\ used}\ x\ 100\%$$

3.2 Film Preparation

Firstly, the film solutions were prepared as a mixture of 1:1 alginate-gelatin ratio. Starting with gelatin, 1 g of gelatin was dissolved in 50 ml distilled water and left for stirring for 30 minutes, then it was heated while stirring for 15 minutes at 60 °C. While for the alginate, 1 g of alginate was dissolved in 50 ml distilled water and left for stirring for 30 min then heated for 30 minutes while stirring at 60 °C. After that, the 2 mixtures were mixed together and left on stirrer.

For the preparation of essential oil solution, tween-80 was used as a surfactant to prepare the oil-in-water emulsion of essential oil and distilled water. Firstly, a solution

of tween-80 and distilled water was prepared by dissolving 10 mL tween-80 in 90 mL of distilled water in order to prepare 10% (v/v) tween-80 solution. Then, the final solution was split into two equal volumes which are 50 mL for each essential oil used. After that, 5 ml of essential oil were added to 45 mL tween-80 solution drop by drop in order to obtain a concentration of 10% (v/v) while stirring and left on stirrer for 30 minutes. Then, the emulsion of essential oil was sonicated for 45 min to obtain its maximum homogeneity.

For the preparation of the final films, the 50 ml of essential oil emulsion was added to 50 ml of gelatin-alginate mixture in order to obtain a concentration of 5% (v/v) while stirring and left on stirrer for 15 minutes. Then, the mixture is sonicated for 15 minutes in order to obtain the maximum homogeneity. The mixture with a concentration of 5% (v/v) was used as a stock to prepare the other concentrations 4, 3, 2, 1, 0.8, 0.6, 0.4, 0.2, and 0.1% by serial dilution. Finally, the final mixtures were cast onto 4 cm diameter Petri dishes with a volume of approximately 5 ml, and left to dry at room temperature for 48 hours.

3.3 Characterization of Film

3.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy was used in order to confirm the formation of essential oil-gelatin-alginate films. Analysis was conducted using a spectrophotometer in a range of 600-4000 cm^{-1}, and the analysis was conducted in the absorbance mode.

3.4 Antioxidant Assay

3.4.1 Determination of Total Phenol Contents

The total phenol contents of the films were determined based on the Folin-Ciocalteau's method (Singleton & Rossi, 1965). Different concentrations of gallic acid were used as a reference in order to prepare a calibration curve. In 96-well microplate, 25 µL of sample which can be the film or gallic acid dilutions were mixed with a specific amount of distilled water, Folin-Ciocalteau's reagent, and sodium carbonate. The 96-well microplate was incubated at 40 °C for 30 min. Afterward, the UV-visible spectrophotometer was used in order to measure the absorbance at 760 nm.

3.4.2 Determination of Total Flavonoid Contents

Aluminum chloride colorimetric method (Woisky & Salatino, 1998) was used in order to calculate the total flavonoid content. Different concentrations of quercetin were used as a reference in order to prepare a calibration curve. In 96-well microplate, 25 µL of sample which can be the film or quercetin dilutions were mixed with specific amount of ethanol, aluminum chloride reagent, sodium acetate, and distilled water. The 96-well microplate was incubated at 25 °C for 30 min. Afterward, the UV-visible spectrophotometer was used in order to measure the absorbance at 415 nm.

3.4.3 Ferric-reducing Antioxidant Power Assay (FRAP)

The assay of (Oyaizu, 1986) was used in order to test the ferric-reducing power of the films. In 96-well microplate, 40 µL of sample which can be the film dilutions were mixed with phosphate buffer (pH 6.6), and 100 µL of potassium ferricyanide. Then, the 96-well microplate was incubated at 50 °C for 20 min. After that, trichloroacetic acid (10%) were added to the mixture and mixed well. Then, 9 µL of sample which can be the film or quercetin dilutions were transferred to another 96-well microplate, and mixed with distilled water, and ferric chloride (0.1%). Afterwards, the 96-well

microplate was incubated at 25 °C for 30 min. Finally, the UV-visible spectrophotometer was used in order to measure the absorbance at 700 nm. The increase in the absorption of the reaction indicates an increase in the reducing power compared with the reference which is chlorogenic acid.

3.4.4 Ferrous ion-chelating Effect

The methodology of (Chua et al., 2008) was used in order to estimate the ferrous ion-chelating effect of the films and reference as well. In 96-well microplate, 10 µL of sample which can be the film or ethylenediamine tetra acetic acid (EDTA) dilutions were mixed with ferrous chloride, ferrozine, and ethanol (80%). Afterwards, the 96-well microplate was incubated at 25 °C for 10 min. Finally, the UV-visible spectrophotometer was used in order to measure the absorbance at 562 nm. The ratio of inhibition was calculated as the following;

$$I\% = \left(\frac{A_{Blank} - A_{Sample}}{A_{Blank}}\right) \times 100$$

3.5 Antibacterial Assay

In order to evaluate the antibacterial activity of the final products, two bacterial strains were utilized including; gram-positive *Staphylococcus aureus* (ATCC 29213), and gram-negative *Escherichia coli* (ATCC 25922). The stock solutions of the two final products of either orange or lemon essential oils were prepared as a concentration of 10% (v/v).

3.5.1 Broth Microdilution

In order to determine the antibacterial activity, Broth Micro-Dilution test (Norouzbahari et al., 2020) was conducted. Initially, 50 µL of Muller Hinton Broth (MHB) solution was poured into the wells. The stock solutions for the final products were diluted serially in a 96-well microplate. The bacterial suspensions were adjusted to 10^6 CFU/mL as 18 hours fresh suspensions. Except the negative control wells, 50

µL of bacterial suspensions were added to each well. After the addition of the bacterial suspensions into the 96-well plate, the plate was incubated for 24 hours aerobically at 37 °C. After 24 hours, the results were read in order to determine the minimum inhibitory concentrations (MICs) of the final products for each bacterial sample.

Chapter 4

RESULTS

4.1 Essential Oil Yield

According to the amount of EO extracted from both species' peels, the yield percentage of EOs of orange (*Citrus sinensis* L.), and orange (*Citrus limon* L.) peels were calculated as 0.53, and 0.61, respectively.

4.2 Characterization of Film

4.2.1 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra was used to indicate the interactions between the chemicals used within this study. The FTIR spectrum of sodium alginate have shown very distinguished peaks for the functional groups (O-H), (C-O), and (C-H) respectively at approximately 3400, 2150, and 1030 cm^{-1}. Also, the carboxylate functional group (C=O) have a prominent peak for the asymmetric stretching at 1620 cm^{-1}, and another notable peak for the symmetric stretching at 1420 cm^{-1}. On the other hand, the FTIR spectrum of gelatin have shown a characteristic peak at 3450 cm^{-1} that resemble the presence of an amino group (N-H) as a stretching peak. Also, the stretching of olefins (C-H) was detected at approximately 3070 cm^{-1}, the stretching of alkanes (C-H) was detected at 2900 cm^{-1} mostly, and the stretching of amide group (C-N, C=O) was observed at approximately 1650 cm^{-1}. Additionally, there are two eminent peaks were

observed for the stretching of (C-N) of amine, and bending of alkanes (C-H) at 1340 cm^{-1}, and 1045 cm^{-1} respectively.

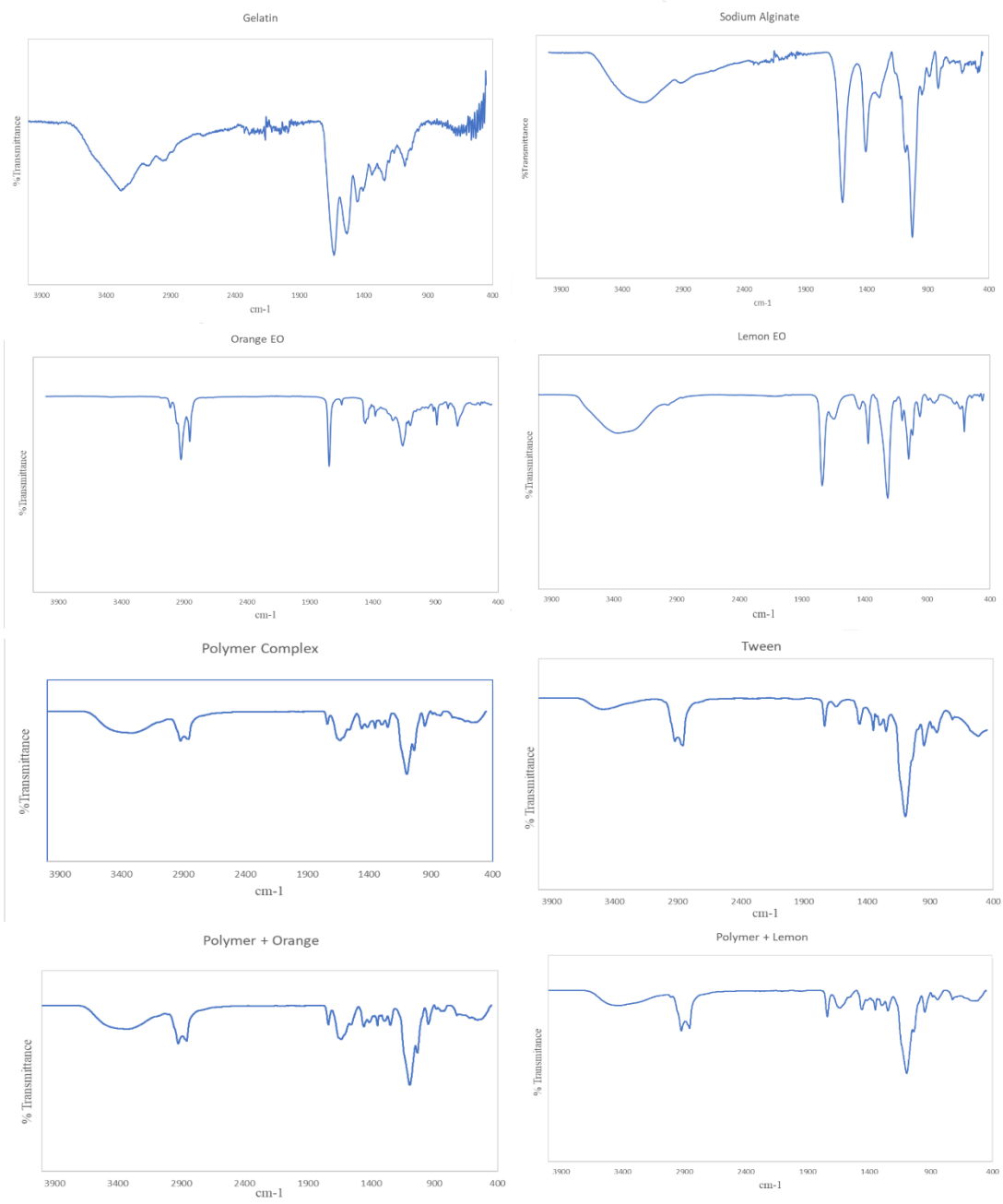

Figure 6: FTIR spectra for Gelatin, Sodium alginate, Orange EO, Lemon EO, Polymer complex, Tween 80, Orange EO complex with Polymer, and Lemon EO complex with Polymer

The FTIR of the polymer complex have shown a characteristic peak at 1630 cm^{-1} that indicate the formation of amide group between both alginate and gelatin. While the amide group that related to gelatin was observed at 1650 cm^{-1}. And that was a confirmation for the formation of amide group as a result of the interactions between the carboxylate group of sodium alginate, and the amine groups from gelatin. Additionally, the comparison between the FTIR spectra of essential oils of either orange or lemon, in addition to polymer complex with the spectra of the final products indicates the chemical interactions between the polymer complex and the constituents of both essential oils.

4.3 Antioxidant Assay

4.3.1 Determination of Total Phenol Contents

The total phenol contents of both products were calculated according to the equations $y = 0.0404x + 0.0076$ ($r^2 = 0.9981$) for both essential oil polymer complexes and the results expressed as µg gallic acid equivalent (GAE) per mg of sample (µg GAE/mg). Figure 7 shows the total phenol contents within both products and compared them with the gallic acid that have been used as a reference.

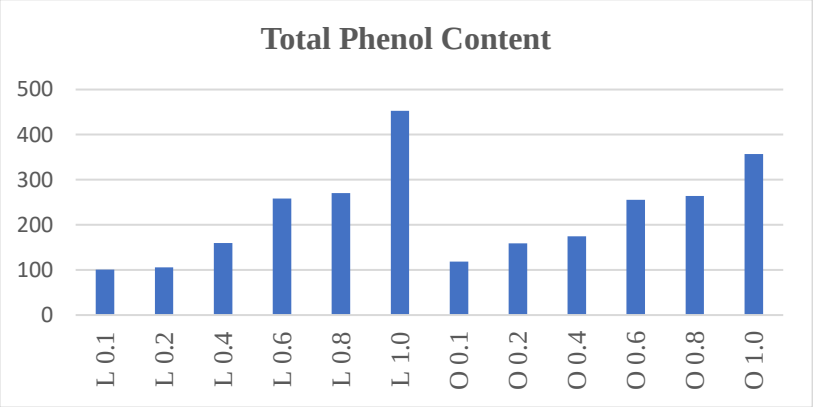

Figure 7: Total phenol contents (L: lemon essential oil polymer complex, O: orange essential oil polymer complex)

Table 5: The results of total phenol contents assay (µg GAE/mg)

Concentration	Total Phenol Contents
L 0.1	101.10 ± 0.01
L 0.2	105.51 ± 0.01
L 0.4	159.73 ± 0.01
L 0.6	258.61 ± 0.03
L 0.8	270.52 ± 0.01
L 1.0	452.69 ± 0.08
O 0.1	118.73 ± 0.02
O 0.2	168.71 ± 0.03
O 0.4	174.52 ± 0.01
O 0.6	255.49 ± 0.02
O 0.8	263.77 ± 0.01
O 1.0	357.19 ± 0.03

4.3.2 Determination of Total Flavonoid Contents

The total flavonoid contents of both products were calculated according to the equations $y = 0.0169x + 0.0467$ ($r^2 = 0.9999$) for both essential oil polymer complexes.

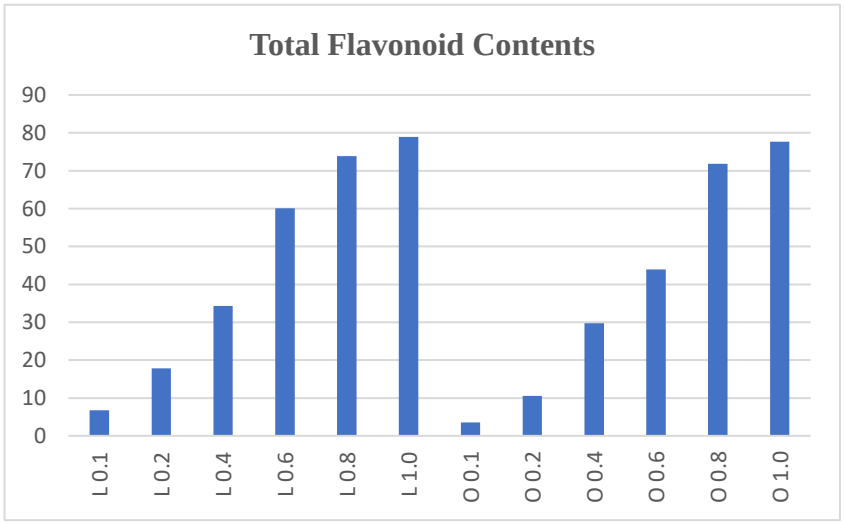

Figure 8: Total flavonoid contents. Graph shows the total phenolic content expressed as µg quercetin/ mg of sample. (L: lemon essential oil polymer complex, O: orange essential oil polymer complex)

Figure 8 shows the total flavonoid contents within both products and compared them with the quercetin that have been used as a reference.

Table 6: The results of total flavonoid contents assay (μg quercetin/mg)

Concentration	Total Flavonoid Contents
L 0.1	6.79 ± 0.01
L 0.2	17.84 ± 0.01
L 0.4	34.26 ± 0.09
L 0.6	60.11 ± 0.11
L 0.8	73.85 ± 0.01
L 1.0	78.91 ± 0.03
O 0.1	3.52 ± 0.01
O 0.2	10.54 ± 0.02
O 0.4	29.7 ± 0.08
O 0.6	43.92 ± 0.01
O 0.8	71.84 ± 0.02
O 1.0	77.66 ± 0.01

4.3.3 Ferric-reducing Antioxidant Power Assay (FRAP)

Both products exerted relatively high activity in the FRAP assay, and as the concentration increases the activity increases as well. The FRAP of both products were calculated according to the equations $y = 0.0131x + 0.1897$ ($r^2 = 1$) for both essential oil polymer complexes. Also, they show a higher activity than the reference used within this assay which was chlorogenic acid (Table 7).

Table 7: FRAP results of products and reference

Product	Concentrations (% (v/v))	FRAP (absorbance at 700 nm)
Orange EO-polymer complex	0.1	12.33 ± 0.01
	0.2	21.46 ± 0.02

	0.4	33.58 ± 0.01
	0.6	40.53 ± 0.04
	0.8	49.16 ± 0.09
	1.0	64.40 ± 0.01
Lemon EO-polymer complex	0.1	10.38 ± 0.01
	0.2	19.78 ± 0.01
	0.4	29.41 ± 0.01
	0.6	29.46 ± 0.03
	0.8	47.66 ± 0.02
	1.0	48.44 ± 0.01
Chlorogenic acid	100.0	9.24 ± 0.01

4.3.4 Ferrous ion-chelating Effect

Both products exerted a relative activity in Ferrous ion-chelating effect assay. However, the lemon EO polymer complex exhibits a relatively high activity, while the orange EO polymer complex exerted a relatively high activity (Table 8).

Table 8: Ferrous ion-chelating effect results for products and reference

Product	Concentrations (% (v/v))	Ferrous ion-chelating effect
Orange EO-polymer complex	0.1	54.76 ± 0.06
	0.2	55.39 ± 0.01
	0.4	59.76 ± 0.02
	0.6	47.56 ± 0.01
	0.8	NA
	1.0	NA
Lemon EO-polymer complex	0.1	63.94 ± 0.01
	0.2	68.60 ± 0.03
	0.4	55.68 ± 0.01
	0.6	44.53 ± 0.05
	0.8	43.52 ± 0.09

	1.0	NA
EDTA	100.0	54.50 ± 0.2

4.4 Antibacterial Assay

Based on the results obtained from the Broth Microdilution assay, The lemon EO polymer complex exerted a higher antibacterial activity than the orange EO polymer complex against both *S. aureus*, and *E. coli*. For the lemon EO polymer complex, standalone MIC was determined as 2.5% (v/v), while it was 5% (v/v) for the orange EO polymer complex against MRSA. Meanwhile, the standalone MIC was determined as 0.3125%% (v/v) for the lemon EO polymer complex, while 1.25% (v/v) for the orange EO polymer complex against *E. coli*. On the other hand, the polymer alone did not show any antibacterial activity.

Chapter 5

DISCUSSION

Essential oils are composed mainly of volatile compounds that are called monoterpenes which is considered as a secondary metabolite. Secondary metabolites can be isolated form different medicinal plants including *Citrus* species such as lemon and orange (Dehsheikh et al., 2020). The interest in the antibacterial activity of essential oil has been increased more than the other natural products due to its promising results (Freires et al., 2015).

Within this study, three different natural products were combined together in order to enhance their biological activities. The main activities that were under investigation are antioxidant activity, and antibacterial activity. A complex of sodium alginate and gelatin polymers were prepared and chemically interact with two different essential oils extracted from orange (*Citrus sinensis*), and lemon (*Citrus limon*).

According to the antibacterial assay results, it was found that the lemon EO polymer complex exhibited higher antibacterial activity than the orange EO polymer complex. On the other hand, both complexes exerted relatively similar antioxidant activity where the activity was correlated with the concentration of EO within the complex. As any the increase of the EO concentration within the polymer complex shows increasing antioxidant activity based on four different antioxidant activity assays.

Chapter 6

CONCLUSION

The complexes of both orange and lemon essential oils loaded on polymer complexes of sodium alginate and gelatin were examined for both antioxidant and antibacterial activities. Our findings indicated that the complex formation leads to a prominent enhancement in the activity of EOs. The final products show a relatively high antioxidant activity, in addition to an excellent antibacterial activity in a comparison with the EOs alone. So, further evaluation must be conducted in order to examine other biological activities including; anticancer activity, anticholinesterase activity, and antiinflammatory activity.

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