ANTIMICROBIAL, ANTIOXIDANT PROPERTIES AND CHEMICAL COMPOSITION OF SOME SPICES/HERBS

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in Food Engineering

by
Hatice YAVUZDURMAZ

December, 2013
İZMİR

We approve the thesis of **Hatice YAVUZDURMAZ**

Examining Committee Members:

Assist. Prof. A. Handan BAYSAL
Department of Food Engineering, İzmir Institute of Technology

Assoc. Prof. Banu ÖZEN
Department of Food Engineering, İzmir Institute of Technology

Assist. Prof. Nükhet N. DEMIREL ZORBA
Department of Food Engineering, Çanakkale Onsekiz Mart University

Assoc. Prof. Duygu KIŞLA
Department of Food Engineering, Ege University

Assoc. Prof. Figen KOREL
Department of Food Engineering, İzmir Institute of Technology

20 December 2013

Assist. Prof. A. Handan BAYSAL
Supervisor, Department of Food Engineering
Izmir Institute of Technology

Prof. Dr.Ahmet YEMENİCİOĞLU
Head of the Department of
Food Engineering

Prof. Dr. R. Tuğrul ŞENGER
Dean of the Graduate Schools of
Engineering and Sciences

ACKNOWLEDGEMENTS

I would like to express my deep appreciation to my supervisor Dr. Handan BAYSAL, for her support, encouragement and helpful guidance throughout my PhD study.

I thank my Committee Members, Dr. Banu ÖZEN, Dr. Nükhet DEMİREL ZORBA, Dr. Figen KOREL, and Dr. Duygu KIŞLA for their comments and their suggestions to make this dissertation more valuable.

I also appreciate the members of the Center for Materials Research, Biotechnology and Bioengineering Application and Research Center, Environmental Development Application and Research Center of Izmir Institute of Technology for their helps.

I would also like to thank to my friend Çelenk MOLVA for her consistent support, being a good listener and advisor and productive discussions. A special thank to Ayşe Burcu AKTAŞ for her help and support for HPLC analysis. Although we have known eachothers for a short period of time, I would like to thank to Esra KAÇAR for sharing her time and materials with me.

Finally, I express my deepest gratitude to my family, my father Ali YAVUZDURMAZ, my mother Beyhan YAVUZDURMAZ and my dear sister Ayşe YAVUZDURMAZ, for their tolerance and patience against my unsteady moods during my doctoral period. Their endless love encouraged me during all the period of my life. In addition, I would express my deep appreciation to my loving, supportive, encouraging and patient life partner Dinçer ZENGİN for holding my hand on the way of life.

Lastly, I would like to acknowledge this study was a part of IYTE Scientific Research Projects (SRP 2012 IYTE07).

ABSTRACT

ANTIMICROBIAL, ANTIOXIDANT AND CHEMICAL COMPOSITION OF SOME SPICES/HERBS

The present study aims to investigate the chemical composition, antimicrobial activity, and mechanism of antimicrobial activity, antioxidant properties of essential oils and extracts and the effects of them when applied to minced beef samples. For this purposes; four essential oils (bay leaf, thyme, clove and cumin), two extracts (grape seed and olive leaf) and constituents of essential oils (eucalyptol, linalool, α-terpineol and α-pinene) were subjected to related tests. Chemical characterization was complemented for all essential oils and extracts. Antimicrobial activity was examined against *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria innocua*, *Shewanella putrefaciens*, *Carnobacterium divergens* and *Serratia liquefaciens*. All tested compounds were effective on the bacteria with different concentrations. Antioxidant activity was proved by FRAP and DPPH methods. Physical disturbance and changes in the structures of bacteria was demonstrated by various techniques. The activity of two most potent essential oils (thyme and clove) was investigated in the minced meat application study. The findings represented that clove essential oil restricted the growth of *S.* Typhimurium and coliform bacteria. They did not have a significant inhibition effect on the aerobic mesophilic bacteria, total yeasts and molds and also psychrotrophic organisms. The results indicated that L* and a* values were maintained during the storage period. The featured effect of essential oils was antioxidant characteristic in meat application study. All treatment showed significant reduction in oxidation comparing with control.

The obtained results may suggest that tested essential oils possess compounds with antimicrobial characteristic as well as antioxidant activity and therefore they can be used as natural preservatives in food especially in meat products.

ÖZET

BAZI BAHARAT/BİTKİLERİN ANTİMİKROBİYAL, ANTİOKSİDAN ÖZELLİKLERİ VE KİMYASAL BİLEŞİMLERİ

Bu çalışma, seçilen bitkisel ürünlerin kimyasal bileşimlerini, antimikrobiyal aktivitelerini ve etki mekanizmalarını, antioksidan özelliklerini ve bunların kıyma örnekleri üzerindeki etkilerini araştırmayı hedeflemektedir. Bu amaçlar doğrultusunda, dört esansiyel yağ (defne yaprağı , kekik , karanfil ve kimyon) , iki ekstrakt (üzüm çekirdeği ve zeytin yaprağı) ve esansiyel yağ bileşenleri (ökaliptol , linalol , α - terpinol ve α - pinen) uygulamalarda kullanılmıştır. Tüm esansiyel yağ ve doğal ekstraktların kimyasal bileşenleri belirlenmiştir. Antimikrobiyal aktivite *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Listeria innocua*, *Shewanella putrefaciens*, *Carnobacterium divergens* ve *Serratia liquefaciens* bakteri suşlarına karşı test edilmiştir. Analiz edilen tüm bileşikler test edilen bakteriler üzerinde farklı konsantrasyonlar ile antimikrobiyal etki göstermiştir. Antioksidan aktiviteleri ise FRAP ve DPPH yöntemleri kullanılarak gösterilmiştir. Bakteri yapısındaki fiziksel değişimler farklı teknikler kullanılarak gösterilmiştir. En belirgin etkiye sahip iki esansiyel yağ (kekik ve karanfil) gıda uygulamasında kullanılmıştır. Bulgular karanfil esansiyel yağının *S*. Typhimurium ve koliform bakteri büyümesini engellediğini göstermiştir. Aerobik mezofilik bakteriler, toplam maya ve küf ve psikrotrofik organizmalar üzerinde önemli bir inhibisyon etkileri yoktur. Sonuçlar esansiyel yağların L * ve a* değerlerini depolama süresince sabit tuttuğunu göstermiştir. Gıda uygulamasında, esansiyel yağların antioksidan etkisi öne çıkan özelliği olmuştur. Tüm uygulamalar oksidasyonda kontrole kıyasla önemli bir azalmaya neden olmuştur.

Bu çalışmada elde edilen sonuçlar test edilen esansiyel yağların antimikrobiyal özellikte ve aynı zamanda antioksidan aktiviteye sahip olduklarını vurgulamaktadır. Bu nedenle de gıdalarda özellikle et ürünlerinde doğal koruyucular olarak kullanılabileceğini göstermiştir.

Dedicated to my family

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

CHAPTER 1

INTRODUCTION

Utilization of medicinal and aromatic plants as natural sources in pharmaceutical, food and cosmetic industries is an increasing trend all over the world. Using plant extracts serve as a “safe” alternative to chemical or synthetic antimicrobials and antioxidants to struggle with the food-borne pathogens or food spoilage organisms, to inhibit lipid oxidation and thus to extend shelf life is an expanding interest in terms of the food industry.

Different plant extracts/essential oils and their derived products have been searched with respect to their antimicrobial, antioxidant activities and application to different types of foods. Selection of these plant extracts and their application depend on their functional properties, availability, cost effectiveness, consumer awareness and their effect on the sensory attributes of the final product (Perumalla and Hettiarachchy, 2011).

Major groups of chemicals present in plant extracts include polyphenols, quinones, flavanols/flavanoids, alkaloids, aldehydes, terpenes, ethers and lectins (Bakkali et al., 2008; Hayes et al., 2011a; Perumalla and Hettiarachchy, 2011; Xia et al., 2010). Beside the plant extracts, their components have been analyzed individually and the interactions of these compounds were also investigated (Friedman et al., 2002; Karaosmanoglu et al., 2010; Obied et al., 2007; Sokmen et al., 2004).

Lipid oxidation is one of the primary problems in food products. It causes formation of off-flavors which directly affect the quality and shelf life of foodstuff. In order to eliminate this problem, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ), are vastly used in food industry. However, health threatening properties like carcinogenic effects of these synthetic antioxidants lead increasing interest to natural sources of antioxidants.

In addition to chemical quality, bacterial quality of food materials serves as an important problem. Either spoilage organisms lead to loss in quality of food or pathogen organisms cause significant health problems. Quick bacterial spoilage of raw meats and

meat products limits their shelf life even though they are stored in refrigerated conditions. Hygienic and safety problems encountered during production or storage of meat and meat products must be eliminated as part of corrective actions such as employing antibacterial agents. Natural plant extracts represent an attractive solution herein, because of their historical safe use and GRAS (Generally recognized as safe) statue or as approved food additives.

Different plants have a rich history of nutritional, medicinal and ceremonial uses. The new trend all over the world is using plant extracts which serve as a “safe” alternative to chemical or synthetic antimicrobials and antioxidants to struggle with the food-borne pathogens or food spoilage organisms, inhibiting lipid oxidation and thus extending shelf life is an expanding interest in terms of the food industry.

The aim of this study is to investigate the various native plant extracts in terms of their biological activities such as; their antimicrobial and antioxidant capacities and to evaluate these activities in raw minced beef samples.

The major goals of this study are as follows:

(1) Determination of chemical composition of plant essential oils (EOs) and extracts.
(2) Evaluation of antimicrobial activity of EOs, EO constituents and extracts against foodborne pathogens and food spoilage bacteria.
(3) Evaluation of antioxidant activity of EOs, EO constituents and extracts.
(4) Investigation of possible mechanisms of antimicrobial activity of EOs and constituents.
(5) Application of thyme and clove EOs to minced beef.

CHAPTER 2

LITERATURE REVIEW

2.1. Chemical Composition

Essential oils are very complex natural mixtures that may contain 20-60 different components with different concentrations. Major components can constitute up to 85% of essential oils and whereas other components are present only as a trace. The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents, all characterized by low molecular weight (Figure 2.1). The monoterpenes are the most representative molecules of EOs and comprising a great variety of structures. Generally these main components are responsible for biological properties (Bakkali et al., 2008; Burt, 2004) and oxygenated monoterpenes are significantly more active than are hydrocarbon monoterpenes (Carson and Riley, 1995). There are functionally and structurally different classes of terpenes. They are made from combinations of several 5-carbon-base (C_5) units called isoprene. The main terpenes are the monoterpenes (C_{10}) and sesquiterpenes (C_{15}), but hemiterpenes (C_5), diterpenes (C_{20}), triterpenes (C_{30}) and tetraterpenes (C_{40}) also exist. A terpene containing oxygen is called a terpenoid. The monoterpenes are also formed from the coupling of two isoprene units (C10). Monoterpenes may be linear (acyclic) or contain rings (moncyclic and bicyclic). Also, biochemical modifications such as oxidation or rearrangement produce the related monoterpenoids. There are different group of monoterpenes ; carbures (sabinene, *p*-cymene, camphene), alcohols (linalool, carveol, α-terpineol), aldehydes (geranial, neral), ketone (tegetone, camphor, carvone), esters (linalyl acetate, α-terpinyl acetate), ethers (1,8 cineole), phenols (thymol, carvacrol) and peroxydes. Aromatic compounds occur less than terpenes. They comprise aldehyde (cinnamaldehyde), alcohol (cinnamic alcohol), phenols (chavicol, eugenol), methoxy derivatives (anethole, estragole, methyleugenols) and methylene dioxy compounds (apiole, myristicine, safrole) (Bakkali et al., 2008).

The phenolic components are primarily responsible for the antibacterial properties of EOs. But, minor components have also critical part in biological

properties, possibly by producing a synergistic effect between other components. The major components of EOs investigated in this study are presented in Table 2.1.

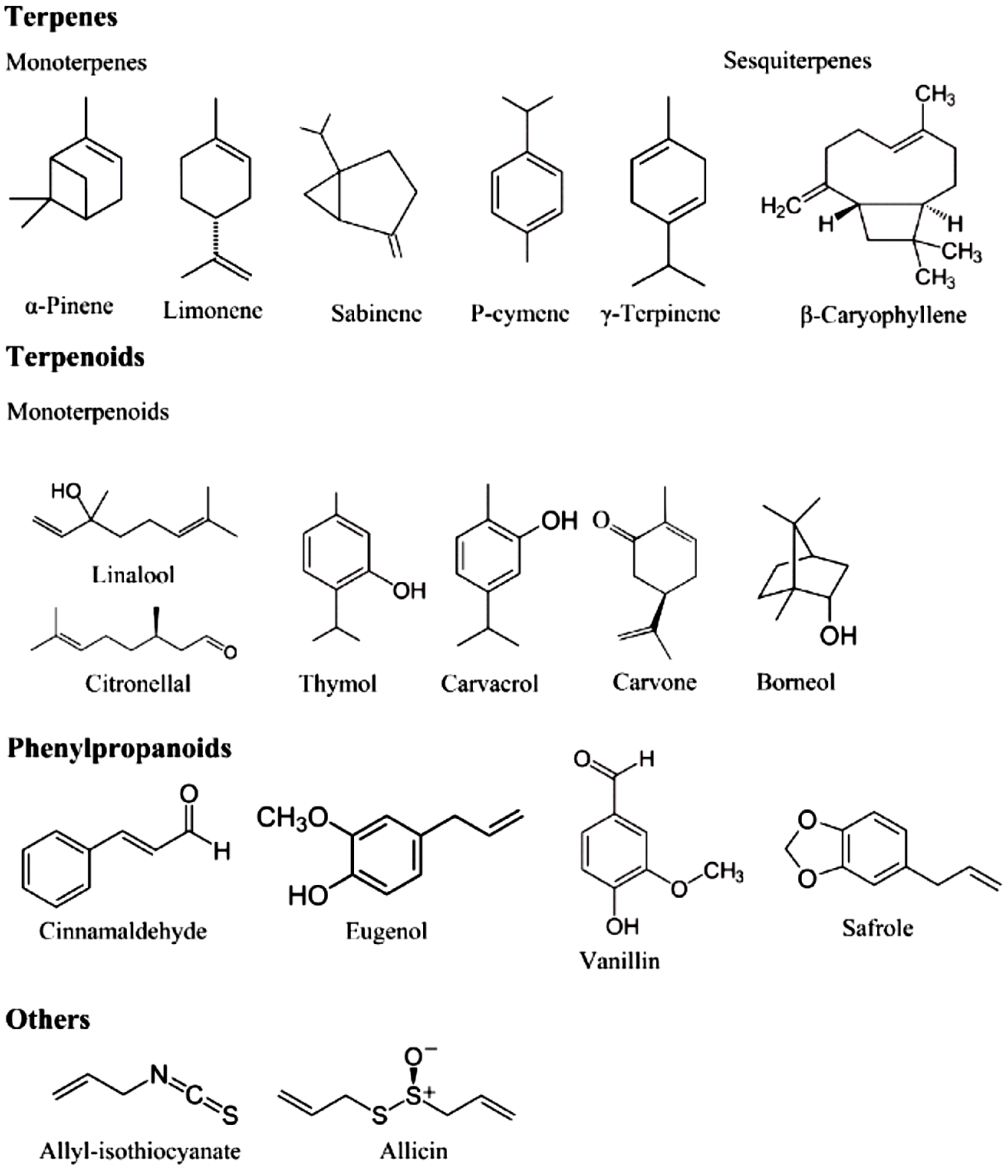


Figure 2.1. Chemical structures of some essential oil components
(Source: Bakkali et al., 2008)

The major components of essential oils are primarily similar. However, composition may change by some effective factors such as soil composition, climate, and geographic origin, the vegetative cycle and seasonal variation etc. The extraction method also affects the organoleptic profile as far as the composition. There are several techniques for extracting essential oils like; use of liquid carbon dioxide or microwaves,

low or high pressure distillation, hot water or steam distillation (Angioni et al., 2006; Behera et al., 2004; Masotti et al., 2003).

Table 2.1. Major components of EOs investigated in the study

Common Name	Major components	References
Bay leaf	1,8-cineole (eucalyptol) linalool sabinene	(Dadalioğlu and Evrendilek 2004; Ozcan and Chalchat 2005; Ramos, Teixeira et al. 2011)
Thyme	thymol carvacrol γ-Terpinene *p*-cymene	(Ait-Ouazzou et al., 2011b; Baydar et al., 2004a; Safaei-Ghomi et al., 2009; Sokmen et al., 2004; Tepe et al., 2005; Verma et al., 2010)
Clove	eugenol eugenol acetate b-caryophyllene	(Dorman et al., 2000; Guan et al., 2007; Lee and Shibamoto, 2001; Martini et al., 1996)
Cumin	cymene cumin aldehyde	(Behera et al., 2004; El-Sawi and Mohamed, 2002; Kedia et al., 2014)

Beside four essential oils, grape seed and olive leaf extracts were also evaluated according to their antimicrobial and antioxidant activities. The grape is one of the fruit crops most widely grown throughout the world. Grape seed extract is a by-product derived from the grape seeds (from grape juice and wine processing) that is extracted, dried and purified to produce a polyphenolic compound rich extract (Lau and King, 2003). This extract sold commercially as dietary supplement and has Generally Recognized as Safe (GRAS) status approved by Food and Drug Administration (FDA).

Grape is a phenol rich plant and these phenols are mainly distributed in the skin, seed and leaf of the grape (Baydar et al., 2004b; Hernández-Jiménez et al., 2009; Katalinić et al., 2010; Pastrana-Bonilla et al., 2003). Grape seed extracts contain mainly proanthocyanidins in the form of monomeric phenolic compounds, such as catechin, epicatechin, epicatechin-3-o-gallate and flavanols such as quercetin, rutin (Fig.2.2). Cultivar, soil composition, climate, geographic origin and cultivation practices or exposure to diseases change the total phenolic content of grape (Delgado Adámez et al., 2012; Pastrana-Bonilla et al., 2003; Perumalla and Hettiarachchy, 2011; Xia et al., 2010).

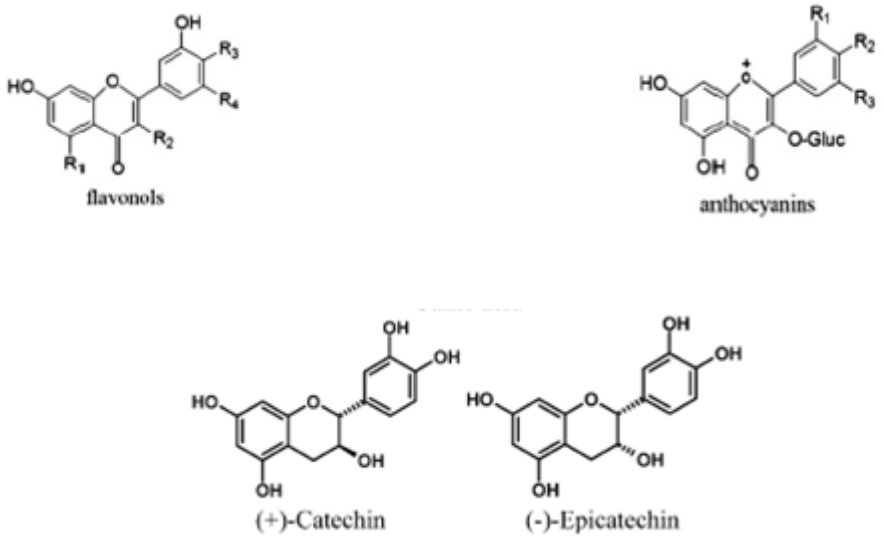


Figure 2.2. Chemical structures of some phenolics from grape seeds
(Source: Yılmaz and Toledo 2004; Perumella and Hettiarachchy, 2011)

Polyphenols of grape seed origin have been reported to have a variety of biological effects, including antioxidant, anticarcinogenic, cardioprotective, estrogenic and/or antiestrogenic activities and antimicrobial activities (Carpenter et al., 2007; Rhodes et al., 2006; Serra et al., 2008; Yilmaz and Toledo, 2004).

The olive fruit, its oil and the leaves of the oil tree have a rich history of nutritional, medicinal and ceremonial uses. Olive oil, table olives and olive products are an important part of the Mediterranean diet, the greatest value of which may be due to olive polyphenols that contribute to the modulation of the oxidative balance in vivo. Beside these olive products olive mill waste serve an important phenol rich source. The biophenolic fraction of olive oil comprises only 2% of the total phenolic content of the olive fruits, with the remaining 98% being lost in olive mill waste (OMW) (Cardinali et al., 2010; Mulinacci et al., 2001; Obied et al., 2005).

Because of high biphenol content olive is recognized as potential antimicrobial and antioxidant target for food and pharmaceutical industries. The most abundant phenolics in olive leaf extract are oleuropein and verbascoside (oleuropeosides); hydroxytyrosol and tyrosol (substituted phenols); apigenin-7-O-glucoside, caffeic acid, rutin and luteolin-7-O-glucoside (flavones) (Fig. 2.3) (Pereira et al., 2007a; Thaipong et al., 2006).

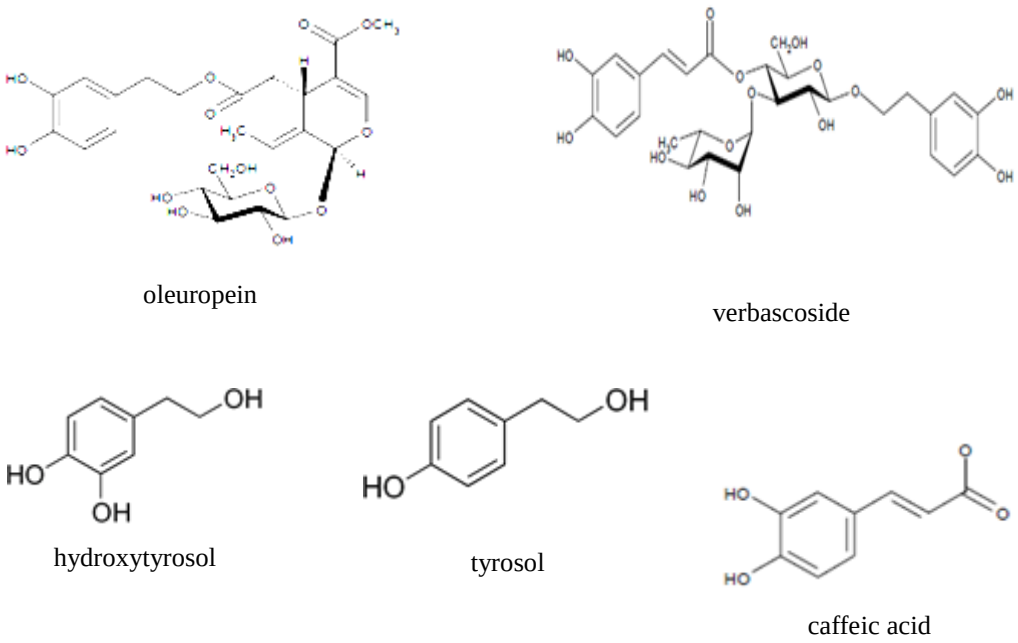


Figure 2.3. Chemical structures of most abundant phenolics in olive leaf extract (Source: Thaipong et al., 2006; Pereira et al., 2007)

2.2. Antimicrobial Activity

Many of the component secondary metabolites in essential oils, and thus the essential oils themselves, exhibit antimicrobial activity and the sensitivity of microorganisms to essential oils vary. The use of EOs as biopreservatives is a matter of great interest for the food industry since consumers prefer natural additives instead of synthetic ones. Many studies have been performed on this subject in the last few years.

Bay laurel (*Laurus nobilis* L.) is an evergreen shrub native to the Mediterranean region, being the only European representative of the Lauraceae family. Its dried leaves and essential oil are used in Italy, France, Turkey, Algeria, Morocco, Spain, Portugal and Mexico as a valuable spice in the culinary and food industry (Ramos et al., 2011). Spices supply secondary compounds that have medicinal, antioxidant and antimicrobial effects. Ramos et al. (2011) searched the essential oil (EO) and extracts (EX) of bay laurel in the means of antimicrobial and antioxidant activity. The EO and EX antibacterial activity was assayed against seven bacterial strains; *B. thermosphacta, E. coli, Listeria innocua, L. monocytogenes, P. putida, S. typhimurium* and *S. putrefaciens* and concluded that EO showed the highest antibacterial properties, being able to inhibit the growth of all tested bacteria (Ramos et al., 2011). In the study performed by

Dadalioğlu and Evrendilek (2004), antimicrobial effects of essential oils including bay laurel, it was demonstrated that bay laurel essential oil had inhibitory effects on common foodborne pathogens in the following order : *E. coli* O157:H7 > *S. aureus* > *S.* Typhimurium > *L. monocytogenes* (Dadalioğlu and Evrendilek, 2004). When the antibacterial activities of leaf essential oils of Himalayan Lauraceae species were observed, Joshi et al. (2010) concluded that activities changed depending to the species. The oils showed moderate to high activity against three Gram negative (*E. coli*, *S. enterica enterica* and *P. multocida*) and one Gram positive (*S. aureus*) bacteria (Joshi et al., 2010). Antimicrobial activity of essential oils of Turkish plant spices including laurel were tested on various microorganisms; *Salmonella typhimurium, Bacillus cereus, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Yersinia enterocolitica, Saccharomyces cerevisiae, Candida rugosa, Rhizopus oryzae* and *Aspergillus niger*. Laurel essential oil was found to be effective against all bacterial species. It showed antimicrobial effects on *B. cereus* and *E. faecalis* at low concentration, in contrast, on other bacterial species at high concentrations (Özcan and Erkmen, 2001). Burt and Reinders (2003) determined at which concentration bay and other essential oils were bacteriostatic and bactericidal to *E. coli* O157:H7 an important foodborne pathogen. Results showed that bay EO was active to a lesser extent than other EOs (Burt and Reinders, 2003). Friedman et al. (2002) evaluated the bactericidal activity levels of a wide range of essential oils (n=96) and oil compounds (n=23) against *Campylobacter jejuni, Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Salmonella enterica*. Oils including bay leaf oil were found to be effective with BA50 values of ≤0.12 for each of the four species organisms (Friedman et al., 2002). In an another study, it was observed that bay leaf oil inhibited all tested organisms; *Acinetobacter baumanii*, *Aeromonas veronii* biogroup *sobria*, *Candida albicans, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enterica* subsp. *enterica* serotype *typhimurium, Serratia marcescens* and *Staphylococcus aureus* at concentrations of ≤2.0 (v/v) (Hammer et al., 1999). In the research, where 21 plant essential oil and two essences were investigated against five important foodborne pathogens; *Campylobacter jejuni, Salmonella enteritidis, Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes*, it was demonstrated that the oils of bay had the one of most inhibitory effect with bacteriostatic concentration of ≤0.075%. The results showed that *Campylobacter jejuni* was the most resistant of the bacteria investigated to plant essential oils, with only the

oils of bay and thyme having a bactericidal concentration of less than 1% (Smith et al., 1998).

Thymus (*Lamiaceae*) is a large genus divided in eight sections, comprising about 215 species particularly prevalent in the Mediterranean area (Hazzit et al., 2009). It is a commonly used culinary herb worldwide. Thyme essential oil is well known with the biological activities. Different species of Algerian Thymus oil were evaluated according to their antimicrobial and antioxidant activity. The susceptibility of different bacteria to Thymus essential oils were tested and it was found that *Salmonella* species were most resistant followed by *S. aureus*, *L. monocytogenes* and *C. albicans*, *B. cereus* was the most susceptible to the tested essential oils (Hazzit et al., 2009). In another study antimicrobial activity of Thymus oil from Morocco was determined. According to the results, Thymus oil showed the best bacteriostatic and bactericidal effect with MIC change between 0.5-10 µl/ml. Strong activity was observed for three of the four Gram positive strains (*S. aureus*, *L. monocytogenes* EGD-e and *L. monocytogenes* 4b) and moderate activity for the other strains: *E. faecium* as Gram-positive and *S. enteritidis*, *E.* coli and P. aeruginosa as Gram-negative strains (Ait-Ouazzou et al., 2011b). The black thyme from Turkey showed also distinctive antimicrobial activity (Özcan and Boyraz, 2000; Sağdıç et al., 2002). The 1/50 concentration showed inhibitory effect on all strains, and the 1/100 concentration inhibited all except *B. cereus* and *E. coli*. Only, *B. amyloliquefaciens* and *P. vulgaris* were sensitive to the 1/200 concentration (Baydar et al., 2004a). Thymus vulgaris is the other important species represents antimicrobial activity. The essential oil of this species showed antibacterial activity against five strains of Gram-positive and eight strains of Gram-negative bacteria as far as antifungal activity against five examined dermatomycetes and *C. albicans* (Bozin et al., 2006). Another species of Thymus (*Thymus spathulifolius)* from Turkey was tested for antimicrobial activity. The results stated that the essential oil of *T. spathulifolius* had great potential of antimicrobial activities against all 25 bacteria, and 9 of 15 molds and a yeast species (Sokmen et al., 2004). Two Thymus species endemic to the southeast of Spain were evaluated according to their antimicrobial activity against bacteria related to food spoilage. *T. piperella* essential oil had an inhibitory effect on 5 of the 11 tested bacteria (*Achromobacter denitrificans*, *Aeromonas hydrophila*), and no antibacterial activity against 6 bacterial species (*Pseudomonas fragi*, *Pseudomonas fluorescens*, *Shewanella putrefaciens*). The *T. moroderi* essential oil had an inhibitory effect on 4 of

the bacteria. This EO showed no activity against 7 tested bacterial species (Ruiz-Navajas et al., 2012).

Clove is an aromatic dried flower bud of a tree in the family Myrtaceae (*Syzygium aromaticum*). Cloves are native to the Maluku islands in Indonesia and used as a spice in cuisines all over the world. The antimicrobial activity of clove essential oil was determined and resulted in the inhibition of all tested bacteria (*Brochothrix thermosphacta, Escherichia coli, Listeria innocua, Listeria monocytogenes, Pseudomonas putida, Salmonella* Typhimurium *and Shewanella putrefaciens*) (Teixeira et al., 2013). Friedman et al. (2002) evaluated the bactericidal activity levels of a wide range of essential oils against *Campylobacter jejuni, Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Salmonella enterica*. Clove bud essential oil showed good antimicrobial activity with BA50 value changed between 0.02-0.013 (Friedman et al., 2002). Clove bud essential oil exhibited lesser antimicrobial activity against comparing with thyme and oregano essential oils *Escherichia coli* O157:H7 (Burt and Reinders, 2003).

Cumin cyminum L. belonging to the family Apaiacae is one of the old cultivated medicinal food herbs in Asia, Africa and Europa. Antimicrobial activity of cumin bud essential oils and extracts has been reported. A species of cumin essential oil, also called as black cumin in Tunisia, was evaluated according to the antimicrobial activity against *S. aureus* and *E. coli.* The activity was expressed as the concentration essential oil inhibiting bacterial growth by 50% (IC50). The results indicated that essential oil was active on both microorganisms with 12-62 μg/ml IC50 value (Bourgou et al., 2010). In a study antimicrobial activity of different plant essential oils were examined against a food spoilage related organism *Pseudomonas putida*. MIC and maximal tolerated concentration (MTC) values were obtained from this study. Cumin essential oil showed antimicrobial activity against tested organism with >0.8% MIC and 0.2% MTC (Oussalah et al., 2006). Antimicrobial activity of essential oils of Turkish plant spices including cumin were tested on various microorganisms. Cumin essential oil possessed greater antimicrobial activity against all bacterial species than other oils. The effective dose changed between 1% and 15% of cumin essential oil (Özcan and Erkmen, 2001). Beside antibacterial activity the effect of cumin essential oil on fungal species was also demonstrated by Kedia et al. (2014). The oil exhibited a broad spectrum of fungal toxicity inhibiting all 19 food borne fungal species (Kedia et al., 2014)

Various bacterial species exhibit different sensitivities towards phenolic compounds of grape seed extracts. It is suggested that, high concentrations of flavonoids and derivatives in grape seeds were responsible for the antimicrobial activity. Delgado Adámez, Gamero Samino et al. (2012) demonstrated that GSE (grape seed extract) exhibited antibacterial action against all bacteria tested; Gram-positive bacteria such as *Listeria innocua*, *Brochothrix thermosphacta*, *Staphylococcus aureus* subsp. *aureus* and three Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica* and *Escherichia coli* in nearly 100-50 µl/ml seed extract dilutions. The results were agreed that GSE showed inhibitory effects were more potent to Gram-positive bacteria than Gram-negative bacteria (Delgado Adámez et al., 2012). In the study of Kao et al. (2010) investigated the antimicrobial activity of GSE against both Gram-positive bacteria; *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., and Gram-negative bacteria; *Enterobacteriaceae* spp., *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia* and concurred with the result that Gram-positive cocci, were more susceptible to GSE inhibition than Gram-negative enterobacteria (Kao et al., 2010). Baydar et al. (2006) concluded that all tested bacteria including both pathogenic and spoilage bacteria; *Aeromonas hydrophila, Bacillus cereus, Enterobacter aerogenes, Enterococcus faecalis, Escherichia coli, E. coli O157:H7, Klebsiella pneumoniae, Mycobacterium smegmatis, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella enteritidis, Salmonella typhimurium, Staphylococcus aureus and Yersinia enterocolitica* were inhibited by GSE where *A. hydrophila* was the most sensitive species (Baydar et al., 2006). Among the tested antimicrobial agent, Serra et al. (2008) decided that grape extract (grape skins and seeds) was the most effective one. Five microbial species; *Escherichia coli, Salmonella poona, Bacillus cereus, Saccharomyces cerevisiae* and *Candida albicans* were investigated according to their sensitivity against grape extract and it was concluded that the inhibitory effect was more efficient on Gram-positive strain *B. cereus* than Gram-negative *E. coli* and *S. poona* (Serra et al., 2008). The polymeric phenolic fractions was found to produce the highest inhibition activity for all *Listerial* species, but not for other bacteria such as *Bacillus cereus, Salmonella menston, E. coli, Staphylococcus aureus* or *Yersinia enterocolitica* by Rhodes et al. (Rhodes et al., 2006). Xu et al. (2007) suggested that the antibacterial effects of GSE were substantial with a greater inhibitory effect on gram-positive (*L. monocytogenes*) organisms than on gram-negative (*Salmonella)* organisms (Xu et al., 2007). The result

of the study carried out by Jayaprakasha et al. (2003) showed that the grape seed extracts exhibited antibacterial effect against all bacteria; *Bacillus cereus, Bacillus coagulans, Bacillus subtilis, Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa,* tested. Extracts were found to be the most effective antibacterial fraction against Gram-positive bacteria when compared to Gram-negative bacteria (Jayaprakasha et al., 2003).

Antimicrobial activity of phenolic compounds of olive leaf individually and the combination of them was examined and concluded that both individual and combined phenolics exhibited good antimicrobial effect against target microorganisms; Gram-positive bacteria, *Bacillus cereus* and *S. aureus*, and the Gram-negative bacteria, *Escherichia coli* and *Salmonella enteritidis*. Furthermore, the antimicrobial effect of combined phenolics was significantly higher (Lee and Lee, 2010). It was also supported by Pereira et al., (2007) that, extracts may be more beneficial than isolated constituents since a bioactive component can change its properties in the presence of other compounds present in the extract. They also reported the antimicrobial capacity order for several concentrations of OLE as follows; *B. cereus > C. albicans > E. coli > S. aureu s> C. neoformans > K. pneumoniae > P. aeruginosa > B. subtilis* (Pereira et al., 2007b). Markin et al. (2003) extracted the ground powdered leaves and observed the antimicrobial activities against both bacteria; *E. coli, Pseudomonas aeruginosa, S. aureus, B. subtilis* and *K. pneumoniae*, and yeast; *Candida albicans*. The results showed that all of tested organisms were inhibited at different concentration of olive leaf extracts (Markin et al., 2003). A wide range of microorganism groups (n=122) including such as *Bacillus cereus, Campylobacter jejuni, Lactobacillus* spp., *Listeria monocytogenes, Salmonella enterica* subsp. *Enterica* were investigated according their susceptibilities against olive leaf extracts. The data indicated that OLE does not show broad-spectrum activity and has appreciable activity only against *C. jejuni*, *H. pylori* and *Staphylococcus* spp. The organisms least susceptible to OLE, with one or more isolates having MICs of ≥50% OLE, were *Bacillus subtilis, Candida* spp., *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Serratia marcescens* whereas *C. jejuni*, with MICs as low as 0.31%, followed by *Helicobacter pylori* with MICs of 0.62% and *S. aureus* with MICs of 0.78% (Sudjana et al., 2009).

2.3. Synergistic Antimicrobial Activity of Essential Oil Components

Essential oils, also known as volatile oils, are complex mixtures of volatile constituents biosynthesized by plants, which mainly include two biosynthetically related groups (Pichersky et al., 2006) .These main groups include terpenes and terpenoids and aromatic and aliphatic constituents, all characterized by low molecular weight. Most of the antimicrobial activity in EOs is found in the oxygenated terpenoids (e.g., alcohols and phenolic terpenes), while some hydrocarbons also exhibit antimicrobial effects (Burt, 2004; Delaquis et al., 2002). Interactions between these components may lead to antagonistic, additive or synergistic effects. Some studies have demonstrated that whole EOs usually have higher antibacterial activity than the mixtures of their major components, suggesting that the minor components are critical to the synergistic activity, though antagonistic and additive effects have also been observed (de Azeredo et al., 2011; Mourey and Canillac, 2002). Usually combinations, either single EOs or artificial mixtures of purified main components, affect multiple biochemical processes in the bacteria, producing a plethora of interactive antibacterial effects (Delaquis et al., 2002). In recent years, there has been an increased interest in the use of natural antimicrobial agents thus the use of these combinations are strategies to control food-borne bacteria and other pathogenic microorganisms (Gutierrez et al., 2008; Karatzas et al., 2001).

There are several techniques to investigate the interaction between essential oils and components. Table 2.2 presents some of the studies related to the methods and interaction effects.

Table 2.2. Combination of components and their antimicrobial interactions against several organisms

Pair combinations	Organism	Methods	Interaction	References
Thymol/carvacrol	*Staphylococcus aureus, Pseudomonas aeruginosa*	Half dilution	Additive	(Lambert et al., 2001; Zhou et al., 2007)
	Escherichia coli	Checkerboard	Synergism	
	S. aureus, Bacillus. cereus, E. coli	Checkerboard	Antagonism	
	S. aureus, P.aeruginosa	Mixture	Additive	
	Salmonella Typhinurium	Mixture	Synergism	
Thymol/eugenol	*E. coli*	Checkerboard	Synergism	(Pei et al., 2009)
Carvacrol/linalool Eugenol/linalool Eugenol/menthol	*Listeria monocytogenes, Enterobacter aerogenes, E. coli, P. aeruginosa*	Checkerboard	Synergism	(Bassolé et al., 2010)

(cont. on next page)

Table 2.2 (cont.)

1,8-Cineole/ Aromadendrene	*methicillin-resistant S. aureus (MRSA) and vancomycin-resistant enterococci (VRE) Enterococcus faecalis*	Checkerboard	Additive	(Mulyaningsih et al., 2010)
Limonene/ 1,8-cineole	*S. aureus, P. aeruginosa*	Mixture	Synergism	(Vuuren and Viljoen, 2007)
α-pinene/Limonene α-pinene/Linalool Linalool/ Terpinen-4-ol	*Saccharomyces cerevisiae*	Checkerboard	Synergism, Additive	(Tserennadmid et al., 2011)
Cinnamaldehyde/ Carvacrol	*E. coli* *S. typhinurium*	Checkerboard Mixture	Additive Synergism	(Zhou et al., 2007; Pei et al., 2009)

Among the test methods checkerboard assay is seemed to be commonly used. The checkerboard test requires determination of the fractional inhibitory concentration (FIC). The FIC of a factor is the concentration that kills when used in combination with another agent divided by the concentration that has the same effect when used alone. Each checkerboard test generates many different combinations, and by convention, the FIC values of the most effective combination are used in calculating the FIC index. The FIC index defines the nature of the interaction. The values of the FIC index used for the definition of the nature of the interaction differ between publications and makes comparison between studies difficult. The definition of the reference concentration differs between publications and this is obstacle at the time of comparing different research studies. Table 2.3 summarizes different studies offers different FIC index used to determine the type of interactions studies.

Table 2.3. FIC index used to determine the type of interactions

FIC index				**References**
Synergy	**Addition**	**Indifference**	**Antagonism**	
<1	1	1-2	>2	(Pei et al., 2009)
<0.5	0.5–1	1–4	>4	(Bassolé et al., 2010; Tserennadmid et al., 2011)
≤0.5	0.5–1	1–4	>4	(Mulyaningsih et al., 2010)
<0.5	0.5–4	-	>4	(Goñi et al., 2009)
≤0.5	0.5–1	-	>1	(Rosato et al., 2007)
≤0.75	0.75–2	-	>2	(Gallucci et al., 2009)
<0.9	0.5–1,1	-	1.1	(Romano et al., 2009)

2.4. Mechanism of Antimicrobial Activity

The exact mechanism of antimicrobial activity of natural compounds is not fully understood. The antimicrobial action of plant extracts/essential oils is due to their phenolic substances, so that their activities are similar to these antimicrobial agents. Membrane disruption by terpenoids and phenolics; metal chelation by phenols and flavonoids; and effect on genetic material by coumarin and alkaloids are thought to inhibit growth of microorganisms (Negi, 2012). The outer cell membrane or cytoplasmic membrane of a bacterium is composed of phospholipids bilayer and proteins and is the major site of interaction with antimicrobial compounds. Physical of disturbance of cytoplasmic membrane; disrupting the proton motive force (PMF), electron flow, active transport and coagulation of cell contents and inhibition of membrane-associated enzyme activity cause damages to vital membrane and result in the death of the bacterium (Fig. 2.4) (Burt, 2004; Perumalla and Hettiarachchy, 2011; Shimamura et al., 2007; Ultee et al., 1998).

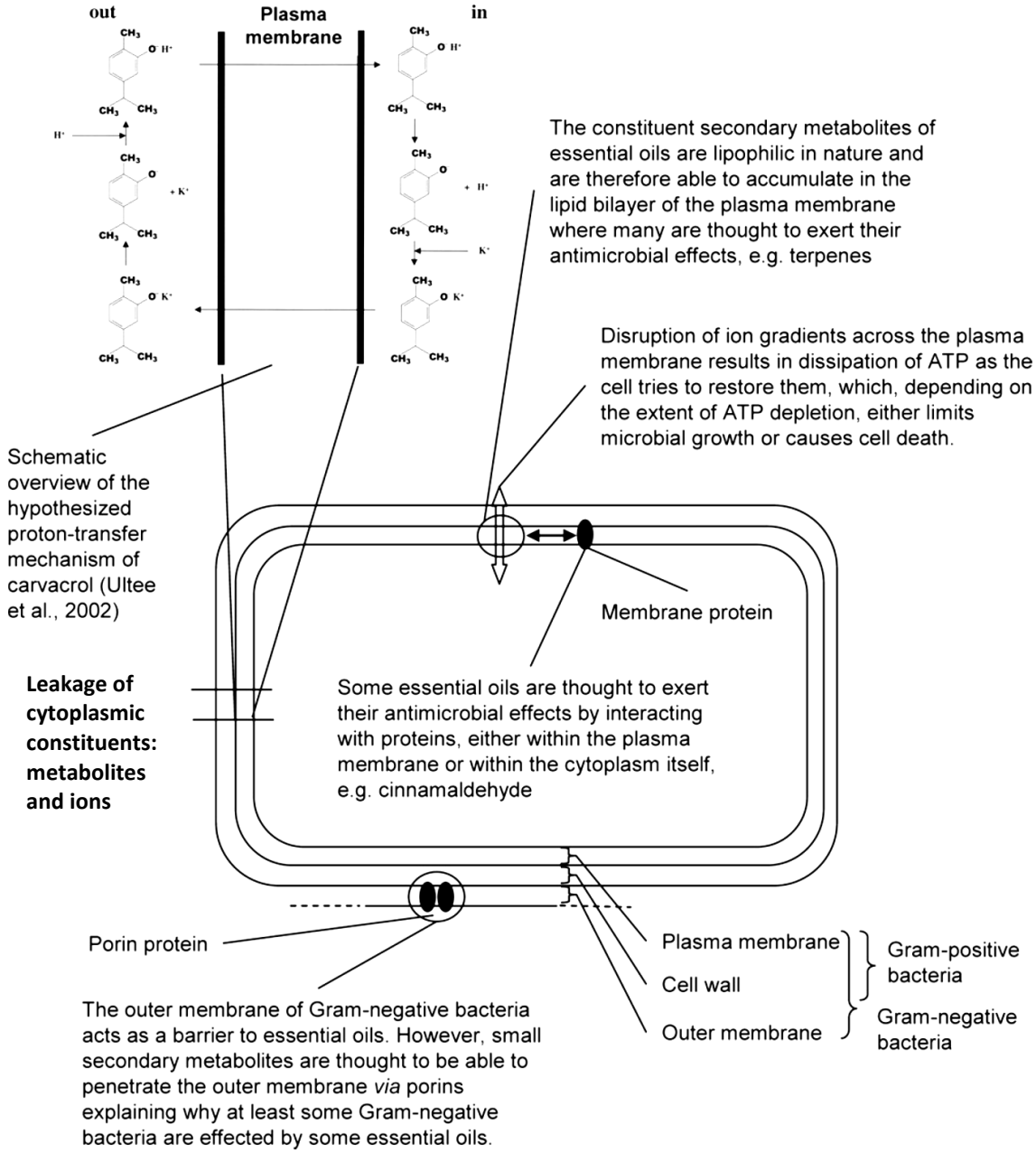


Figure 2.4. Some of the sites and mechanisms of antimicrobial activity of essential oils in the bacterial cell (Source: Benchaar and Greathead, 2011)

There are different studies that have demonstrated the effectiveness of antimicrobials and their effective compounds to control or inhibit the growth of pathogenic and spoilage microorganisms. Degradation of the cell wall, damage to cytoplasmic membrane and membrane proteins, leakage of intracellular contents, coagulation of cytoplasm and depletion of proton motif force can cause cell death (Benchaar and Greathead, 2011; Burt, 2004; Nychas et al.; Tiwari et al., 2009). Most of the studies showed that essential oils are slightly more active against gram-positive than

gram-negative bacteria. It is due to the hydrophilic cell wall structure of Gram-negative bacteria surrounding the cell membrane (Al-Reza et al., 2010) which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Al-Reza et al., 2010; Rahman and Kang, 2009).

Helander et al. (1998) studied Carvacrol, (+)-carvone, thymol, and *trans*-cinnamaldehyde for their inhibitory activity against *Escherichia coli* O157:H7 and *Salmonella typhimurium*. It was investigated that thymol and carvacrol both have prominent ouyer membrane (OM) disintegrating properties, as indicated by their enhancing effect on 1-*N*-phenylnaphthylamine (NPN) uptake and (lipopolysaccharide) LPS release. These compounds also inhibited bacterial growth at concentrations similar to those required for OM disintegration and increased the permeability of the cytoplasmic membrane to ATP (El-Abbassi et al., 2012). In the study of observation the antimicrobial mechanism of carvacrol and thymol against *Escherichia coli*, it was concluded that the mechanism of the action of carvacrol and thymol is the disruption of the cytoplasmic membrane, which increases its permeability and depolarizes its potential (Bouaziz et al., 2004). Burt and Reinders (2003) observed the oregano EO treated *E. coli* O157:H7 cells and showed that after loss of contents cells collapsed which enabled them to pass more easily through the pores of the membrane than the untreated control cells (Fig. 2.5) (Burt and Reinders, 2003).

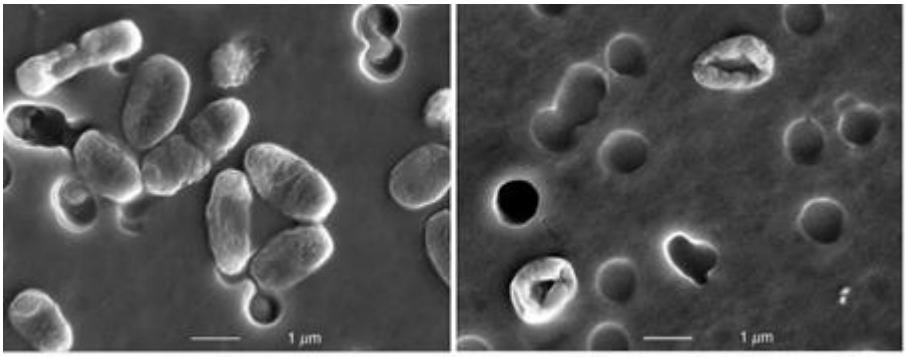


Figure 2.5. Scanning electron microscope images of *E. coli* O157:H7 cells after treatment with oregano essential oil (Source: Burt and Reinders, 2003)

Markin et al. (2003) observed the effect of olive leaf extracts on *Candida albicans* and *Escherichia coli* cells. The non-treated yeast cells showed oval or regularly spherical forms whereas; modifications to yeasts which were subjected to olive leaf extract appeared 24-h incubation. Cells became amorphous and deformations of the cell wall were manifested as inward invaginations (Fig. 2.6). In addition to that observation,

while, the non-treated *Escherichia coli* cells appeared as symmetrical rods, the treated ones begun to lose their symmetrical appearance and cell wall invaginations after 6 h. Following 24-h incubation in olive leaf extract, *E. coli* cells exhibited complete destruction (Markin et al., 2003).

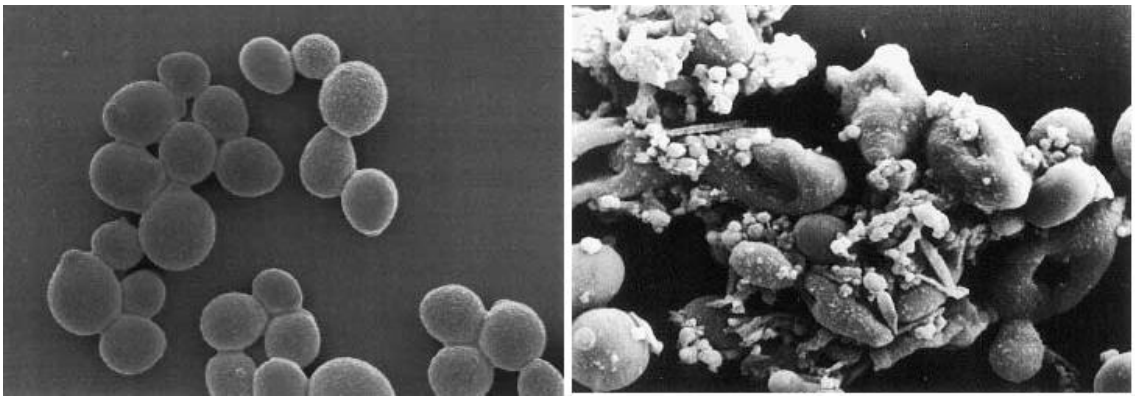


Figure 2.6. Untreated and treated with olive leaf extract *Candida albicans* cells (Source: Markin et al., 2003)

The antimicrobial efficacy of three monoterpenes (linalyl acetate, (+) menthol, and thymol) was determined against the gram-positive bacteria *Staphylococcus aureus* and the gram-negative bacterium *Escherichia coli*. The data speculated that the antimicrobial effect of (+) menthol, thymol, and linalyl acetate partially due to a perturbation of the lipid fraction of bacterial plasma membranes, resulting in alterations of membrane permeability and in leakage of intracellular materials. This effect appeared to be dependent on the lipid composition and net surface charge of the bacterial membranes as well as being related to physicochemical characteristics of the agents (Trombetta et al., 2005). Treatment of *E. coli* cells with thymol and carvacrol resulted in the disruption of the cytoplasmic membrane, which increases its permeability and depolarizes its potential (Xu et al., 2008).

If there is no effects observed on cell structure and membrane functionality, it is assumed that the site of action is intracellular. In general, the target can be proteins and enzymes or it is possibly essential cellular processes involved in biosynthesis or energy generation (Hyldgaard et al., 2012).

2.5. Antioxidant Activity

Lipid oxidation in food products is considered to be one of the important factor limiting product quality and acceptability due to the production of potentially toxic reactive oxygen species and off-flavors from unsaturated fatty acids. The addition of antioxidants is required to preserve flavour and colour and to avoid vitamin destruction. Synthetic antioxidants begin to replace with natural ones due to the toxic and carcinogenic effects on humans and also abnormal effects on enzyme systems (Cui et al., 2012; Göktürk Baydar et al., 2007).

The lipid oxidation mechanism in food can be discussed in 3 steps namely; Initiation, propogation and termination.

Initiation: When an unsaturated lipid contact with oxygen this produces free radicals.

$RH \rightarrow R^{\bullet} + H^{\bullet}$

$ROOH \rightarrow RO^{\bullet} + HO^{-}$

$2ROOH \rightarrow RO^{\bullet} + ROO^{\bullet} + H_2O$

$R^{\bullet}$ = lipid radicals

$RO^{\bullet}$ = alkoxy radicals

$ROO^{\bullet}$ = lipid peroxyradicals

Propogation: Propagation reactions generate different type of radicals. Previously formed free radicals in the initiation reactions take part in the chain reactions and as a result of consuming of oxygen by lipids new free radical species occurs such as peroxy radicals ($ROO^{\bullet}$) and peroxides (ROOH).

$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$

$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$

ROOH: lipid peroxides

$R^{\bullet}$: lipid radicals

$ROO^{\bullet}$: lipid peroxy radicals

Termination: In the further steps of propagation, the amount of unsaturated lipids (or fatty acids) is reduced and free radicals react with each other, resulting in stable non radical compounds

$R^{\bullet} + R^{\bullet} \rightarrow RR$

$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR$

$ROO\bullet + ROO\bullet \rightarrow ROOR + O_2$

Antioxidant activity (AH) refers to the inhibition of oxidation of lipids or other molecules by inhibiting the initiation or propogation step of the oxidative chain reactions or forming stable radicals ($A\bullet$) which are either unreactive or form non-radical products (Tyagi and Malik, 2010).

$ROO\bullet + AH \rightarrow ROOH + A\bullet$

The antioxidant activities of phenolics are related to a number of different mechanisms, such as free radical-scavenging, hydrogen-donation, singlet oxygen quenching, metal ion-chelation, and acting as substrates for radicals such as superoxide and hydroxyl. A direct relationship has been found between the phenolic content and antioxidant capacity of plants (Bassolé et al., 2010; Cao et al., 2011; Djenane et al., 2012). Antioxidants can deactivate radicals by two major mechanisms, HAT (Hydrogen Atom Transfer) and SET (Single Electron Transfer). Antioxidants with the HAT mechanism quench free radicals by hydrogen donation and with SET mechanism antioxidants transfer one electron to reduce any compound, including metals, carbonyls, and radicals (Schwarz et al., 2001).

Natural antioxidants, particularly in herbs and plants have gained increasing interest among consumers and the scientific practices because lots of studies have indicated that frequent consumption of natural antioxidants resulted in a lower risk of cardiovascular disease and cancer (Thaipong et al., 2006). In the food industry, an antioxidant is defined as a substance that, in small amounts, is capable of preventing or delaying, in a significant way, the oxidation of easily oxidizable materials, such as fats. The antioxidant properties of different plant extracts, essential oils and pure compounds can be evaluated using various in vitro assays. Antioxidant assays in foods and biological systems can be divided in two groups, that evaluate lipid peroxidation and that measure free radical scavenging activity (Miguel, 2010). There are many developed methods that have been still discussed according to their advantages and limitations. There is not still an agreement on the convenient method as a standard method for claiming total antioxidant capacity. The limitations for determination of hydrophilic antioxidants, the problems occurring in determination of reaction end point, the concern on light sensitivity of initiators, possible interference from certain food components, and the use of different standards for expressing results cause problems to compare the findings (Karadag et al., 2009).

Several assays have been used to examine the antioxidant capacities of essential oils and plant extracts including; DPPH, ABTS, TEAC, ORAC, FRAP, TBARS and etc. These methods are summarized in Table 2.4 according to their principles (Singh and Singh, 2008).

Table 2.4. Methods used to evaluate antioxidant capacity
(Source: Singh and Singh, 2008)

Method	Principle
DPPH (2,3-diphenyl-1-picrylhydrazyl) assay	Evaluation of scavenging activity of antioxidants by measurement of change in absorbance at 515-517 nm
TEAC (Trolox Equivalent Antioxidant Capacity) ABTS [(2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] assay	Measurement of inhibition of the absorbance of ABTS radical cation by antioxidants at 415 nm
ORAC (Oxygen Radical Absorbance Capacity) assay	Calculating the net protection area under the time recorded fluorescence decay curve of Red-phycoerythrin or β-phycoerythrin
FRAP (Ferric Reducing Ability of Plasma) assay	Measurement of blue color of reduced (Fe+2-TPTZ Tripyridyltriazine) at 593 nm at low pH.
TRAP (Total Radical Trapping Antioxidant Parameter)	Measuring the oxygen consumed
PCL (Photochemiluminescence) assay	Measurement of chemiluminiscence of luminonol radical
TBARS (Thiobarbituric Acid Reactive Substances) assay	Measurement of lipid peroxides formed after peroxidation with TBA
Carotene linoleate system	Measurement of bleaching of Carotene

Bay laurel extract was assessed for the antioxidant (iron(III) reduction, inhibition of linoleic acid peroxidation, iron (II) chelation, DPPH radical-scavenging and inhibition of hydroxyl radical-mediated 2-deoxy-D-ribose degradation, site and non-site specific) activities. The extracts from basil and laurel possessed the highest antioxidant activities except for iron chelation and they were found to be significantly better inhibitors of lipid peroxidation than the other spices (Hinneburg et al., 2006). Scavenging activity of DPPH radical and chelation power on ferrous ions of Indian bay leaf was analyzed and it was found that extracts had high radical scavenging activity. However, the chelation power was higher in methanol extract of bark than extract of bay leaf (Sudan et al.).

Clove essential oil has been reported as one of the strongest antioxidant. The highest antioxidant activity was reported for clove essential oil among thyme, origanum and citronella with respect to the results of DPPH and ferric reducing power assays. The EC_{50} (35.7 μg/ml) value was found to be lowest for clove essential oil, thus being classified as very strong antioxidant (Teixeira et al., 2013). Scherer and Godoy (2009) came up with the results of clove essential oil had very strong activity, because of the presence of eugenol (Scherer and Godoy, 2009). When the antioxidant activity was tested by using four different assay, clove essential oil exhibited high antioxidant activity for all methods (Wei and Shibamoto, 2010). Clove essential oil demonstrated scavenging activity against DPPH radical at lower concentration than the concentrations of standards (eugenol, BHT, BHA). There was also a significant inhibitory effect against hydroxyl radicals (Jirovetz et al., 2006).

Two types of *Thymus* species were evaluated according to their antioxidant activity by using DPPH and β-carotene/linoleic acid assays. Both of the species was active for free radical scavenging activity with 220 and 2670 μg/ml IC_{50} values, whereas, oxidation of linoleic acid was effectively inhibited only by one of them (Tepe et al., 2005). Thyme essential oil showed also the highest activity in terms of the inhibition of the degradation of deoxyribose even more than standard BHT used for the assay (Bozin et al., 2006). Thyme essential oils were investigated for their possible antioxidant activities by four complementary assays, namely DPPH free radical scavenging, hydroxyl radical scavenging, inhibition of lipid peroxidation and reducing power. The two new chemotypes exhibited strong hydroxyl radical scavenging, but were not or only slightly active against the other radicals and exhibited a weak reducing power (Hazzit et al., 2009). A *Thymus* species that was grown in different regions of Iran was subjected to DPPH and β-carotene/linoleic acid assays. The essential oil showed a comparable antioxidant activity in both methods (Safaei-Ghomi et al., 2009).

Cumin essential oil was tested by using a cell based assay and it was observed that essential oil exhibited strong *ex vivo* antioxidant activity, inhibiting reactive oxygen species and thus exhibited the ability to protect cells from oxidative stress (Bourgou et al., 2010). Cumin essential oils obtained from different distillation periods examined by β-carotene bleaching and DPPH methods. All cumin oils showed strong antioxidant activity in both assay with good correlation (Chen et al.). Six cold pressed cumin seed oils were evaluated according to their antioxidant activity by using DPPH assay. All oils

displayed DPPH scavenging capacity within the range of IC_{50} 76-83 μmol/100 μmol (Lutterodt et al., 2010).

Flavonoids of GSE are the reason of antioxidant properties. Mechanism is due to scavenging action on free radicals, metal chelating activity, reduction of hydroperoxide formation and their effects on cell signaling pathways and gene expression (Caillet et al., 2006; Jacob et al., 2008). Delgado Adámez, Gamero Samino et al. (2012) examined the antioxidant capacity of GSE by free radical scavenging ability (1,1-diphenyl-2-picrylhydrazyl, DPPH) and ferric reducing antioxidant power (FRAP). According to the observation, antioxidant capacity of extracts was related to the presence of a mixture of polyphenolic compounds. Significant higher values were found in GSE due to their higher concentration of phenolic compounds (Delgado Adámez et al., 2012). When DPD colorimetric method was used to examine the free radical scavenging capacity, the data obtained revealed that the grape extracts were free radical-scavengers and primary antioxidants, which react with free radicals. However, these results indicated that the phenols of the seed extract have free radical scavenging activities which are more significant than those of phenols present in the skin extract (Caillet et al., 2006). In the study where DPPH radical scavenging, hydrogen peroxide scavenging and phosphomolybdenum methods were used, it was determined that the grape seed extracts contained a higher amount of total phenolic content as they showed high-antioxidant activity than synthetic ones (BHA and BHT) (Göktürk Baydar et al., 2007). In another comparison study the antioxidant potential of GSE was found to be twenty and fifty fold greater than those of vitamins E and C respectively (Shi et al., 2003). The determination of the antioxidant activities of grape seeds (FRAP and TEAC assays) was performed by Maier et al. (2009). It was concluded that the press residues of grape seed oil production was to be a polyphenol-rich by-product with high antioxidant activity (Maier et al., 2009).

High phenolic contents give the olive leaf extract potential antioxidant agent properties. The antioxidant capacity of olive leaf extract was observed using the DPPH, ABTS, ferric reducing antioxidant capacity (FRAP), oxygen reducing antioxidant capacity (ORAC) and b-carotene-linoleic acid assays by Hayes et al. (2011). All of the methods showed compatible results supported that olive leaf extract was capable of scavenging free radicals at physiological pH and it was observed that OLE was a potential antioxidant agent (Hayes et al., 2011a). Benavente-García et al. (2000) described the differential antioxidant activities of main phenolic compounds of olive

leaf through the extent of their abilities to scavenge the ABTS radical cation. It was suggested that although all of individual phenolics were quenchers for the ABTS radical cation, they showed a synergic behaviour in their radical scavenging capacity when mixed, as occurs in the OL (Benavente-García et al., 2000). Electron-donating ability (EDA) by DPPH radical, superoxide dismutase (SOD)-like activity, antioxidant activity in the linoleic acid system, inhibition of auto-oxidation in linoleic acid emulsion (LAE) system and antioxidant activity on thermal oxidation system of olive leaf extract and its fractions were evaluated by Lee et al. (2009) and data suggested that olive leaf extract and its various fractions were effective in scavenging radicals and protecting lipid-oxidations when assessed by DPPH assay, SOD-like activity, POV and thermal oxidation assay (Lee et al., 2009).

2.6. Application of Essential Oils to Meat and Meat Products

Meat and meat products are convenient for bacterial contamination since they are rich in nutrients and perishable in nature. Because of the contamination with pathogenic organisms or their toxins, meat and meat products cause problem for public health. Essential oils represent a source of natural antimicrobial substances and have potential to be used in food industry as preservative to prevent spoilage to increase the shelf life and also decontaminate foods from pathogenic organisms.

The antimicrobial activity of natural compounds change among in vivo and in vitro studies. High amounts of these compounds are required when they are used in food systems. It is because of complex structure of food products. The greater availability of nutrients in foods compared to laboratory media may enable bacteria to repair damaged cells faster. Beside the intrinsic properties of the food (fat/protein/water content, antioxidants, preservatives, pH, salt and other additives) the extrinsic determinants (temperature, packaging, characteristics of microorganisms) can also influence bacterial sensitivity (Burt, 2004). The suseptibiltiy of bacteria to the antimicrobial activity of essential oils is highly influenced by pH, protein/fat content and storage temperature. The activity appears to increase with a decrease in pH of the food and the storage temperature (Hao et al., 1998). Because, the hydrophobicity of an EO increases at low pH enabling it to more easily dissolve in the lipids of the cell membrane of target bacteria (Juven et al., 1994). The high protein content in the food supports the inhibitory

properties of the EOs used whereas carbohydrates and fat diminish it (Gutierrez et al., 2009).

The antimicrobial effect of thyme EO at addition levels of 0.3%, 0.6%, or 0.9% on *E. coli* O157:H7 was investigated in minced beef by Solomakos et al. (2008). Treatment of minced beef with thyme EO (0.6%) inhibited *E. coli* O157:H7 growth during storage at 10 °C but not at 4 °C (Solomakos et al., 2008).

Krisch, Pardi, Tserennadmid, Papp, and Vagvolgyi (2010) suggested that the shelf life of minced pork can be prolonged by adding thyme or marjoram EOs, which decreased *E. coli* counts in minced pork by 1 log cfu after 24 h of storage at 5 °C. Further, 0.5 ml/ ml and 2 ml/ml were found to be the MIC values for marjoram and thyme EOs, respectively. Additionally, Chouliara and Kontominas (2006) observed a small but statistically significant effect of thyme oil (0.1%) on the extension of shelf life of fresh chicken breast meat (Chouliara et al., 2007) .

Menon and Garg (2001) examined the antimicrobial effect of clove oil on *L. monocytogenes* in minced mutton at 30 °C and 7 °C and revealed the potential use of clove oil as a natural preservative of meat. At both the concentrations (0.5% and 1%) tested, clove oil restricted the growth of *L. monocytogenes* in minced mutton at both temperatures compared to that for the control samples. However, treatment of minced mutton with 1% clove oil showed greater inhibitory activity compared to that obtained for minced mutton treated with 0.5% clove oil. Further, it was suggested that lower levels of clove oil may be sufficient for ensuring meat safety in actual situations in which bacterial loads are low (Vrinda Menon and Garg, 2001).

Essential oils are also applied as part of a hurdle system which improves the microbial stability and the sensory quality of meat and meat products (Jayasena and Jo). Lower levels of EOs can be combined with technologies including low temperature and acidity (Al-Reza et al., 2010; Skandamis and Nychas, 2000), modified atmosphere packaging (MAP) (Gomez and Lorenzo, 2012; Marino et al., 1999), high hydrostatic pressure (Devlieghere et al., 2004), preservatives (e.g., lactic acid, nisin etc.) (Naveena et al., 2006; Solomakos et al., 2008).

Use of essential oils mainly restricted since they affect the organoleptic quality of foods. Further, they are required in high concentrations in food has been limited in order to achieve the sufficient antimicrobial activity. Because of these problems it is better to use essential oils in hurdle system.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Microbial Strains

Pathogenic, non-pathogenic and spoilage bacteria (Table 3.1) were handled during the study. The bacterial strains included both Gram negative and Gram positive bacteria. *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium were chosen as pathogenic microorganisms, whereas, *Listeria innocua* were used as non-pathonic organism. *Shewanella putrefaciens*, *Carnobacterium divergens* and *Serratia liquefaciens* were picked out since they are associated with meat spoilage.

Shewanella putrefaciens, *Carnobacterium divergens* and *Serratia liquefaciens* strains were obtained from USDA, ARS Culture Collection (NRRL). In addition, *Staphylococcus aureus* strain was kindly provided by Dr. Figen Korel (Department of Food Engineering, İzmir Institute of Technology), *Listeria innocua* strain was also kindly provided by Dr. Nükhet Demirel Zorba (Department of Food Engineering, Çanakkale Onsekiz Mart University).

Escherichia coli O157:H7, *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria innocua* strains were grown in Nutrient broth. *Shewanella putrefaciens* and *Serratia liquefaciens* were firstly propagated in Tryptic Soy Broth (TSB), whereas *Carnobacterium divergens* was grown in YG broth (25 g nutrient broth no:2, 3 g yeast extract, 5 g glucose, 1 L of water; pH 6.8). After first propagation all bacteria were transferred to Nutrient broth and the growth was observed. Nutrient medium was used for all the experiments. All bacterial strains were preserved in nutrient broth containing glycerol (20%) at -80 °C.

Table 3.1. Bacterial strains used in the study

Gram Positive Bacteria	Origin	Gram Negative Bacteria	Origin
Staphylococcus aureus RSSK 01009	Clinical	*Escherichia coli* O157:H7 ATCC 700728	unknown
Listeria innocua NRRL B-33314	Turkey/ham deli sticks	*Salmonella* Typhimurium CCM5445	unknown
Carnobacterium divergens NRRL B-14830	Minced-beef	*Shewanella putrefaciens* NRRL B-951	unknown
		Serratia liquefaciens NRRL B-41553	Ground beef

3.1.2. Essential oils, Extracts and Components

Commercial grape seed liquid extract (*Vitis vinifera*), olive leaf liquid extract (*Olea europaea*), thyme essential oil (*Thymus* sp.), clove essential oil (*Syzygium aromaticum*) and cumin essential oil (*Cuminum cyminum*) were purchased from native producers. Bay leaf essential oil was kindly provided by Turer Tarim Ltd. Sti. All EO components (eucalyptol, linalool, α-terpineol and α-pinene) were obtained from Sigma-Aldrich.

Oleuropein, hydroxytyrosol, tyrosol, apigenin, verbascoside, rutin, luteolin-7-glucoside, luteolin, quercetin-3-galactoside, o-coumarin, p-coumaric acid, ferulic acid, vanillic acid, caffeic acid, vanillin, epicatechin, epicatechin gallate, gallic acid, oleuropein aglycon, syringic acid, catechin gallate, quercetin and resveratrol were used as standards for HPLC analysis of extracts.

Different concentrations of essential oils were prepared in ethanol with the highest concentration of 1%.

3.2. Chemical Characterization

3.2.1. GC-MS Analyses of Essential Oils

Gas chromatography coupled with mass spectrometry (GC–MS) analysis was carried out in an Agilent 6890 gas chromatograph interfaced to an Agilent 5973N mass selective detector MSD (Agilent Technologies, Palo Alto, USA) according to the method suggested by Ramos et al. (Ramos et al., 2011). The GC was equipped with a (5%-phenyl)-methyl polysiloxane HP-5MS column (30 m length * 0.25 mm internal

diameter * 0.25 μm film thickness). Helium was used as the carrier gas at a flow rate of 1 mL/min constant flow. The oven temperature was programmed from 45 °C (hold for 1 min) to 250 °C (5 min) at 5 °C /min in a 47 min total running time. 100-400 amu was used as scanning mass range. Identification of components in essential oils was carried out by the comparison of the mass spectra characteristic features with the NIST 05 Mass Spectral Library.

3.2.2. HPLC Analyses of Extracts

Phenolic compounds identifications of grape seed and olive leaf extracts were performed by HPLC analysis (Aktas et al., 2013; Bouaziz et al., 2004). Chromatographic analyses were carried out with Agilent 1200 high-performance liquid chromatograph (Agilent, Santa Clara, CA, USA) with a refractive index detector. The stationary phase was a Hypresil Gold (Thermo, USA) analytical column (250 mm*4 mm i.d.) with a particle size of 5 μm. The mobile phases used for separation were 0.1% acetic acid in water (A) versus methanol–acetonitrile–acetic acid (50:50:0.1) (B) for a total running time of 70 min. The gradient elution procedure was applied as follows: initial concentrations of mobile phase B was 5 %, then it was increased to 55% in 45 min and kept for 10 min, in further step the concentration of B was increased to 100% in 5 min and maintained for 5 min, ultimately, phase B was reduced to 5% in 5 min and resumed for 5 min at this concentration. The injection volume was 20 μl, where the flow rate was 1 ml/min. Chromatograms at 280 nm were used to identify phenolic compounds by comparing the retention times with commercial standards analyzed in the same conditions. Quantification was performed by using their respective calibration curves and expressed as ppm and the percentages of components.

3.3. Antimicrobial Activity

3.3.1. Bacterial Suspension

Bacterial cultures were grown in appropriate media and incubation conditions. Then bacterial suspensions were adjusted equivalent to 0.5 McFarland standard

(Densitometer, HVD DEN-1) (approximately 10^7-10^8 cfu/ml) and one more tenfold dilution was performed in broth medium.

3.3.2. Disk Diffusion Method

100 µl of bacterial suspension prepared as above, was spread on Nutrient Agar plates with sterile cotton swabs to form an even lawn. Sterile paper disks (6mm in diameter, Oxoid) were impregnated with 20 µl diluted essential oils and extracts in different concentrations were placed onto the surface of agar plates. Then plates were incubated aerobically in appropriate conditions for each organism for 24 h (Ramos et al., 2011). At the end of incubation period the absence or presence of inhibition zones were evaluated.

3.3.3. Broth Microdilution Method

For broth microdilution assay, 20 µl of bacterial suspension was added into the wells of a sterile 96-well microtitre plate containing 180 µl of two-fold diluted essential oils and plant extracts. Control wells were prepared with medium inoculated with bacterial suspension and also essential oils and plant extracts without inoculation. Plates were incubated for 24 h and the turbidity was determined by a microplate reader (Thermo, Vario Skan Flash) at 600 nm with 30 min interval (Klančnik et al., 2010). After the incubation period a 100 µl samples were taken from each wells and spread onto agar plates to check the bacterial growth. The MICs of essential oils and extracts were recorded as the lowest concentration where no viability was observed in the wells of 96-microwell plates after incubation for 24 h.

3.3.4. Broth Macrodilution Method

100 µl bacterial suspensions were inoculated to 900 µl growth media already containing desired concentration of essential oils and plant extracts. Eppendorf tubes were incubated by shaking for 24 h at appropriate incubation temperatures. After 24 h, 100 µl samples were directly spread onto agar plates and plates were checked for

presence or absence of colonies after incubation for 24 and 48 h. The absence of colonies on plates of a treatment was considered the MIC values.

3.3.5. Time-kill Assay

The plant extracts were added to 900 µl of growth medium to give a final concentration in accordance with the results obtained by broth macrodilution method. 100 µl bacterial suspensions were inoculated in growth media already containing desired concentration of essential oils, shaken and incubated. Bacterial growth was followed by sampling at 0, 0.5, 1, 3, 5 and 24 h and plating on cultivation media after serial sample dilutions in Maximum Recovery Diluent (MRD). After incubation of plates at proper temperatures for 24 h, number of bacteria was counted. Positive controls were performed in the same way, except without adding essential oils. The MIC was defined as the lowest concentration of essential oils in solid media where no growth was observed after 24 h or 48 h incubation (Burt, 2004). All experiments were repeated at lest two times independently and results were given as the average log cfu/ml (± standard deviations).

3.3.6. Synergism Testing of Constituents of Essential Oils

After the determination of chemical composition of essential oils, the antimicrobial activity of some major constituents was examined. For this purpose; eucalyptol, linalool, α-terpineol and α-pinene were analyzed alone or in combination to evaluate the potential interaction among them.

The checkerboard assay was performed to determine potential synergistic, additive or even antagonistic effects of combination of individual compounds by defining Fractional Inhibitory Concentration (FIC) (Bevilacqua et al.) index using 96-well microtitre plates (Bassolé et al., 2010; Mulyaningsih et al., 2010). The combinations were designed by using the concentration of constituents ranging from MIC to 1/8 MIC. The final volume of each well was 100 µl including 50 µl of each constituent dilution. Subsequently, 100 µl of bacterial suspension were added into the wells. The plates were then incubated at 37 °C for 24 h and the turbidity was determined by a microplate reader (Thermo, Vario Skan Flash) at 600 nm with 30 min interval. The

FIC indices were calculated as FICA + FICB where each of them was the minimum concentrations that inhibited the bacterial growth. FIC index was calculated as follows;

FICA= MIC of component A in combination / MIC of component A alone

FICB= MIC of component B in combination / MIC of component B alone

FIC= FICA + FICB

The combination of two compounds was considered to be synergistic when the FIC index was ≤ 0.5, additive when it was > 0.5 to 4 and antagonistic when it was > 4 (de Azeredo et al., 2011; de Oliveira et al., 2010; Mackay et al., 2000).

3.4. Investigation of Mechanism of Antimicrobial Activity

3.4.1. Structural Observations by Scanning Electron Microscope

Overnight cultures were adjusted to McFarland 1 standard and then treated to essential oils and extracts at determined MIC and FIC values. After appropriate incubation period for each bacterium, cells were harvested by centrifugation, washed 2 or 3 times and resuspended in sterile distilled water or PBS. 20 µl of suspension was spread onto a microscope slide and air dried. Another portion of cultures were used as untreated control. Then samples were coated with gold under vacuum followed by microscopic examinations using scanning electron microscope (SEM) (Philips XL 30SFEG) (Burt and Reinders, 2003; Lv et al., 2011).

3.4.2. Structural Observations by Atomic Force Microscope

For AFM imaging, samples were prepared according to method of (Cui et al., 2012; Tyagi and Malik, 2010) in which aliquots of untreated and treated bacterial suspension, adjusted to McFarland 1 standard, then washed and resuspended with deionized water and spread on glass cover. The glass cover slips were then dried in a covered Petri dish and examined by using atomic forced microscopy in tapping mode (AFM) (MMSPM Nanoscope IV).

3.4.3. Release of Cellular Material

Overnight broth cultures were adjusted to McFarland 2 standard. Cells were collected by centrifugation at 5000 rpm for 5 min. Pellet was washed twice and resuspended in PBS (pH 7.2) containing 2MIC and MIC value of essential oils and also FIC values of constituents. Samples were incubated at appropriate temperature for each bacterium under agitation for 1 or 2 h. After treatment cell suspensions were centrifuged at 10000 rpm for 10 min. Supernatant was used to measure UV absorption at 260 nm to determine the concentration of release of cellular constituents (Devi et al., 2010; Lv et al., 2011). Results were expressed as the ratio of OD260 of incubated samples to initial measurements of OD260.

3.4.4. FTIR Observation

The alteration in structural features of tested bacteria at the molecular level upon treatment was analyzed by FT-IR spectroscopy (Mackay et al., 2000). Essential oils were added to cell suspensions of overnight culture. Treatment was performed for 4 h. The cells were then washed twice and resuspended with sterile deionized water. Samples were mounted on the plate and then analyzed with FT-IR spectroscopy. Control samples without treatment were prepared and subjected to analysis as mentioned above. IR spectrometer (Perkin Elmer Spectrum 100 FT-IR spectrometer, Wellesley, MA) was within the range of 650-4000 cm^{-1} wave number. This equipment has a horizontal attenuated total reflectance (HATR) accessory with ZnSe crystal (45 deg. Trough Plate) and deuterated tri-glycine sulphate (DTGS) detector. The scanning was carried out at 4 cm^{-1} resolution, and 0.50 cm/s scan speed. The number of scans for each spectrum was adjusted to 64.

3.4.5. Measurement of Bacterial Membrane Potential

The BacLight™ Bacterial Membrane Potential Kit (Invitrogen) was used to measure the bacterial membrane potential in *S. aureus*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium treated with essential oils at MIC and 2MIC values. Diethyloxacarbocyanine ($DiOC_2$) exhibits green fluorescence in all bacterial cells, but

the fluorescence shifts toward red emission as the dye molecules self-associate at higher cytosolic concentrations caused by larger membrane potentials. Proton ionophores such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP) could destroy membrane potential by eliminating the proton gradient, thus it was used as a positive control in the study. The ratio of red to green fluorescence provides a measure of membrane potential that is largely independent of cell size, with a low coefficient of variation (CV) (Cao et al., 2011).

Overnight bacterial cultures were adjusted to Mc Farland 2 standard and treated with essential oils for 4 h. At the end of incubation period cells were harvested by centrifugation and resuspended in filtered PBS. Assay was performed according to the manufacturer's instruction with slight modification suggested by Cao et al. (2011). Aliquots of 1 ml bacterial suspension were taken into flow cytometry tubes for staining treatments. Two additional tubes were also prepared for each bacterium for depolarized and unstained controls. 10 µl of 500 µM CCCP was added to depolarized control samples and then incubated 30 min at 4 °C, followed by adding 5 µl 0f 3 mM $DiOC_2$ to each flow cytometry tubes except the unstained control tubes. Afterwards, all stained tubes were mixed well and incubated at room temperature for 15 min in a dark place. Stained bacteria were assayed in a flow cytometer (Facscanto, BD) with a laser emitting at 488 nm. Fluorescence was collected in the green and red channels ("GC" and "RC"); the unstained controls were used to designate bacterial populations in the forward and side scatter channels. The bacterial population was gated using forward versus side scatter and fluorescence photomultiplier tube voltages were adjusted such that the green and red mean fluorescence intensity (MFI) values were approximately equal without compensation. While the relative amount of red and green fluorescence intensity varied with respect to cell size and aggregation, the ratio of red to green florescence intensity can be used as a size-independent indicator of membrane potential. For a dot plot of red versus green fluorescence, the regions around the populations of interest were set and red and green MFI values for each recorded. The change of the membrane potential was expressed as MFI of the red population divided by the green population MFI.

3.5. Antioxidant Activivity

3.5.1. Total Phenol Content

Total phenolic content was determined by Folin-Ciocalteu assay. Briefly, diluted samples were mixed with Folin-Ciocalteu's reagent (1/10 in distilled water) and left to stand 2-3 min at room temperature. After adding sodium carbonate solution (7.5% w/v) to the mixture, tubes were allowed to stand for 60 min in a dark place at room temperature then the absorbance of each solution was read against the blank at 765 nm. The same procedure was applied to gallic acid with different concentrations to obtain a calibration curve. Results were expressed as mg gallic acid equivalents (GAE) per ml (Slinkard and Singleton, 1977).

3.5.2. Antioxidant Acitivity by FRAP Method

Ferric reducing antioxidant power (FRAP) was performed according to Thaipong et al. (Thaipong et al., 2006). The fresh working FRAP solution was prepared by mixing 10 ml acetate buffer (300 mM, pH 3.6), 1 ml TPTZ (10 mM 2, 4, 6-tripyridyl-s-triazine) and 1 ml $FeCl_3$.$6H_2O$ solution and then warmed at 37 °C before using. 150 µl of diluted essential oils and extracts were allowed to react in FRAP solution in a 3 ml total volume for 30 min in the dark condition. Readings were then taken at 593 nm and results were expressed as milimol Trolox equivalent per ml.

3.5.3. Antioxidant Acitivity by DPPH Method

This assay measure the antioxidant activity in terms of hydrogen-donating or radical scavenging ability, using the stable radical DPPH (Sangun et al., 2007). DPPH radical was determined by the method with some modifications (Ojeda-Sana et al., 2013), briefly 20 µL of each sample in triplicate and six different concentrations and 180 µL of DPPH solution (160 mM) in ethanol, were added to a well in a 96-well flat-bottom microtitration plate. A DPPH solution was used as blank sample. Plate was incubated for 24h and absorbance was measured at 515 nm with 10 min time intervals.

The antioxidant activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to the formula;

$$IC\ (\%) = [(Ab - As)/Ab]x100$$

Where *Ab* = absorbance of blank sample and *As* = absorbance of a tested sample at the end of the reaction (Delgado Adámez et al., 2012; Ojeda-Sana et al., 2013; Ramos et al., 2011). Percent inhibition after where the reaction gone to completion ("plateau") (Schwarz et al., 2001) was plotted against concentration and a linear regression was applied to obtain the IC_{50} value.

3.6. Antimicrobial Effect of Essential Oils in Minced Beef

3.6.1. Preperation of Minced Beef Samples

Salmonella Typhimurium bacterial suspension was prepared using McFarland standard and further diluted to yield a final concentration of 4 log cfu/g. After inoculation and homogenization in a stomacher of meat samples, thyme essential oil and clove essential oil were added at 2MIC and 4MIC values. Afterwards, mixtures were homogenized again. Minced meat samples were then divided into 10 g of portion for each sampling time and for each analysis. Samples were kept at 4 °C and examined during 9 days.

3.6.2. Microbiological Analyses

Microbiological analyses of samples included the determination the population of *Salmonella* Typhimurium, Aerobic mesophilic microorganisms (AMM), Enterobacteriaceae, yeasts and molds and psychrotrophic microorganisms. Analyses were carried out at 3 days intervals up to the 9th day of 4 °C. All of microbial counts were expressed as log10 colony forming units (CFU) per g of sample. The media used in the study were obtained from Difco, BD, Dickinson.

At each sampling time 10 g of samples were homogenized in a stomacher bag for 2 min in 90 ml of sterile buffered peptone water (BPW). Serial dilutions were made in BPW and used for enumeration of microroganisms.

Brillant Green Agar (BGA) and Bismuth Sulphite Agar (BSA) were used to evaluate the number of *Salmonella* Typhimurium after 24 h incubation at 37 °C.

Enterobacteriaceae were determined using Violet Red Bile agar (VRBA) as a medium after 24 h incubation at 37 °C.

Number of aerobic mesophilic microorganisms was determined on plate count agar (PCA) incubated at 30 °C for 48 h.

Potato Dextrose Agar (PDA) was used to determine yeasts and molds after 48 h incubation at 25 °C.

Determination of psychrotrophic microorganisms was carried out on PCA with10 day incubation at 7 °C.

3.6.3. Determination of Lipid Oxidation

Lipid oxidation was measured by the 2-thiobarbituric acid (TBA) method as described by (Djenane et al., 2012). 5 g of minced beef samples were taken and mixed well with 10 ml of trichloroacetic acid (10%). Samples were then centrifuged at 10000 rpm for 30 min at 5 °C and supernatants were filtered through quantitative paper. 2 ml of filtered supernatant was mixed with 2 ml of thiobarbituric acid (20 mM) (99%, Sigma); tube contents were mixed well by vortex vigorously and incubated at 97 °C for 20 min in a boiling water bath. After incubation tubes were cooled immediately and absorbance was measured at 532 nm. Average of three absorbance values was used to determine the oxidative stability.

3.6.4. Color Evaluation

Color was instrumentally measured by L*a*b* system by using Minolta CR400 (Tokyo, Japan) colorimeter. The instrument was firstly standardized against a white reference plate. Five measurements were taken from each sample. The colorimeter directly calculated three color features of L* (lightness), a* (red–green component), and b* (yellow–blue component).

3.7. Statistical Analyses

Data of minced beef application study were analyzed by ANOVA (Minitab 16). Means with a significant difference ($p<0.05$) were compared using Tukey test.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Chemical Characterization

4.1.1. GC-MS Analyses of Essential Oils

The chemical compositions of essential oils from bay leaf, thyme, clove and cumin were determined by comparing the relative retention times and the mass spectra of oil components with mass spectra from data library. The essential oils were characterized by one or two dominant components.

The results of chemical analysis of essential oils are presented in Table 4.1, 4.2, 4.3 and 4.4. The components are listed according to their retention time and percentage contribution.

The GC-MS analyses resulted in the identification of 30 components of bay leaf essential oil (Table 4.1). The main components were 1,8-cineole (eucalyptol) (36 %), α-terpineol acetate (14.93 %), sabinene (9.87 %), α-pinene (6.18 %), β-pinene (4.72 %), 4-terpineol (4.58 %), α-terpineol (3.88 %), linalool (1.42 %). 1,8-cineole with a concentration of about 36 % was found to be the major component in bay leaf essential oil. The major components of bay leaf essential oil were demonstrated also as 1,8-cineole by many other researchers (Cimanga et al., 2002; Kilic et al., 2004; Sangun et al., 2007). Although the composition of bay leaf essential oil changes by factors such as soil composition, climate, and geographic origin, our results support that the major 11 constituents are 1,8-cineole (eucalyptol), linalool, sabinene, α-terpineol, α-pinene (Dadalioğlu and Evrendilek 2004; Ozcan and Chalchat 2005; Ramos, Teixeira et al. 2011).

Table 4.1. Volatile compounds of bay leaf essential oil determined by GC-MS analyses

Compound	**R. T. (min)**	**%**
1,8 Cineole (eucalyptol)	10.143	36.00
α-terpineol acetate	19.04	14.93
sabinene	8.363	9.87
α-pinene	7.237	6.18
β-pinene	8.436	4.72
(-)-4-terpineol	14.278	4.58
α-terpineol	14.662	3.88
teta-terpinene	10.767	1.68
linalool	11.982	1.42
β-pinene	8.789	1.29
eugenol methylether	20.393	1.22
p-cymene	9.837	0.93
α-terpinene	9.536	0.87
eugenol	19.219	0.76
L-trans-pinocarveol	13.133	0.64
α-thujene	7.029	0.57
(1R)-(-)-myrtenal	14.806	0.52
β-caryophyllene	20.799	0.50
caryophyllene oxide	24.818	0.49
cis-β-terpineol	11.032	0.43
terpinolene	11.611	0.41
α-phellandrene	9.172	0.38
limonene	12.609	0.37
camphene	7.605	0.35
β-elemene	20.069	0.33
geraniol acetate	19.303	0.31
(-)-trans-pinocarvylacetate	17.427	0.24
pinocarvone	13.813	0.23
α-pinene	9.338	0.11
3-hexen-1-ol	5.33	0.10

The major components of thyme essential oil were determined by GC-MS were carvacrol (75.27%) following by cymene (7.84 %), thymol (4.51), γ-terpinene (2.96 %) and borneol (1.44%). The others comprise terpinene, terpineol, limonene, linalool and eucalyptol (Table 4.2).

There is a great variability and diversity of chemical composition of *Thymus* species due to factors such as origin, climatic and soil variation, vegetative cycle, seasonal variation etc. (Ait-Ouazzou et al., 2011b; Baydar et al., 2004a; Safaei-Ghomi et al., 2009; Sokmen et al., 2004; Tepe et al., 2005; Verma et al., 2010). Some thymus oils are characterized by the increased percentages of thymol, carvacrol, borneol, linalool or α-terpineol (Tepe et al., 2005).

The high carvacrol content of thymus EO following by cymene, thymol, γ-terpinene and borneol, is in good agreement with the findings of chemical composition of Iran originated thyme oil (Safaei-Ghomi et al., 2009). The studies associated with essential oils of Turkish origin *Thymus* species were also in a good agrrement with our findings (Baydar et al., 2004a; Sokmen et al., 2004). Most of the identified components have also previously been reported as the major components of thyme essential oils from Tunisia (Hosni et al., 2013), Sicily (Napoli et al., 2010) and Greece origins (Economou et al., 2011).

Table 4.2. Volatile compounds of thyme essential oil determined by GC-MS analyses

Compound	**R. T. (min)**	**%**
carvacrol	17.623	75.27
cymene	9.643	7.84
thymol	17.248	4.51
γ-terpinene	10.632	2.96
borneol	13.751	1.44
β-bisabolene	22.808	1.33
α-terpinene	9.409	1.25
β-murcene	8.687	1.10
4-terpineol	14.079	0.9
caryophylene	20.649	0.85
α-pinene	7.126	0.60
D-limonene	9.76	0.469
linalool	11.827	0.39
α-terpinolene	11.488	0.27
camphene	7.522	0.25
α-phellandrene	9.058	0.15
eucalyptol	9.851	0.15
1-octen-3 -ol	8.378	0.138

Clove essential oil consists of eugenol (75.2%), benzyl salicylate (14.74%), propylene glycol (6.02%) and also β-caryophyllene (3.21%) (Table 4.3). Clove essential oils have different origins and extracted by different methods have been analyzed by various researchers. Eugenol seems to have the highest proportion of tested clove essential oils as it was also observed from our results. Besides eugenol, eugenol acetate and β-caryophyllene are the major constituents (Dorman et al., 2000; Guan et al., 2007; Lee and Shibamoto, 2001; Martini et al., 1996) . GC-MS results differ in the presence and the amount of benzyl salicylate from the literature.

Table 4.3. Volatile compounds of clove essential oil determined by GC-MS analyses

Compound	**R. T. (min)**	**%**
eugenol	19.103	75.20
benzyl salicylate	30.78	14.75
propylene glycol	3.126	6.02
β-caryophyllene	20.653	3.21
α-caryophyllene	21.505	0.69
caryophyllene oxide	24.642	0.122

The GC-MS analyses showed that the major components of cumin essential oil were 3-caren-10-ol (29.69 %), γ-terpinene (22.73 %), L-β-pinene (17.7 %), p-cumic aldehyde (11.84 %), cymene (6.47 %), α-phellandrene (3.028 %), 2-caren-10-ol (1.13 %), D-limonene (1.116%), β-pinene (1.107 %), α-pinene (1.08 %) (Table 4.4). GC analysis has confirmed the presence of major constituents are alcohols, terpene hydrocarbons and aldehydes that showed similarity with the findings of Behera et al. (Behera et al., 2004). Cymene was identified as the major component by Kedia et al. (2014) whereas, cumin aldehyde was the main constituent notified by (El-Sawi and Mohamed, 2002) in a conformity with Egyptian and Turkish origin cumin essential oils. The variation in essential oil composition may be caused by ecological and geographical distribution and also climatic and soil variation condition.

Table 4.4. Volatile compounds of cumin essential oil determined by GC-MS analyses

Compound	**R. T. (min)**	**%**
3-caren-10-ol	17.282	29.69
γ-terpinene	10.648	22.73
L-β-pinene	8.295	17.70
p-cumic aldehyde	15.856	11.84
cymene	9.638	6.47
α-phellandrene	9.059	3.028
2-caren-10-ol	17.077	1.13
D-limonene	9.761	1.116
β-pinene	8.687	1.107
α-pinene	7.126	1.08
carotol	24.946	1.067
β-phellandrene	8.209	0.954
β-farnesene	21.504	0.485
β-gurjunene	19.608	0.46
α-thujene	6.95	0.406
corymbolone	29.6	0.355
β-caryophllene	20.645	0.3467

4.1.2. . HPLC Analyses of Extracts

Identification of each component of grape seed and olive leaf extracts was performed by HPLC by comparing the retention times with commercial standards. The amount of compounds was determined by using their respective calibration curves and expressed as ppm and the percentage of constituents.

HPLC profiles of major phenolic compounds present in olive leaf extract are shown in Fig. 4.1. Retention times and abundance of the components are also presented in Table 4.5. HPLC analysis of olive leaf extract showed several peaks corresponding to different phenols which were identified from their retention times (Fig. 4.1).

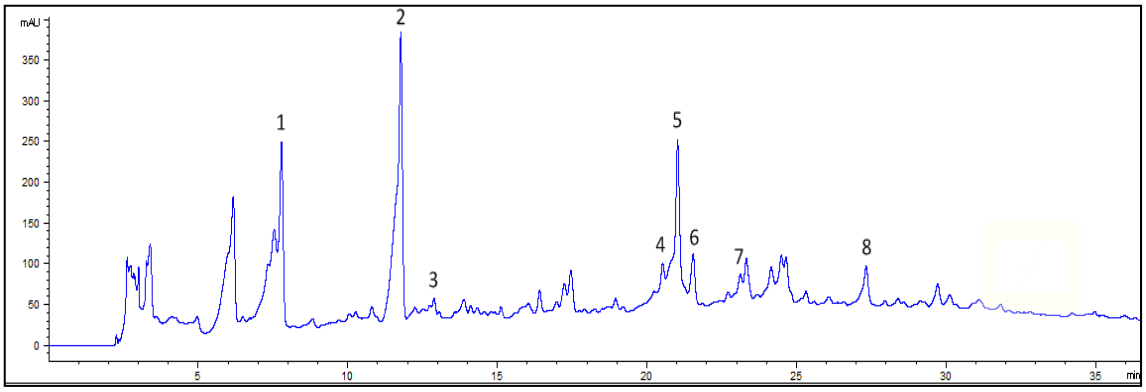


Figure 4.1. HPLC phenol profiles of olive leaf extract 1, hydroxytyrosol; 2, tyrosol; 3, oleuropein aglycon; 4, rutin; 5, ferulic acid; 6, luteolin-7-glucoside; 7, verbascoside; 8, oleuropein

The major polyphenols were identified as tyrosol, hydroxytyrosol, oleuropein, ferulic acid, oleuropein aglycon, verbascoside and luteolin-7-glucoside (Table 4.5). The most abundant one is tyrosol with a high percentage of 44.26%, following by hydroxytyrosol (20.13%) and oleuropein (17.47%). All these phenolic compounds have previously been reported to be present in olive leaf extract (Ahmad-Qasem et al., 2013; Hayes et al., 2011b; Mylonaki et al., 2008). The report submitted by (Benavente-García et al., 2000) showed that polyphenolic compounds in olive leaves consists of oleuroside (oleuropein and verbascoside), flavones (luteolin, diosmetin, apigenin-7-glucose, luteolin-7-glucose, and diosmetin-7- glucose), flavonols (rutin), flavan-3-ols (catechin), and substituted phenol (tyrosol, hydroxytyrosol, vanillin, vanillic acid, and caffeic acid) and oleuropein and hydroxytyrosol were the most abundant polyphenols. Besides, (Lee et al., 2009) found that oleuropein (102.11 mg/100 g) was major phenolic compound,

whereas, rutin (1.38 mg/100 g), vanillin (0.66 mg/100 g) and caffeic acid (0.31 mg/100 g) were minor phenolic compounds. Our findings are also in agreement that the minor components were vanillin and caffeic acid. On the other hand, rutin was seemed to be one of the major one. This result is also supported by which showed that oleuropein and rutin were the main components (Altıok et al., 2008). Considering the differences in extraction methods, sample origin, in terms of both geographic provenance and year of collection (Pereira et al., 2007a) and even type of the trees (Altıok et al., 2008), the distinction between the phenolic profiles of different olive leaf extracts is possible.

Table 4.5. Retention times and concentration of components of olive leaf extract

Compound	**Retention time**	**Amount (ppm)**
tyrosol	11.76	7286.44
hydroxytyrosol	7.77	3314.02
oleuropein	27.32	2876.24
ferulic acid	21.01	723.40
oleuropein aglycon	12.87	580.69
verbascoside	23.11	498.27
luteolin-7-glucoside	21.53	432.04
rutin	20.51	310.51
apigenin	34.95	254.25
o-coumarin	24.64	51.65
vanillic acid	13.87	42.38
syringic acid	14.09	36.91
p-coumaric acid	17.45	23.58
vanillin	16.40	16.59
caffeic acid	13.57	15.56

Figure 4.2 represents the profiles of major phenolic compounds found in grape seed extracts. The amount, retention times and the percentage of phenolic is also demonstrated in Table 4.6.

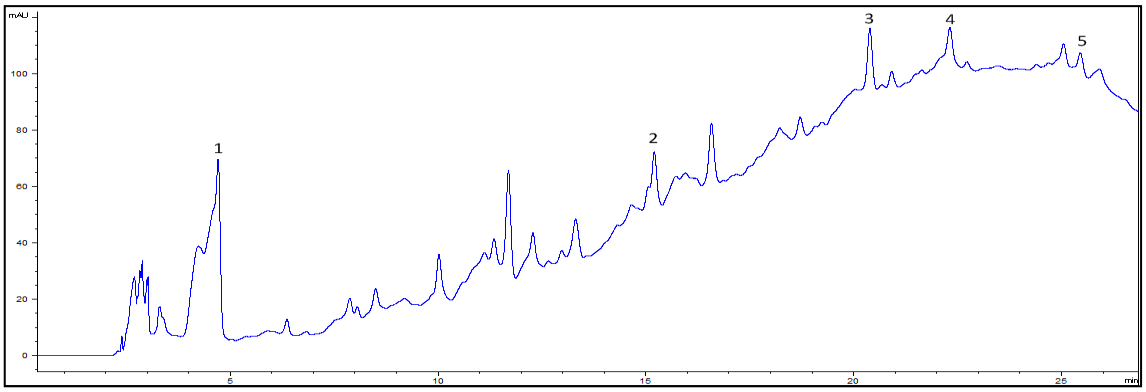


Figure 4.2. HPLC phenolic profiles of grape seed extract 1, gallic acid; 2, epicatechin; 3, rutin; 4, epicatechin gallate; 5, quercetin-3-galactoside

Epicatechin, gallic acid, quercetin-3-galactoside, rutin and epicatechin gallate were determined as the major components of grape seed extract (Table 4.6). It was observed that epicatechin was the main constituent with a high proportion of 9827.07 ppm. The compounds mainly included in grape seed extract are proanthocyanidins, anthocyanins, flavonols, flavanols, resveratrols and phenolic acids (Perumalla and Hettiarachchy, 2011; Xia et al., 2010). Flavonoids like the dimers and trimmers of catechin, epicatechin and epicatechin-3-O-gallate have been found mostly in the extract (Caillet et al., 2006; Delgado Adámez et al., 2012). The results of HPLC analysis of grape seed extract support that epicatechin is he major constituent (Iacopini et al., 2008; Revilla and Ryan, 2000; Rodríguez Montealegre et al., 2006; Yilmaz and Toledo, 2004). (Rockenbach et al., 2011) quantified rutin in some of the red grape varieties and also results showed similarities that resveratrol was existed as a minor element.

Table 4.6. Retention times and concentration of components of grape seed extract

Compound	**Retention time**	**Amount (ppm)**
epicatechin	15.19	9827.07
gallic acid	4.70	700.93
quercetin-3-galactoside	25.44	536.52
rutin	20.38	277.25
epicatechin gallate	22.30	249.21
quercetin	31.17	74.13
ferulic acid	20.02	65.07
catechin gallate	23.46	44.14
caffeic acid	14.31	28.87
vanillic acid	13.30	25.17

(cont. on next page)

Table 4.6 (cont.)

syringic acid	14.65	22.53
p-coumaric acid	18.22	17.31
vanillin	16.57	16.09
resveratrol	28.49	9.29

4.2. Antimicrobial Activity

4.2.1. Disk Diffusion Method

A wide range of concentrations were tested against bacteria, but only high concentrations resulted in inhibition zones according to disk diffusion method (Fig 4.3). Among the methods that were used in this study, disk diffusion technique seems less suitable for quantification purposes such as the determination of MIC value and it is also difficult to examine the susceptibility of fastidious and slow-growing bacteria (Dickert et al., 1981; Wilkins and Thiel, 1973). Although this method allows testing a large number of antimicrobials in a relatively easy and inexpensive manner, the results are considered as qualitative. Because this method can only reveal the susceptibility of antimicrobials against the bacteria tested, and this susceptibility could be described as susceptible, intermediate, and resistant according to correllation with diameter of inhibition zone (Faleiro 2011, Jiang 2011). Also, it has been reported (Klancnik *et al.*, 2010) that this method is not always reliable for determining the antimicrobial activity of 18 natural antimicrobials, i.e., plant extract, because the polarity of the natural compounds can affect the diffusion of compounds onto the culture medium. This technique is appropriate to use as a preliminary observation only prior to more detailed studies, because, method is affected easily by volume of extract impregnated to the paper disks, the thickness of the agar layer, the diffusion rate of extracts. Compounds with less polarity diffused slower than more polar ones (Moreno et al., 2006). Due to these concerns, disk diffusion may not be a suitable one to determine the antimicrobial activity of natural compounds.

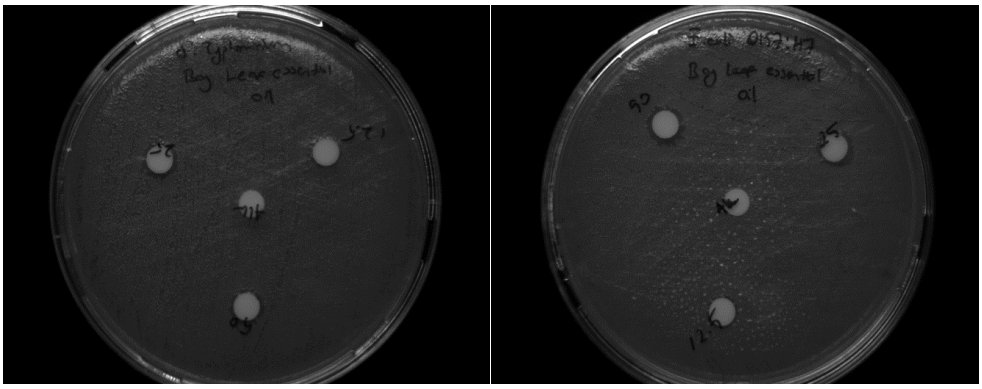


Figure 4.3. Inhibition zones of bay leaf EO on *S.* Typhimurium and *E.coli* O157:H7

4.2.2. Broth Dilution Methods

Four essential oils; bay leaf, thyme, clove and cumin and two extracts; grape seed and olive leaf were examined according to their antimicrobial activities by using broth microdilution and macrodilution assays.

A wide range of concentration of essential oil and extracts (Table 4.7, Table 4.8) was examined by considering the findings in the literature. Results showed obviously that antimicrobial effects of the plant essential oil and the extracts were concentration dependent for all tested bacteria. It was determined that bay leaf essential oil was highly effective on Gram negative bacteria except from *S. putrefaciens*, and especially foodborne pathogens with a MIC value of 1%. *L. innocua* and *S. putrefaciens* were the most resistant to bay leaf essential oil with a MIC value of 8%. *C. divergens* differed from the other tested organisms that have a MIC value of 2%.

24 h observation of bacterial growth indicated that the MIC values were 0.05% for all *S. aureus*, *E.coli* O157:H7 and *Salmonella* Typhimurium for thyme essential oil as it is shown in representative graphs (Fig. 4.4, Fig A). *Shewanella putrefaciens* was the most resistant bacteria to thyme essential oil with a MIC value of 0.25%. In general, food spoilage organisms were observed to be more resistant to thyme essential oil than foodborne pathogen organisms. *Serretia liquefaciens, Carnobacterium divergens* have the MIC value of 0.06% for thyme essential oil whereas, *L. innocua* was the most resistant one with the MIC value of 0.125% among all the tested bacteria. A significant retardation was observed in *S. aureus* growth when 0.04% thyme EO was used. 0.03% concentration also elongated the lag phase of *S.* Typhimurium. It was determined that thyme essential oil was highly effective on Gram negative bacteria except from *S. putrefaciens*, and especially foodborne pathogens with a MIC value of 0.05%.

Clove bud essential oil was seemed to be effective on both Gram positive and negative bacteria within the range of MIC values between 0.5% and 2% (Fig. 4.5) .

All tested bacteria were resistant to cumin EO below the concentration of 2%. Among the tested bacteria *S.aureus* and *E.coli* O157:H7 were the most sensitive strains to all essential oils. MIC values of these strains were 0.05% and 0.5% for thyme and clove essential oil, respectively.

Table 4.7. Antimicrobial activity of extracts expressed as MIC (%) determined by broth microdilution and broth macrodilution

Bacteria	**Plant extracts (vol/vol)**			
	GS extract %		**OL Extract %**	
	Concentration Ranges	**MIC**	**Concentration Ranges**	**MIC**
Gram positive bacteria				
Staphylococcus aureus	2.0 - 50.0	3	5.0 - 25.0	10
Carnobacterium divergens	5.0 - 50.0	10	5.0 - 25.0	15
Listeria innocua	5.0 - 50.0	40	5.0 - 25.0	15
Gram negative bacteria				
Shewanella putrefaciens	5.0 - 50.0	40	5.0 - 25.0	20
Serretia liquefaciens	5.0 - 50.0	>50	5.0 - 25.0	>20
E.coli O157:H7	2.0 - 50.0	40	5.0 - 25.0	15
Salmonella Typhimurium	2.0 - 50.0	50	5.0 - 25.0	15

Grape seed extract was more effective on Gram positive bacteria with MIC values of 3%, 10% and 40% for *S.aureus*, *C. divergens* and *L. innocua*, respectively.MIC values were 40% for *S. putrefaciens* and *E.coli* O157:H7, whereas *S. liquefaciens* showed resistance to GS extract even at the concentration of 50%.

All tested bacteria were totally inhibited by the same MIC value of 15% for olive leaf extract with the exception of *S.aureus* (10%), *S. putrefaciens* (20%) and *S. liquefaciens* (>20%). Results obtained in this study showed that *S. liquefaciens* growth was only inhibited by the bay leaf EO with a low MIC value of 1%. However, GS extract and OL extract were not effective with MIC values more than 50% and 20%, respectively on this gram negative food spoilage bacterium.

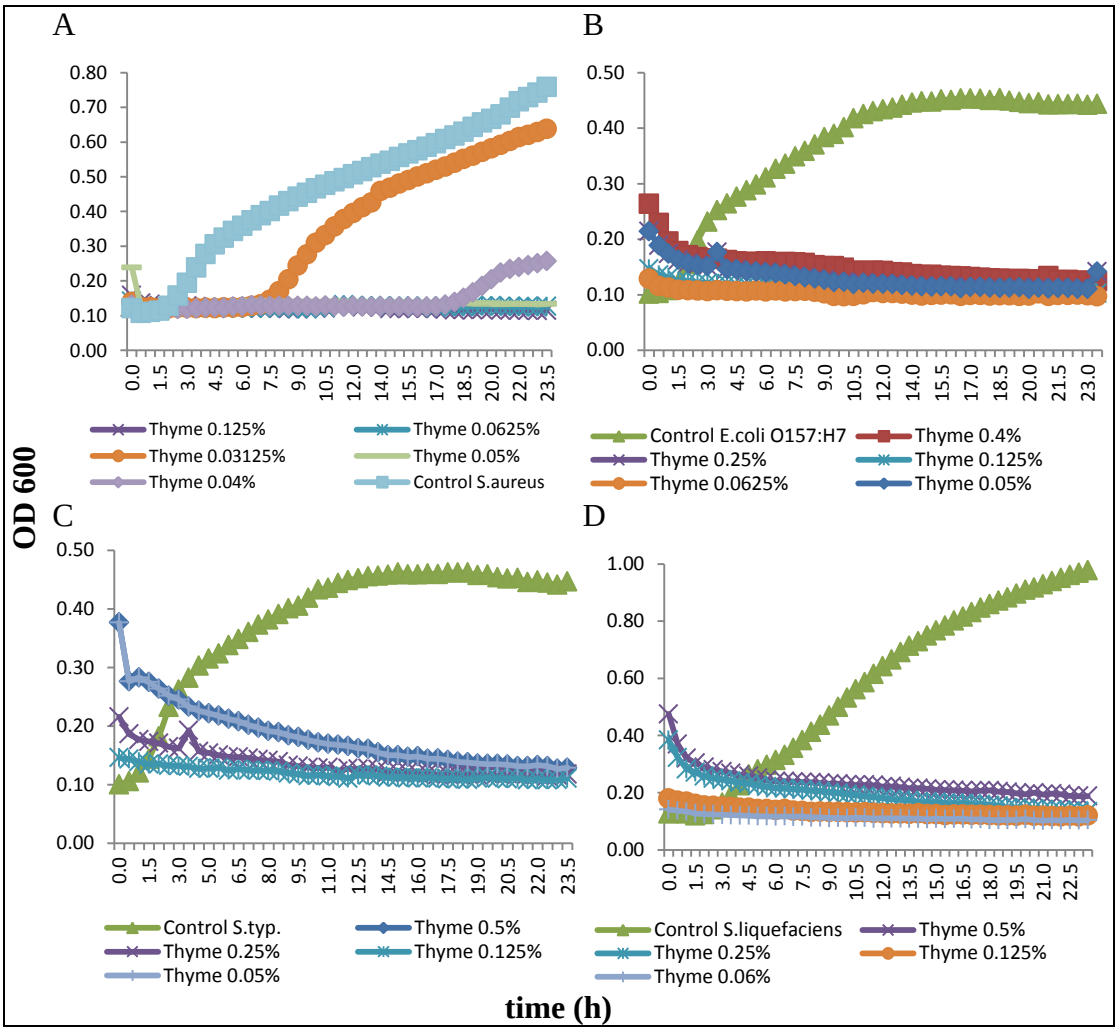


Figure 4.4. The growth and inhibition of A) *S. aureus*; B) *E. coli* O157:H7; C) *S.*Typhimurium; D) *S.liquefaciens* on exposure to thyme essential oil

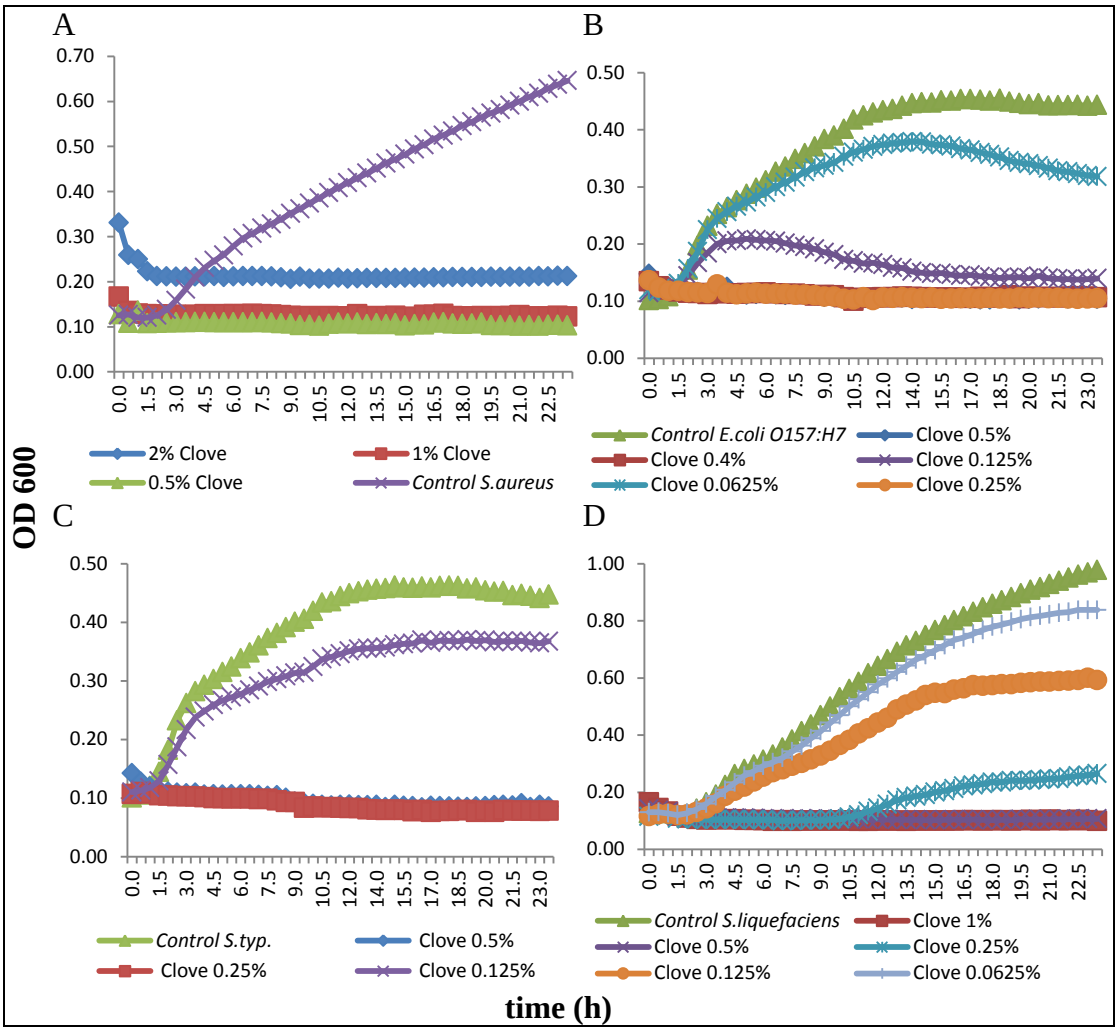


Figure 4.5. The growth and inhibition of A) *S. aureus*; B) *E. coli* O157:H7; C) *S.*Typhimurium; D) *S.liquefaciens* on exposure to clove essential oil

Table 4.8. Antimicrobial activity of essential oils expressed as MIC (%) determined by broth microdilution and broth macrodilution

Bacteria	**Essential oils (vol/vol)**							
	Thyme EO %		**Clove EO %**		**Cumin EO %**		**Bay leaf EO %**	
	Concentration Ranges	**MIC**	**Concentration Ranges**	**MIC**	**Concentration Ranges**	**MIC**	**Concentration Ranges**	**MIC**
Gram positive bacteria								
Staphylococcus aureus	0.03 - 2	0.05	0.03 – 2	0.5	0.03 - 2	>2	10 - 0.25	1
Carnobacterium divergens	0.03 - 2	0.06	0.03 - 2	1	0.03 - 2	>2	10 - 0.25	2
Listeria innocua	0.03 - 2	0.125	0.03 - 2	2	0.03 - 2	>2	10 - 0.25	8
Gram negative bacteria								
Shewanella putrefaciens	0.03 - 2	0.25	0.03 - 2	1	0.03 - 2	>2	10 - 0.25	8
Serretia liquefaciens	0.03 - 2	0.06	0.03 - 2	0.5	0.03 - 2	>2	10 - 0.25	1
E.coli O157:H7	0.03 - 2	0.05	0.03 - 2	0.5	0.03 - 2	>2	10 - 0.25	1
Salmonella Typhimurium	0.03 - 2	0.05	0.03 - 2	1	0.03 - 2	>2	10 - 0.25	1

4.2.3. Time-kill Assay

Based on the test results of broth macrodilution method, survival and dead curves for different concentrations of bay leaf essential oil were obtained by time-kill assay. *S. aureus*, *E.coli* O157:H7 and *Salmonella Typhimurium* were examined as foodborne pathogens and representative *Serretia liquefaciens* was chosen as foodborne spoilage microorganism for this assay.

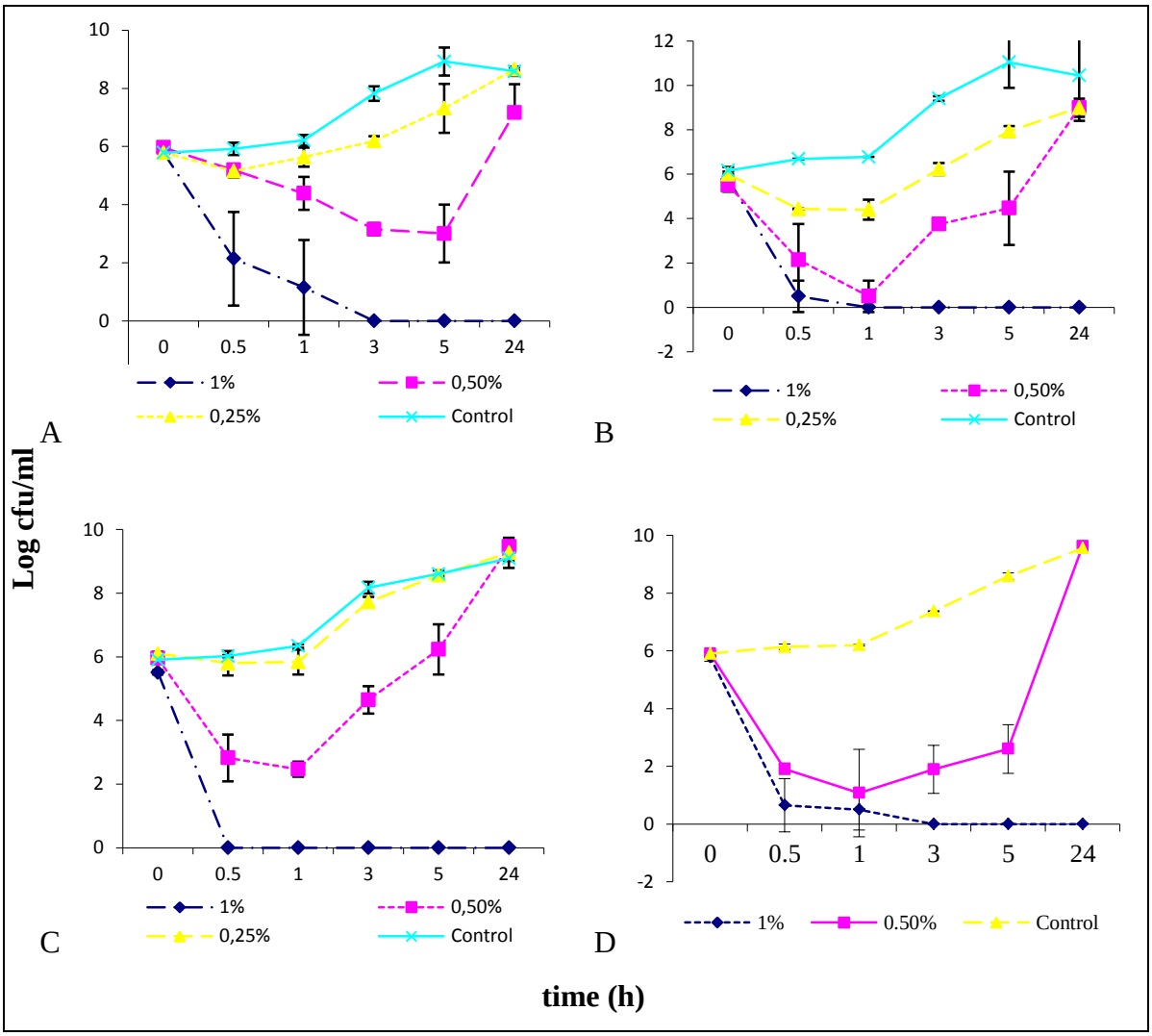


Figure 4.6. Time-kill curves of A) *S.aureus* ; B) *S.* Typhimurium; C) *E.coli* O157:H7; D) *S. liquefaciens* on exposure to bay leaf essential oil

The MIC values of these bacteria were 1% of bay leaf essential oil. Although the effective concentration was the same, the affecting time differs for the tested bacteria. Approximately a 6 log reduction was observed after 3 h of bay leaf EO exposure for

S.aureus and *S. liquefaciens* exposed to bay leaf EO at 1%, whereas *E.coli* O157:H7 and *S.* Typhimurium were completely inhibited after 0.5 h and 1 h, respectively (Fig. 4.6).

All tested bacteria showed apparent decrease while they were treated by bay leaf EO with a 1/2 MIC value, but after a while, they started to grow up. This may be due to the ability of bacteria to develop resistance against the antimicrobial agent.

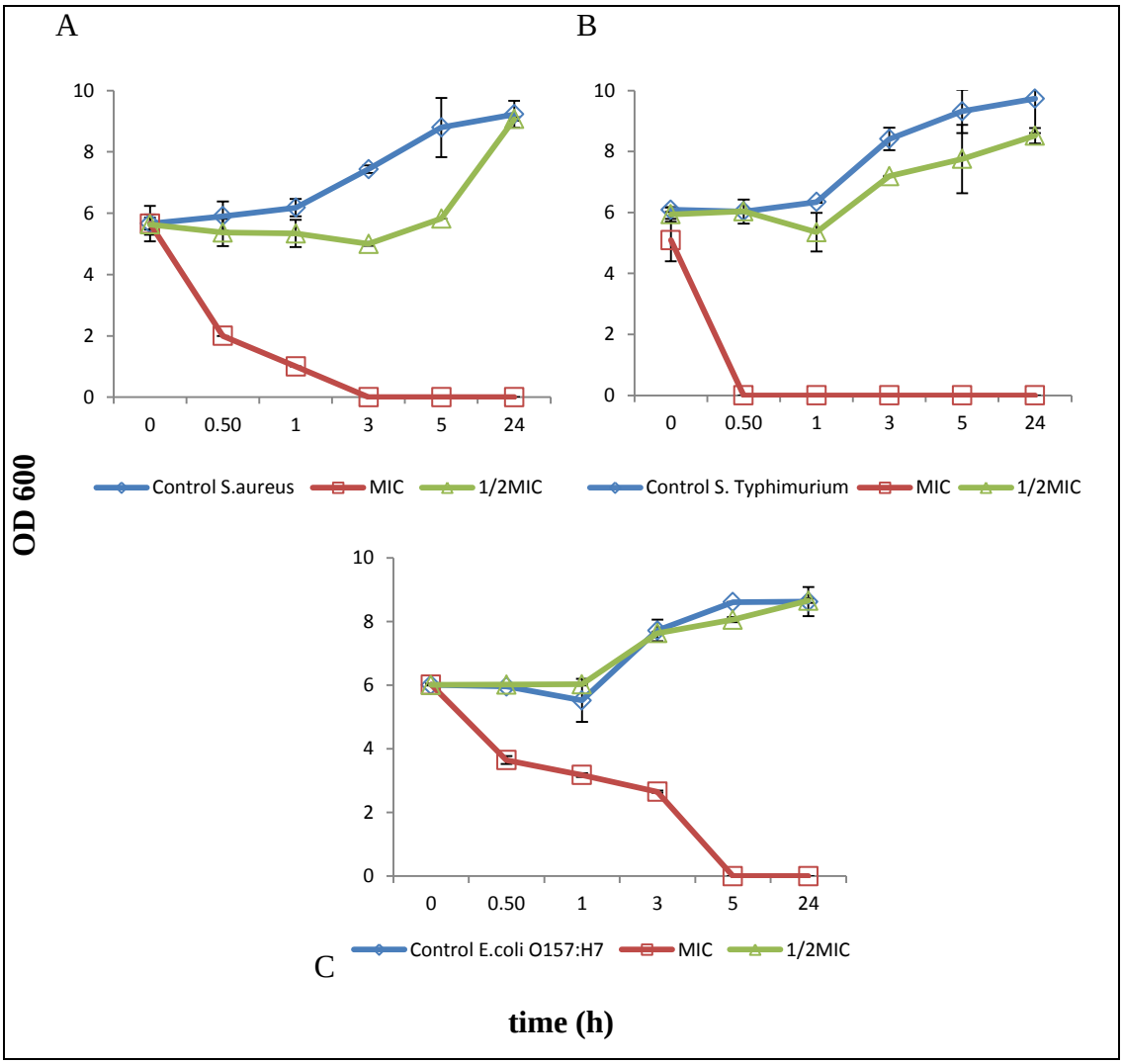


Figure 4.7. Time-kill curves of A) *S.aureus* ; B) *S.* Typhimurium C) *E.coli* O157:H7 on exposure to thyme essential oil

Among the tested essential oils thyme EO seems to be most effective ones. *S. aureus, E.coli* O157:H7 and *Salmonella* Typhimurium were examined as the most sensitive microorganisms for this assay.

The MIC values of these bacteria were 0.05% of thyme essential oil. Although the effective concentration was the same, the affecting time differs for the tested bacteria. Approximately a 6 log reduction was observed after 24 h of thyme EO exposure for *S.aureus*, whereas *S.* Typhimurium and *E.coli* O157:H7 were completely inhibited after 0.5 h and 5 h respectively (Fig. 4.7).

Among tested bacteria only *S.aureus* showed a slight decrease while it was treated with thyme EO with a 1/2 MIC value.

4.2.4. Synergism Testing of Constituents of Essential Oils

Before the interaction study, all significant constituents were subjected to broth microdilution assay to determine the MIC values for *S. aureus*, *S.* Typhimurium, *E.coli* O157:H7, *Serretia liquefaciens*, *Carnobacterium divergens, L. innocua* and *Shewanella putrefaciens*. Table 4.9 and representative Figure 4.8 (A-H) show the concentration ranges during the study and MIC values of examined essential oil components for tested bacteria by broth microdilution assay.

Table 4.9. Antimicrobial activity of essential oil components expressed as MIC (%) determined by broth microdilution

Bacteria	**Essential oil components (vol/vol)**					
	α- terpineol (T) %		**Linalool (L) %**		**Eucalyptol (E) %**	
	Concentration Ranges	**MIC**	**Concentration Ranges**	**MIC**	**Concentration Ranges**	**MIC**
Gram positive bacteria						
Staphylococcus aureus	0.03 - 2	0.7	0.03 - 2	1	0.03 - 2	2
Carnobacterium divergens	0.03 - 2	0.6	0.03 - 2	2	0.03 - 2	2
Listeria innocua	0.03 - 2	0.6	0.03 - 2	1	0.03 - 2	>2
Gram negative bacteria						
Shewanella putrefaciens	0.03 - 2	2	0.03 - 2	>2	0.03 - 2	>2
Serretia liquefaciens	0.03 - 2	0.6	0.03 - 2	1	0.03 - 2	>2
E.coli O157:H7	0.03 - 2	0.6	0.03 - 2	0.6	0.03 - 2	0.7
Salmonella Typhimurium	0.03 - 2	0.7	0.03 - 2	0.7	0.03 - 2	0.8

24 h observation of bacterial growth indicated that the MIC values were 0.6% for all *E.coli* O157:H7, *Serretia liquefaciens*, *Carnobacterium divergens* and *L. innocua*

for α-terpinol (Table 4.9). *S. aureus* and *S. Typhimurium* were inhibited with the concentration of 0.7% of α-terpinol. *S. aureus, L. innocua* and *Serretia liquefaciens* showed the same MIC value of 1% for linalool, while *E.coli* O157:H7 had the MIC value of 0.6% following by *S.* Typhimurium (0.7%). It is obvious that all tested bacteria were comparably resistant to eucalyptol with the MIC values of 2 or >2. The most sensitive bacteria to eucalyptol were *E.coli* O157:H7 (0.7%) and *S.* Typhimurium (0.8%). *Shewanella putrefaciens* was the most resistant bacteria to all tested components with the MIC values of 2% or higher. Whereas, *E.coli* O157:H7 was the most sensitive strains to all phenolic compounds among the tested bacteria.

All tested bacteria were resistant to α-pinene with the MIC values above the concentration of 2% (data not shown). Among the tested bacteria *E.coli* O157:H7 was the most sensitive strains to all extracts.

Linalool, 1,8-cineole, α-terpinol and α-pinene were previously tested according to their antimicrobial activities and MIC and MBC (minimum bactericidal concentration) values were also determined by other authors (de Sousa et al., 2012a; de Sousa et al., 2012b; de Sousa et al., 2012c; Gomes Neto et al., 2012; Park et al., 2012). When α-pinene and 1,8-cineole was tested against four human pathogenic bacteria (*S. aureus*, *Enterococcus faecalis*, *E.coli* and *Klebsiella pneumonia*), MIC values changed between 0.8 µl/ml and 20 µl/ml (Ojeda-Sana et al., 2013). α-pinene was found to be active aagainst four bacteria, while 1,8-cineole showed no activity against *S. aureus* and *Enterococcus faecalis* in contrast to our results. In another study, α-pinene did not show any inhibitory activity against tested bacteria, supporting our results (Ait-Ouazzou et al., 2011a). Linalool and 1,8-cineole were also examined. Linalool showed bacteriostatic properties with MIC ≤ 0.2 µl/ml, whereas MIC value of 1,8-cineole was greater than 2 µl/ml. The results of this study also indicated that linalool was more active than 1,8-cineole on tested bacteria. Bactericidal activity of 23 oil components were determined as BA50 value that was a 50% decrease in the number of cfu (Friedman et al., 2002). Although, results changed according to the tested organisms and components; BA50 values changed between 0.10-0.56, 0.35-0.67, >0.67 for α-terpineol, linalool and 1,8-cineole respectively.

There are some researches studying the antimicrobial activities of essential oil constituents. However, there has been no synergistic activity study concerning the selected components in this study. Combinations of terpenes such as; thymol, carvacrol, eugenol, menthol and etc. (Bassolé et al., 2010; Gallucci et al., 2009; Pei et al., 2009),

combinations of essential oils (de Azeredo et al., 2011; Goñi et al., 2009; Gutierrez et al., 2008), combinations of essential oils/phenolics and nisin/bacteriocin (Moosavy et al., 2008; Turgis et al., 2012) and also combination of essential oil components and food processing technique (Karatzas et al., 2001) were mostly studied.

A significant retardation was observed during the growth of *S. aureus* when 0.6% α-terpinol was used (Fig. 4.8 F). Eucalyptol elongated the lag phase of *S.* Typhimurium, *E.coli* O157:H7, *S. aureus* at the concentration of 0.7%, 0.6% and 1% respectively (Fig. 4.8 A, C, F).

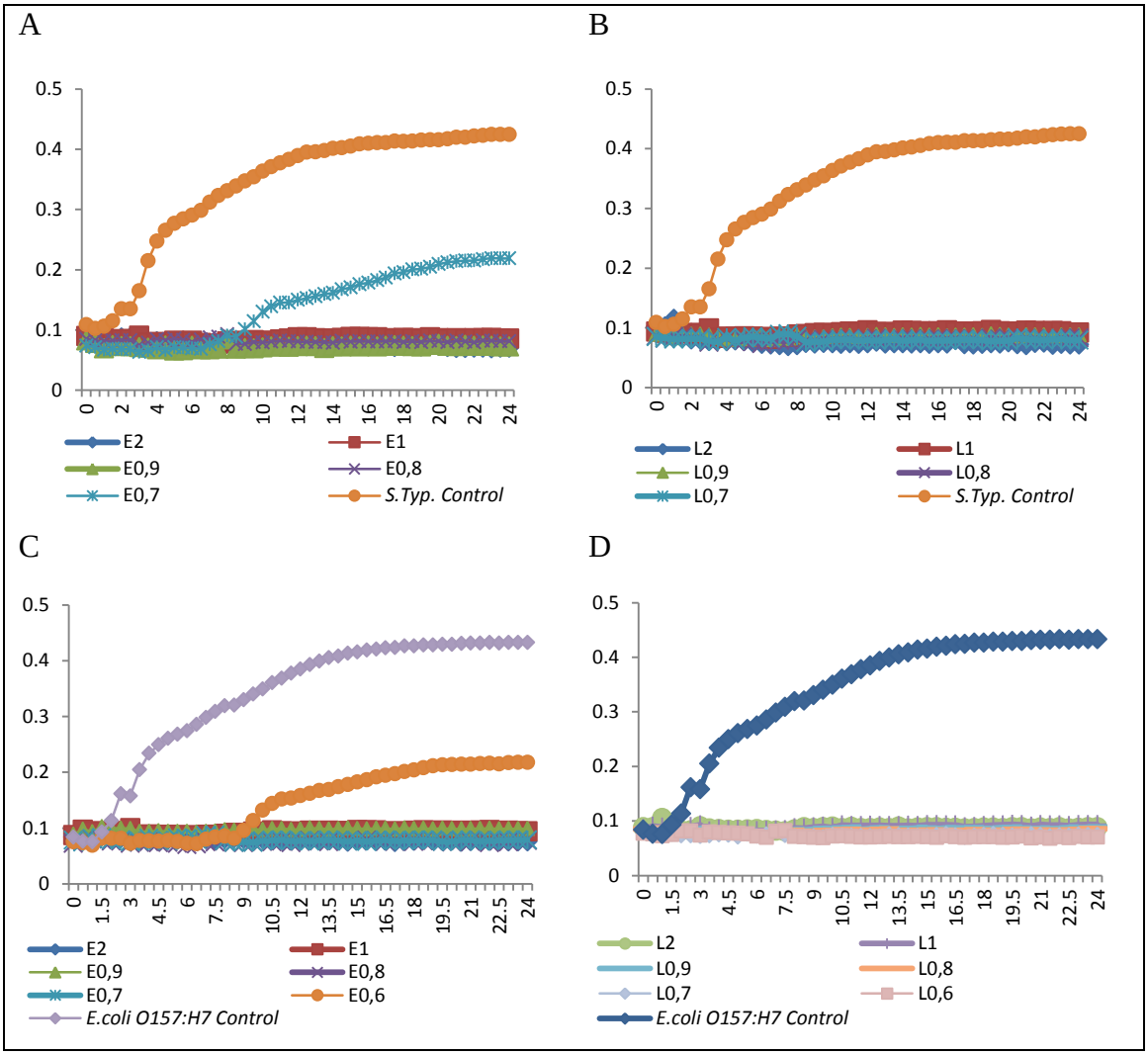


Figure 4.8. Inhibition of cells treated with (A) eucalyptol, *S.*Typhimurium; (B) linalool, *S.* Typhimurium; (C) eucalyptol, *E.coli* O157:H7; (D) linalool, *E.coli* O157:H7; (E) α-terpineol, *S.aureus*; (F) eucalyptol, *S.aureus*; (G) linalool, *S.aureus*; (H) α-terpineol, *S.* Typhimurium

(**cont. on next page**)

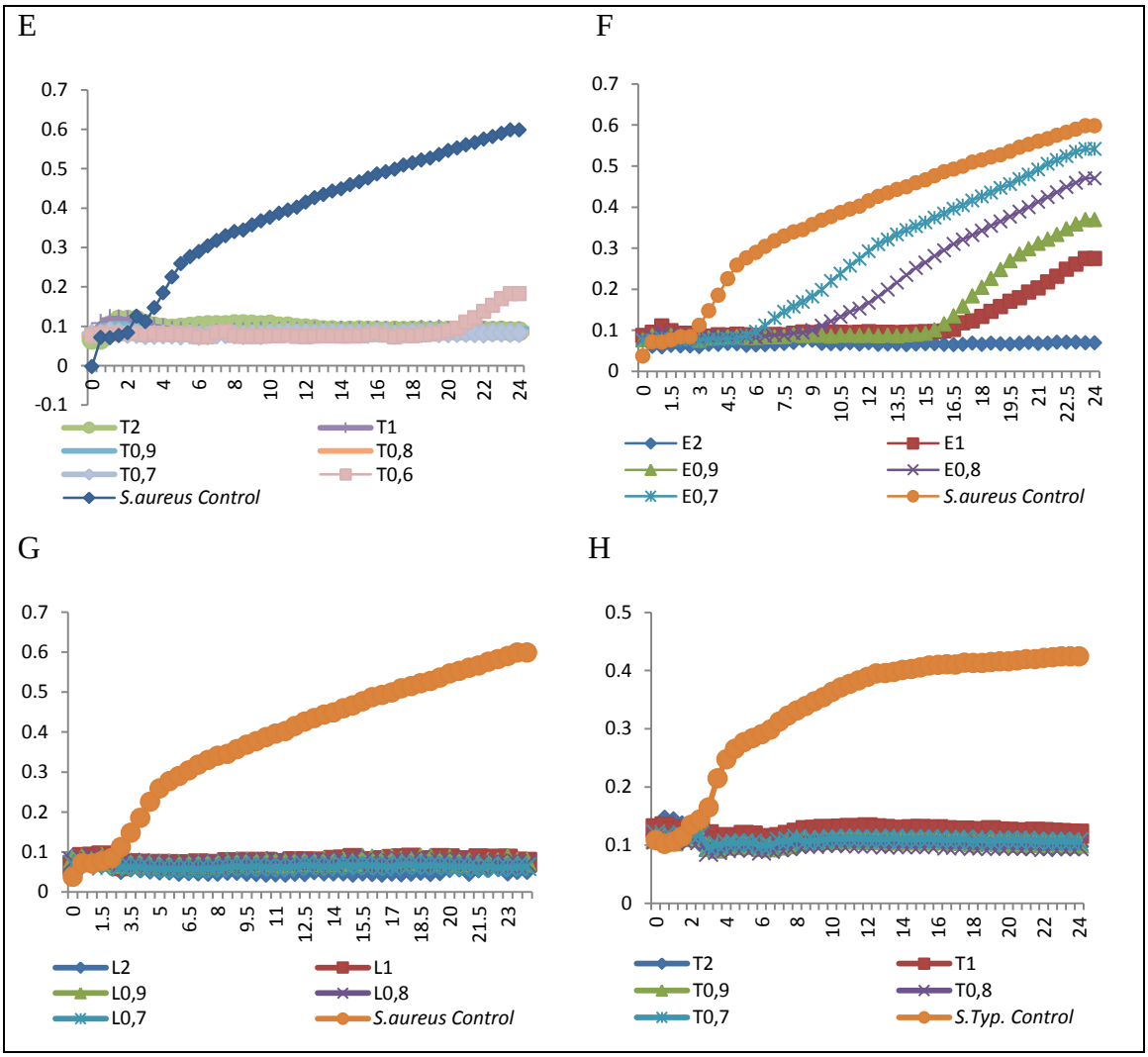


Figure 4.8. (cont.)

Three pathogen bacteria showed more sensitivity to essential oil constituents. Therefore, the interactions between the constituents were examined against these bacteria. Checkerboard assays of all three tested bacteria gave additive or synergistic profiles when components were combined at sub-inhibitory concentrations (Table 4.10). Synergistic effects were observed at least one dose pair of combination against *S.* Typhimurium, *E.coli* O157:H7 and *S.aureus* (Fig.4.9/15). Synergy was noted when the components of α- terpineol and linalool were combined at 1/4 MIC + 1/4 MIC ; 1/4 MIC + 1/8 MIC; 1/8 MIC + 1/4 MIC and 1/8 MIC + 1/8 MIC respectively. All other dose pair combinations resulted in additive effects. α- terpineol/eucalyptol and linalool/eucalyptol combinations showed additive effects against all tested bacteria. Antagonistic effect was not found in these combinations.

Table 4.10. Effect of treatments with combined components according to FICI

	Components		MIC (%)		Combined MIC (%)			
	A	B	A	B	A	B	FIC	Effect
***E.coli* O157:H7**	*α- terpineol*	*linalool*	*0.6*	*0.6*	*0.3*	*0.3*	*1*	*Additive*
	α- terpineol	*linalool*	*0.6*	*0.6*	*0.15*	*0.15*	*0.5*	*Synergy*
	α- terpineol	*eucalyptol*	*0.6*	*0.7*	*0.3*	*0.35*	*1*	*Additive*
	linalool	*eucalyptol*	*0.6*	*0.7*	*0.3*	*0.35*	*1*	*Additive*
S. Typhimurium	*α- terpineol*	*linalool*	*0.7*	*0.7*	*0.35*	*0.35*	*1*	*Additive*
	α- terpineol	*linalool*	*0.7*	*0.7*	*0.175*	*0.175*	*0.5*	*Synergy*
	α- terpineol	*eucalyptol*	*0.7*	*0.8*	*0.35*	*0.4*	*1*	*Additive*
	linalool	*eucalyptol*	*0.7*	*0.8*	*0.35*	*0.4*	*1*	*Additive*
S.aureus	*α- terpineol*	*linalool*	*0.7*	*1*	*0.35*	*0.5*	*1*	*Additive*
	α- terpineol	*linalool*	*0.7*	*1*	*0.175*	*0.25*	*0.5*	*Synergy*
	α- terpineol	*eucalyptol*	*0.7*	*2*	*0.35*	*1*	*1*	*Additive*
	linalool	*eucalyptol*	*1*	*2*	*0.5*	*1*	*1*	*Additive*

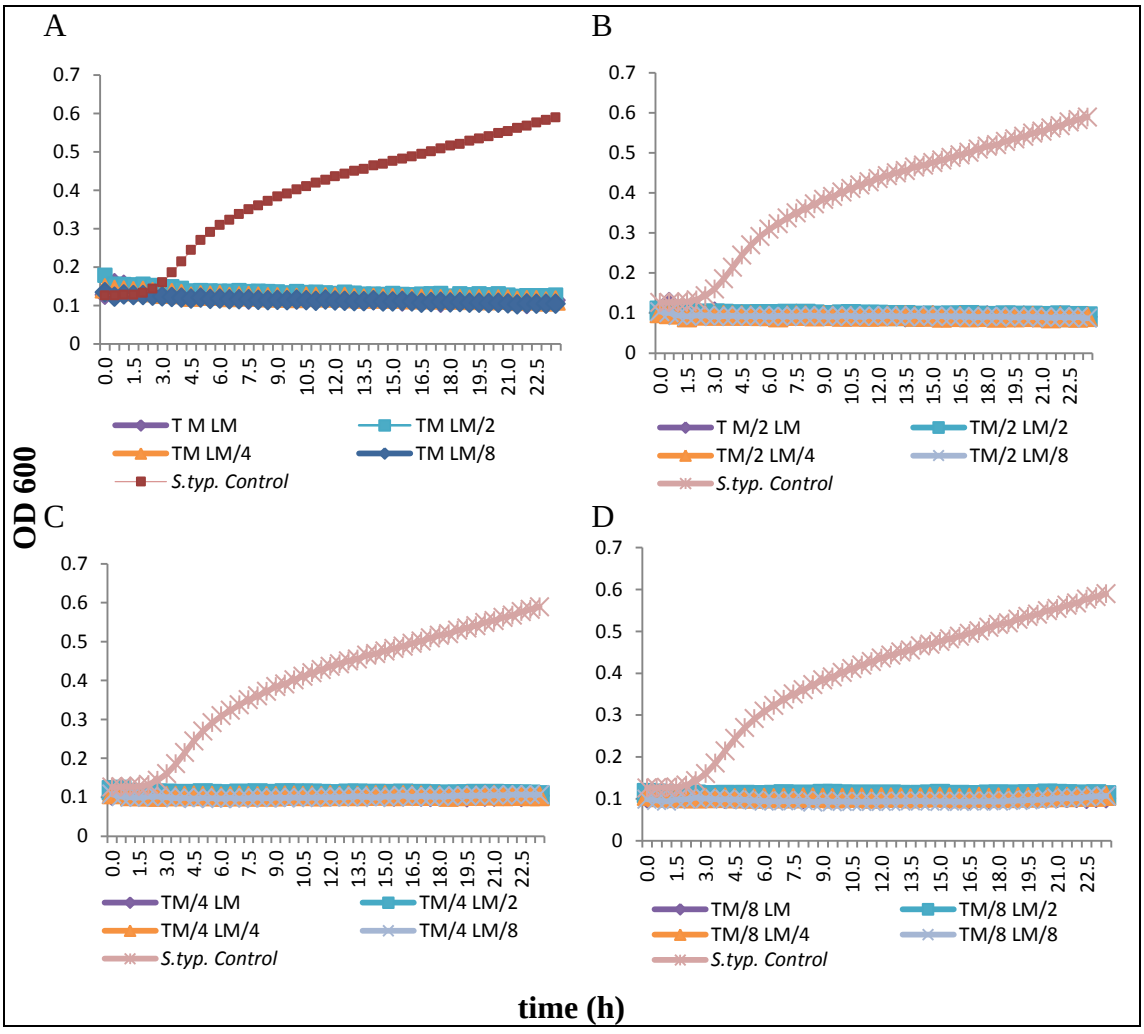


Figure 4.9. Inhibition of *S.* Typhimurium treated with α-terpineol/linalool (T/L) combination at FIC index values

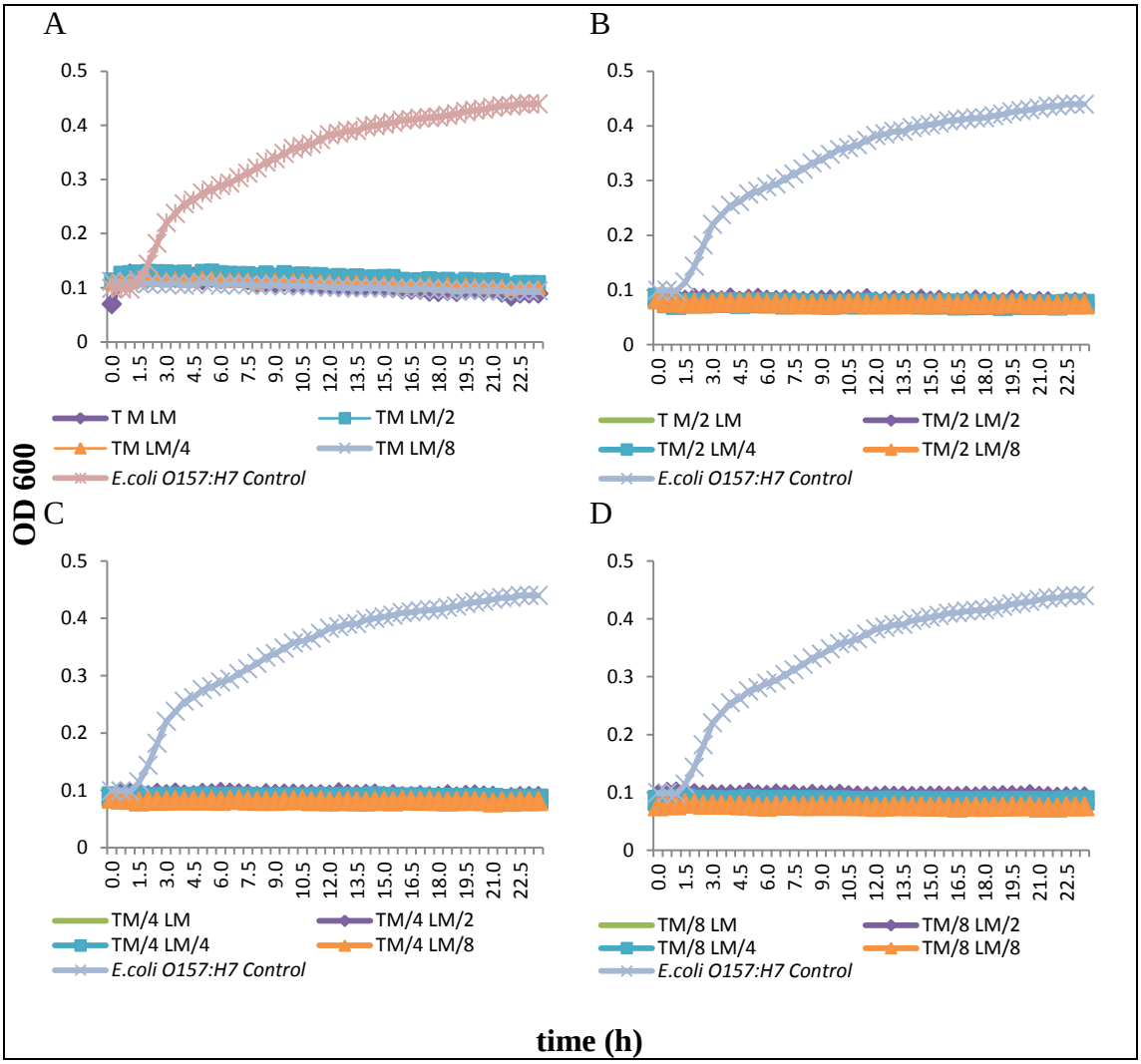


Figure 4.10. Inhibition of *E.coli* O157:H7 treated with α-terpineol/linalool (T/L) combination at FIC index value

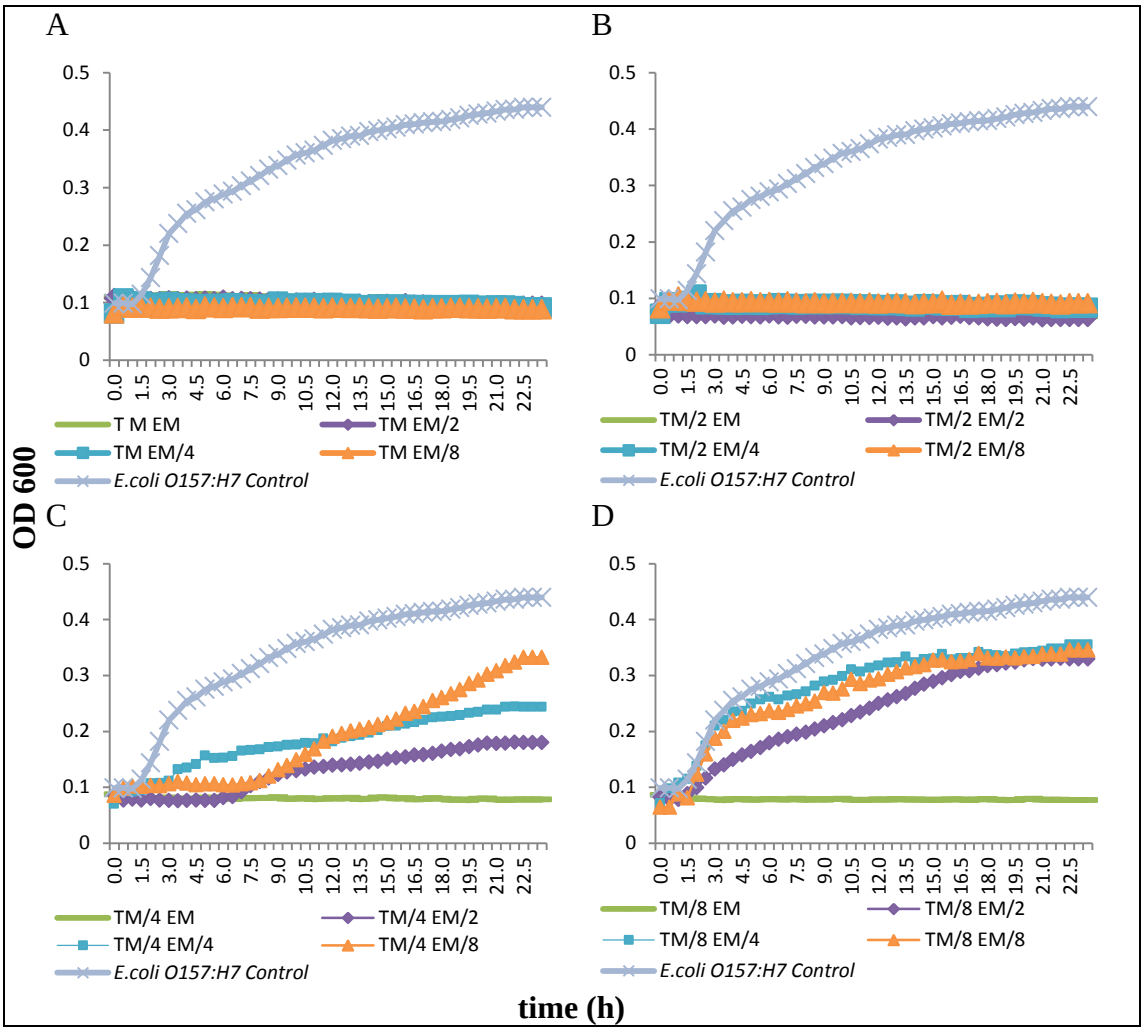


Figure 4.11. Inhibition of *E.coli* O157:H7 treated with α-terpineol/eucalyptol (T/E) combination at FIC index values

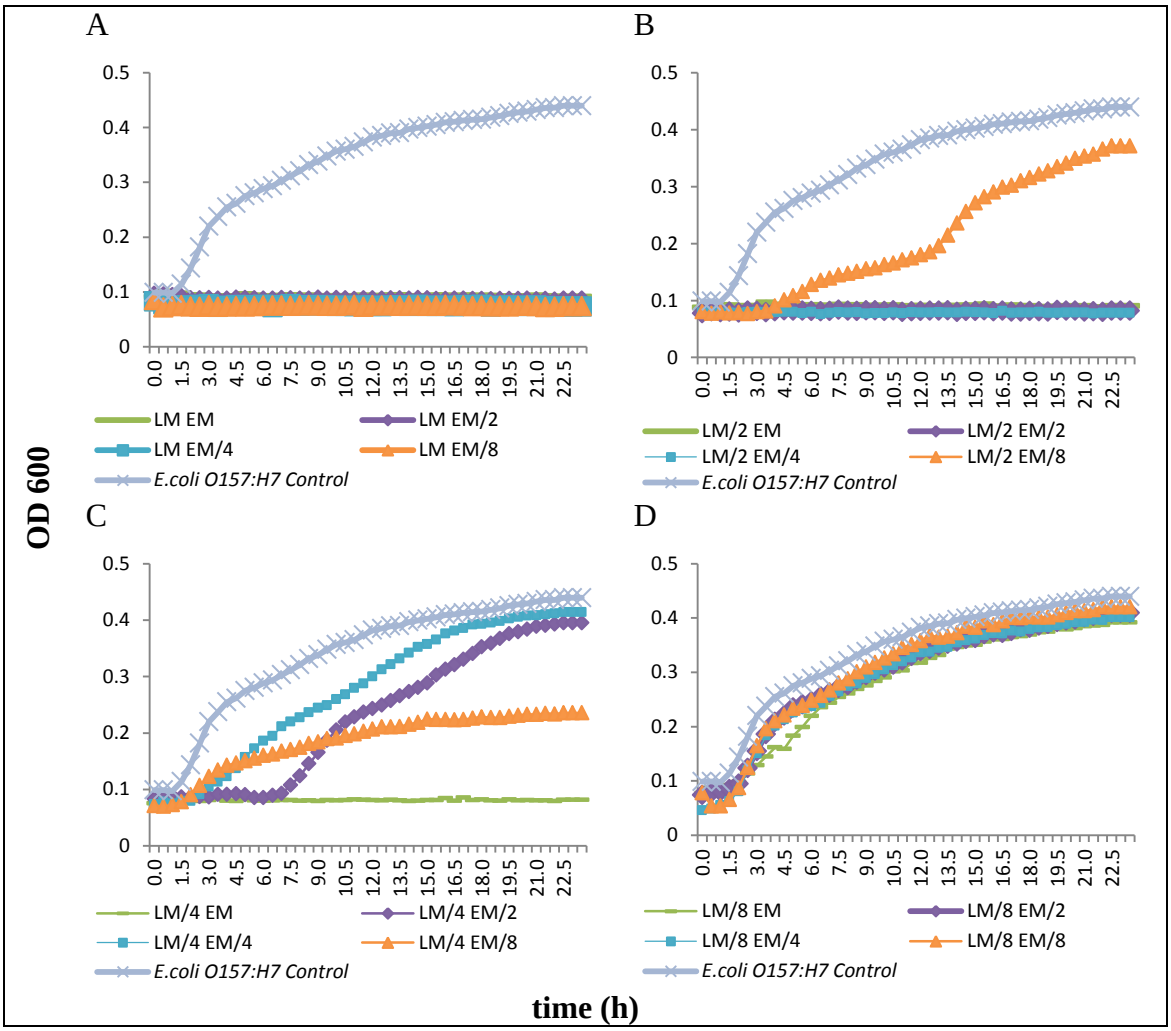


Figure 4.12. Inhibition of *E.coli* O157:H7 treated with linalool/eucalyptol (L/E) combination at FIC index values

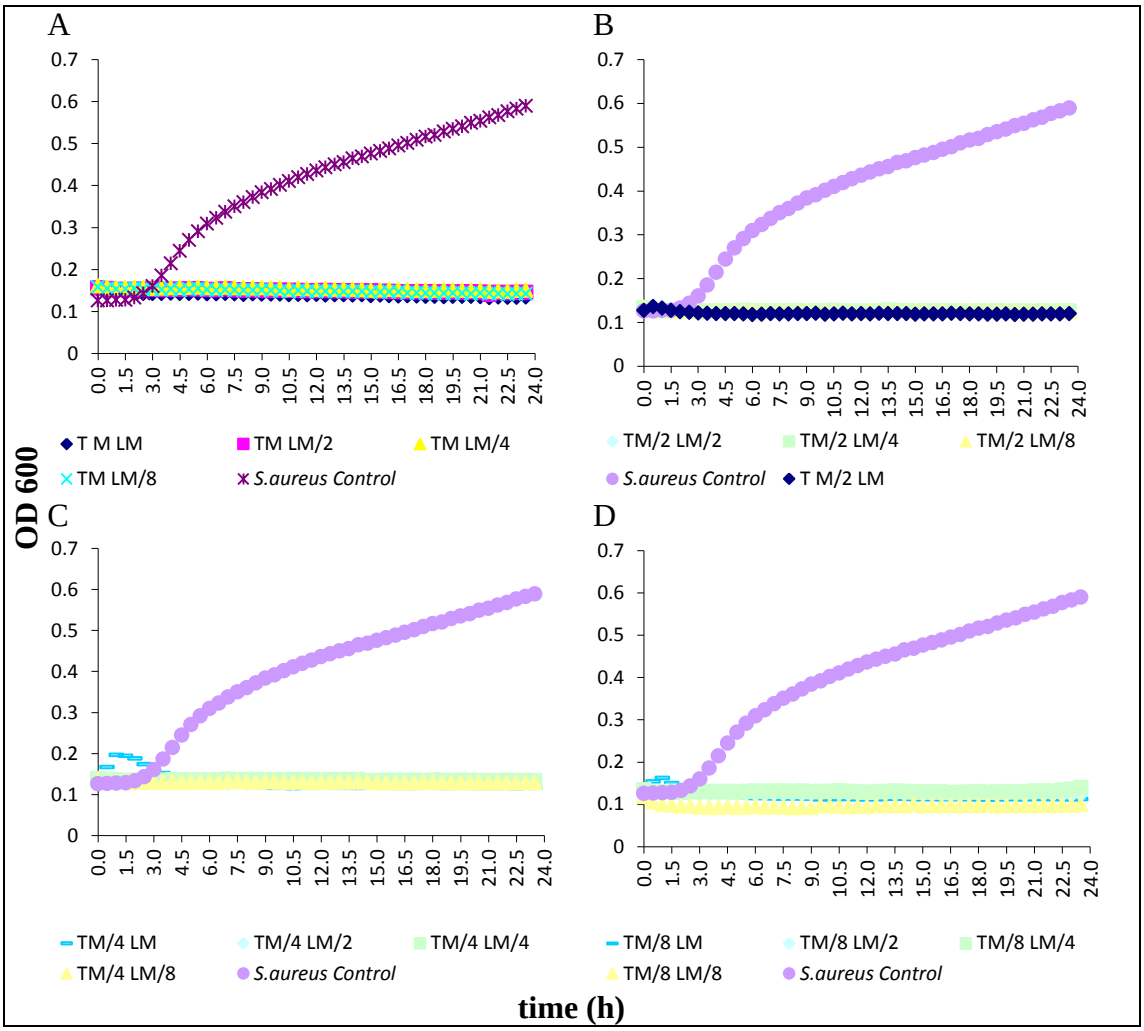


Figure 4.13. Inhibition of *S.aureus* treated with α-terpineol/linalool (T/L) combination at FIC index values

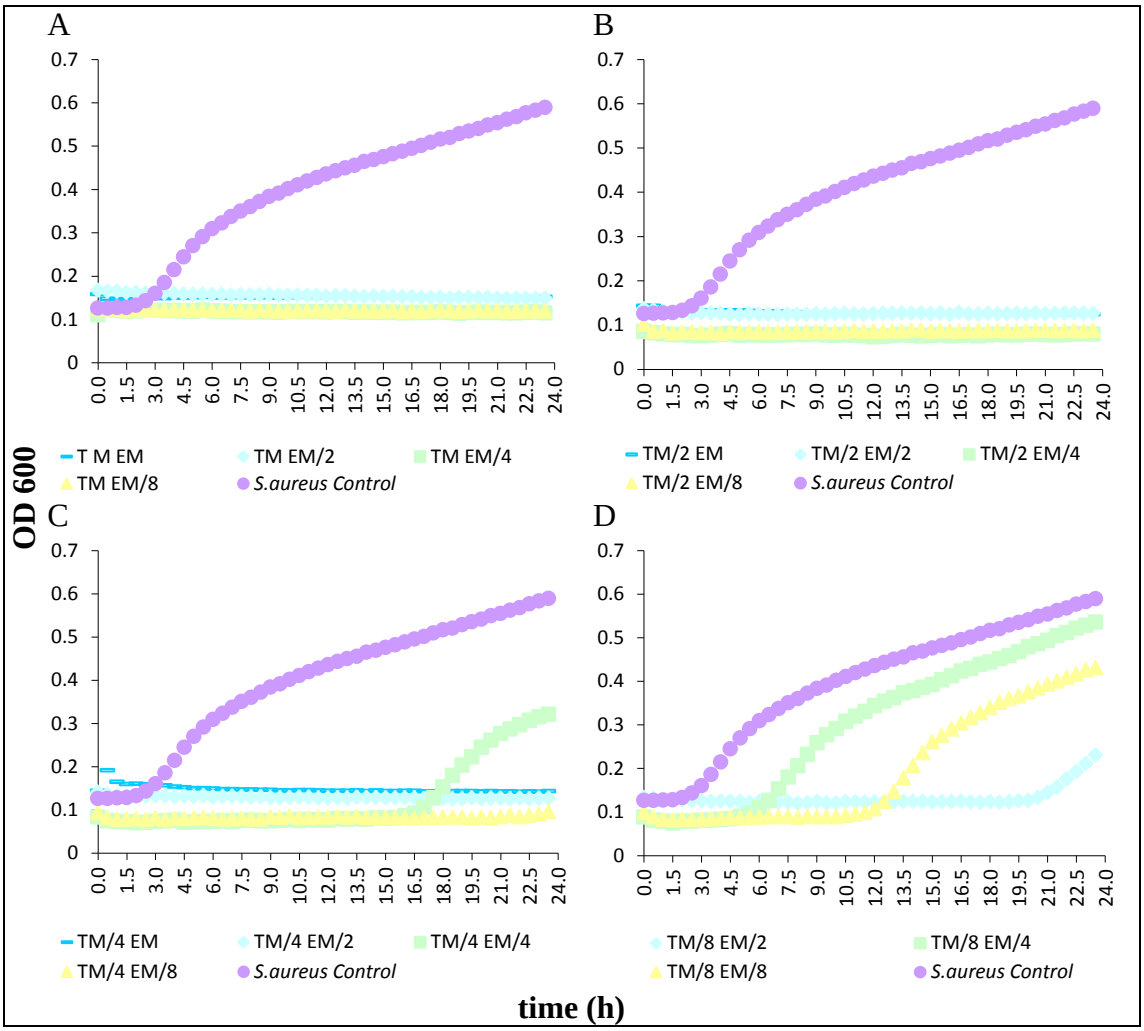


Figure 4.14. Inhibition of *S.aureus* treated with α-terpineol/eucalyptol (T/E) combination at FIC index values

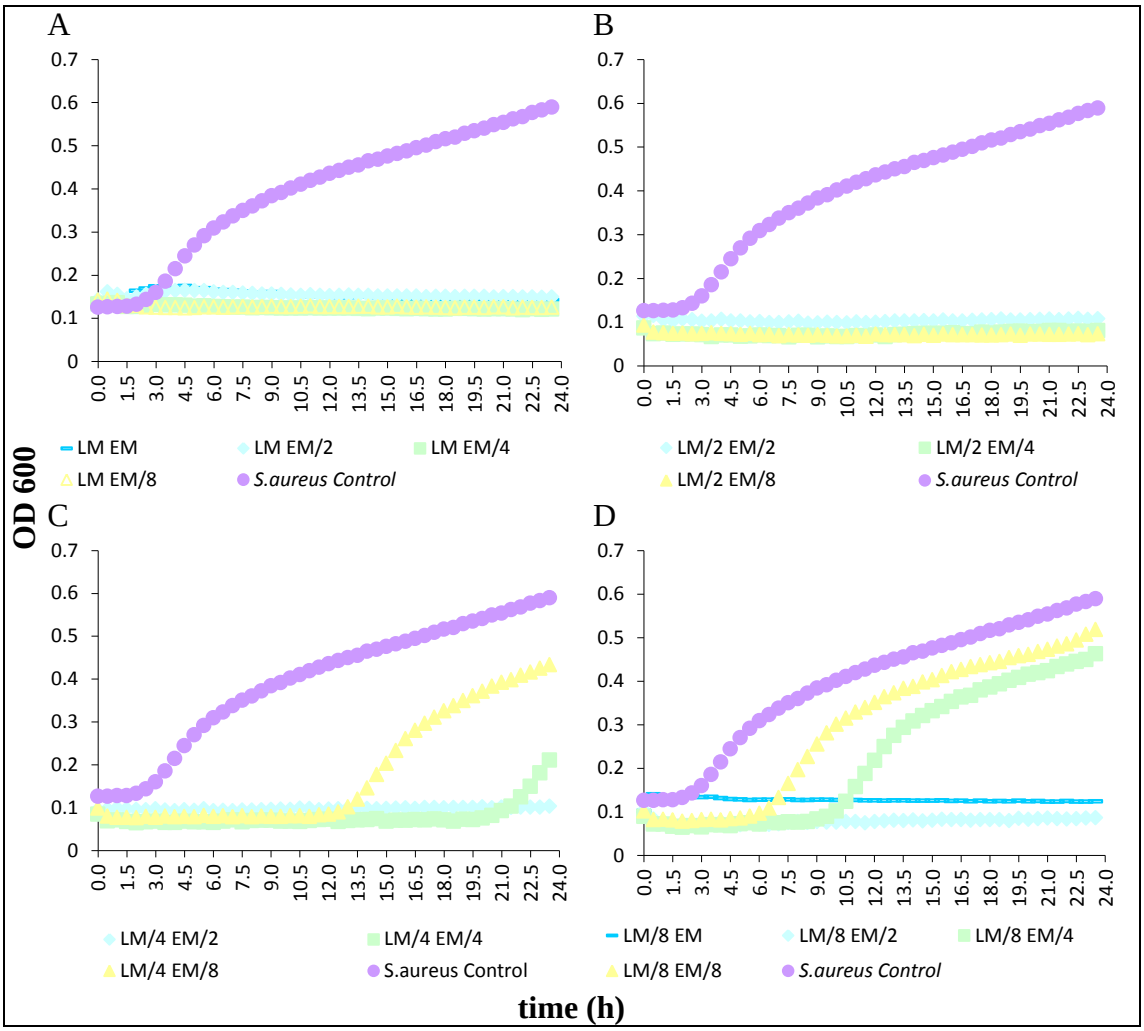


Figure 4.15. Inhibition of *S.aureus* treated with linalool/eucalyptol (L/E) combination at FIC index values

4.3. Investigation of Mechanism of Antimicrobial Activity

4.3.1. Structural Observations by Scanning Electron Microscope

Treatment with essential oils:

Pathogenic bacteria (*S.*aureus, *S.* Typhimurium, *E. coli* O157:H7) and also a represantative food spoilage bacterium (*Serretia liquefaciens*) were treated with relevant MICs for each and then incubated for 1 hours at appropriate incubation temperature and conditions. They were observed by SEM to investigate the morphological changes in the appearance of the cells. SEM observations confirmed the physical damage and considerable morphological alteration to all tested Gram-positive and Gram-negative bacteria treated with EOs.

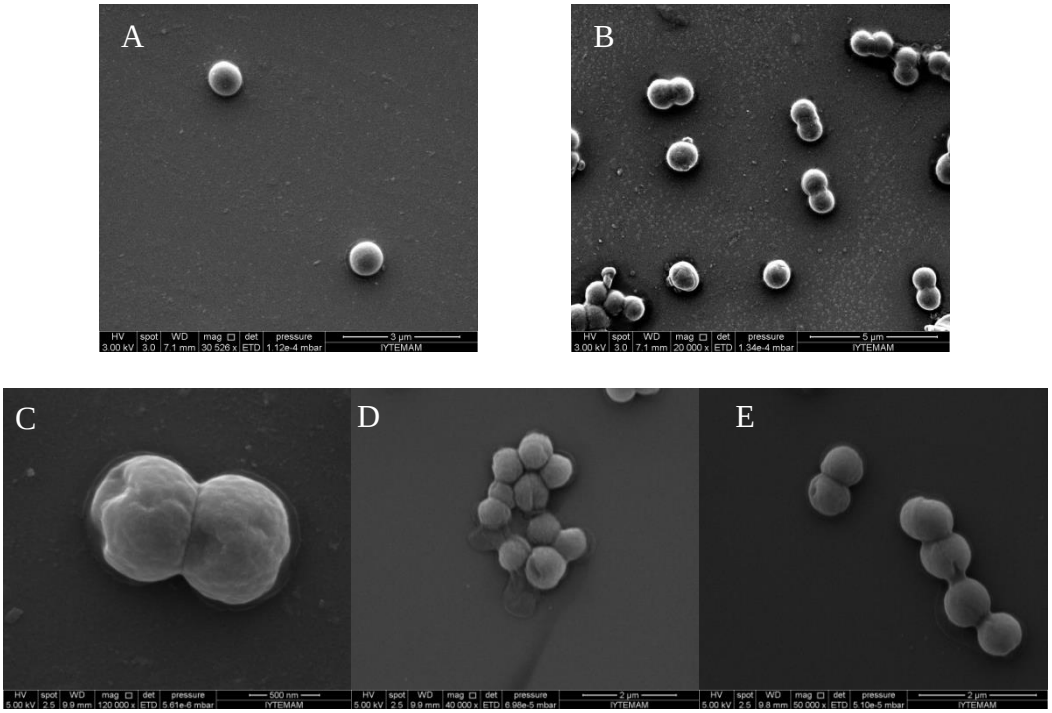


Figure 4.16. SEM micrographs of *S.aureus* cells: (A,B) untreated; (C) treated with BL EO; (D) treated with thyme EO ; (E) treated with clove EO

These images directly illustrate the destructive effects of the essential oils on the tested bacteria. Non-treated cells (control) were intact and showed a smooth surface (Fig. 4.16 (A,B); Fig. 4.17 (A); Fig. 4.18 (A); Fig. 4.19 (A)) while bacterial cells treated with the essential oils underwent considerable damage (Fig. 4.16 (C,D,E); Fig. 4.17 (B,C,D); Fig. 4.18 (B, C, D); Fig. 4.19 (B,C)). Although the samples were not prepared quantitatively, it was clearly observed that the number of the damaged cells was significantly greater in the treatments than in the control.

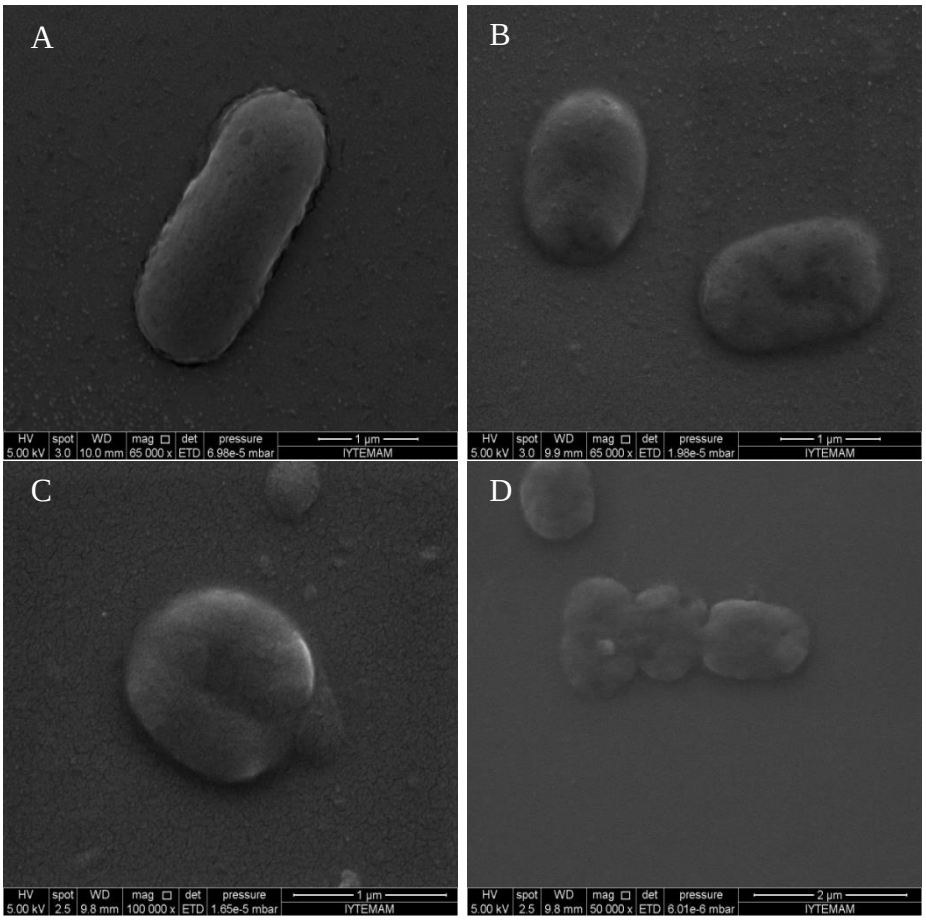


Figure 4.17. SEM micrographs of *S*. Typhimurium cells: (A) untreated; (B) treated with BL EO; (C) treated with thyme EO; (D) treated with clove EO

Most of the cells were observed to get clustered and stick to each other (Fig. 4.16 (C, D, E); Fig. 4.17 (C, D); Fig. 4.18 (B) and Fig. 4.19 (B, C)). The distortion of the cell physical structure would cause the expansion and destabilization of the membrane and would increase membrane fluidity, which in turn would increase passive permeability (Ultee et al., 2002) and manifest itself as a leakage of various vital intracellular constituents, such as ions, ATP, nucleic acids, and amino acids (Cox et al., 1998; Helander et al., 1998; Kim et al., 1995).

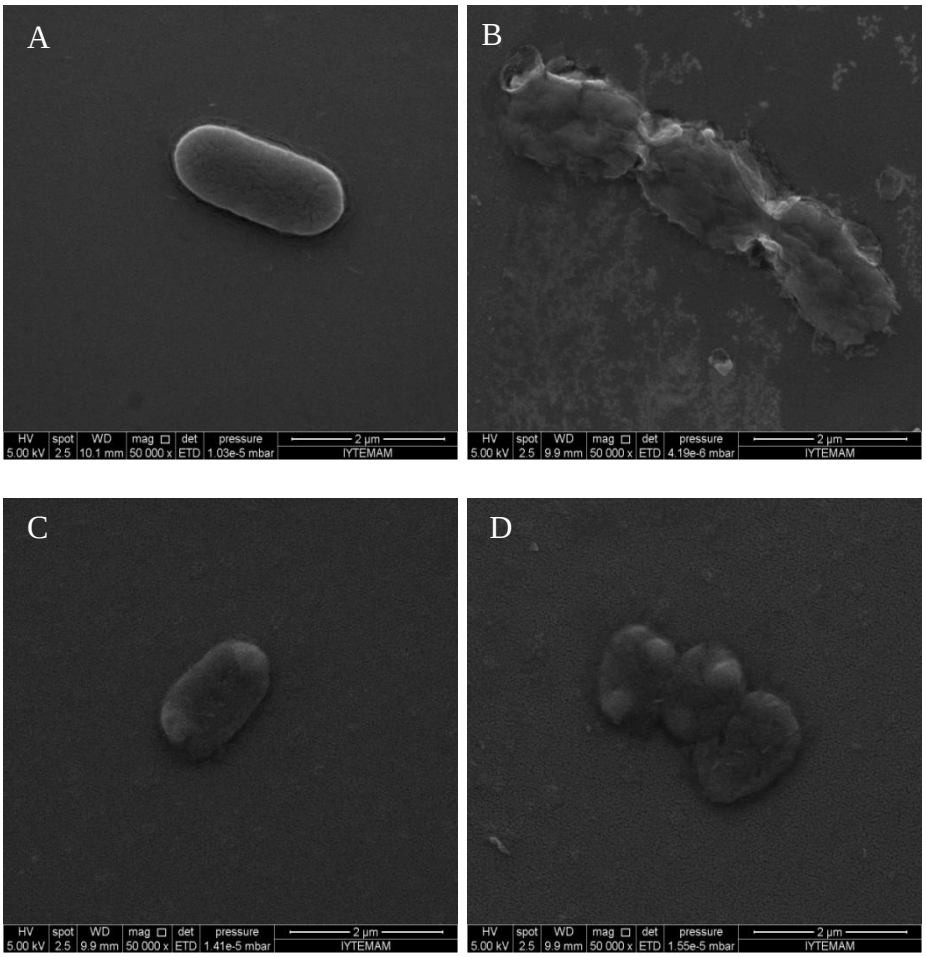


Figure 4.18. SEM micrographs of *E. coli* O157:H7 cells: (A) untreated; (B) treated with BL EO; (C) treated with thyme EO; (D) treated with clove EO

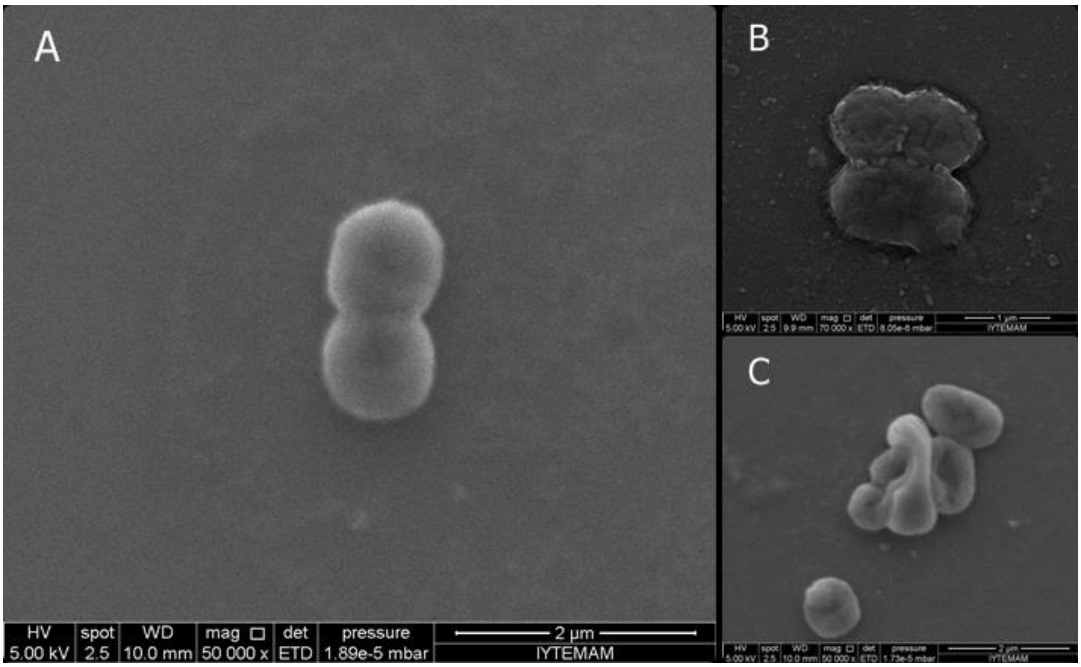
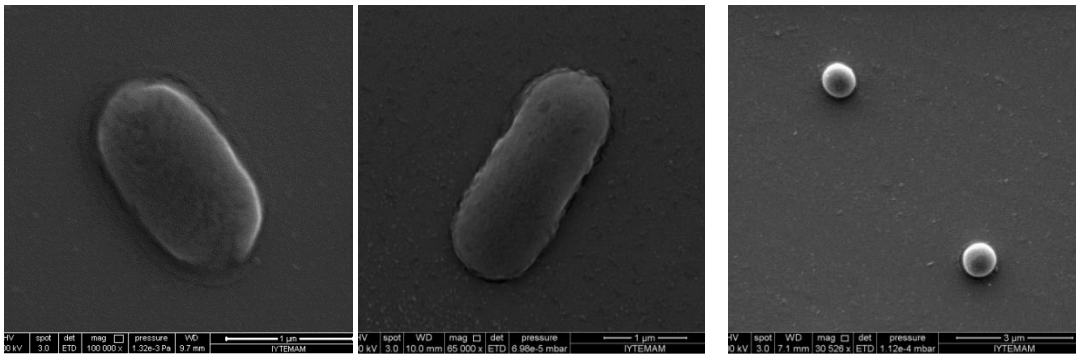


Figure 4.19. SEM micrographs of *Serretia liquefaciens* cells: (A) untreated; (B) treated with BL EO; (C) treated with thyme EO

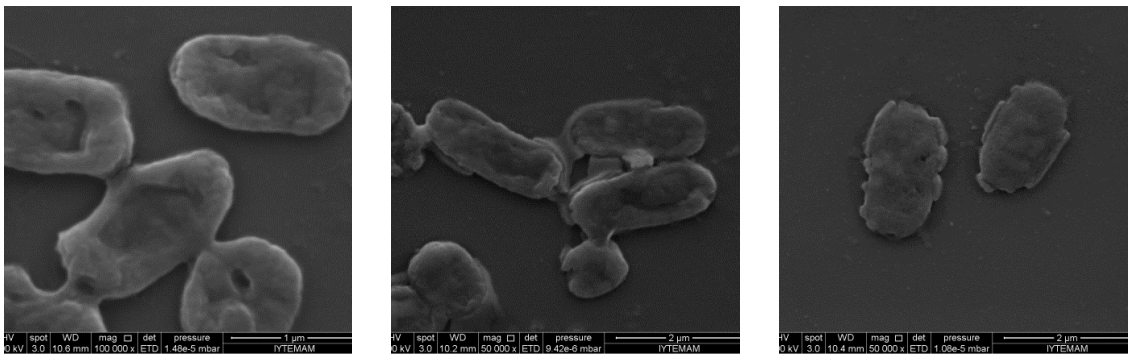
Treatment with essential oil components:

Pathogenic bacteria (*S.aureus, S.* Typhimurium, *E. coli* O157:H7) were treated for 2 h with each essential oil components (eucalyptol, α-terpineol and linalool) using relevant MIC values. They were then, observed by SEM to investigate the morphological changes in the appearance of the cells. SEM observations confirmed the physical damage and considerable morphological alteration to all tested Gram-positive and Gram-negative bacteria. Non-treated cells were intact and showed a smooth surface as it was shown in Fig. 4.20, while bacterial cells treated with the phenolics underwent considerable damage as it is obviously discriminated in Fig 4.21 and Fig 4.22.



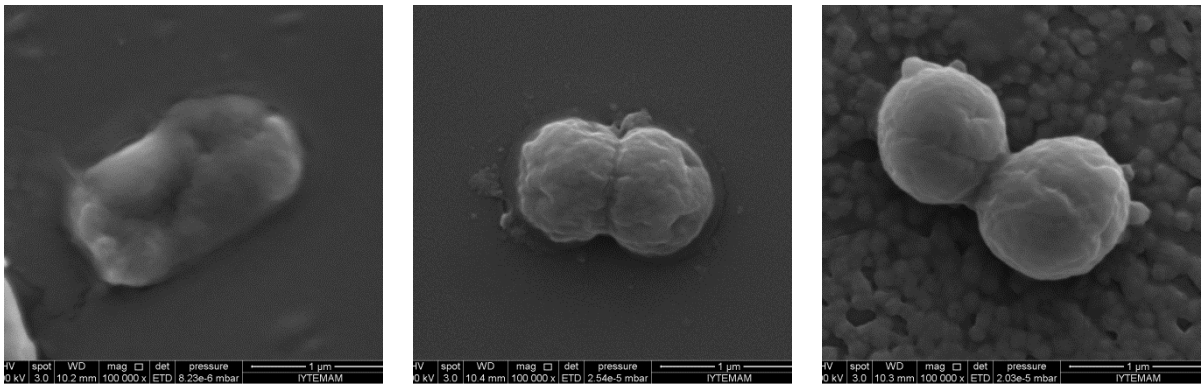
A B C

Figure 4.20. SEM micrographs of untreated bacterial cells of (A) *E.coli* O157:H7; (B) *S.* Typhimurium; (C) *S.aureus*



A B C

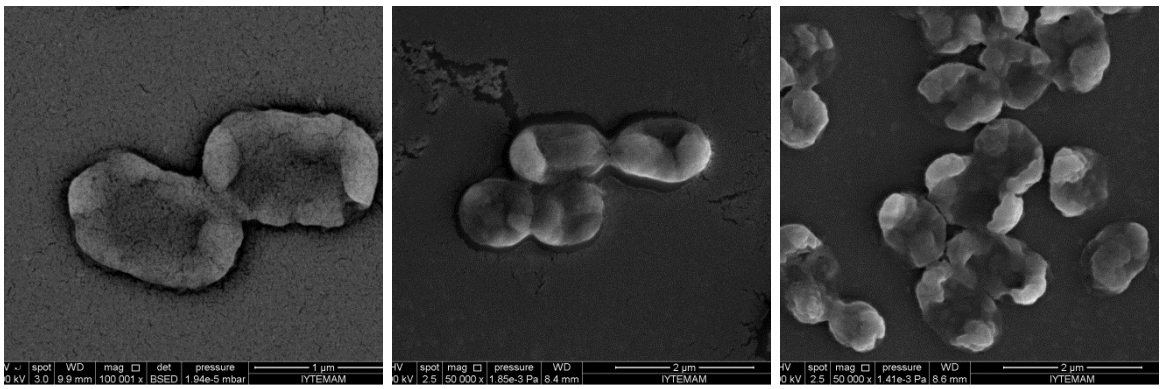
Figure 4.21. SEM micrographs of treated bacterial cells of *E.coli* O157:H7 (A) Eucalyptol; (B) Linalool; (C) α- terpineol



A B C

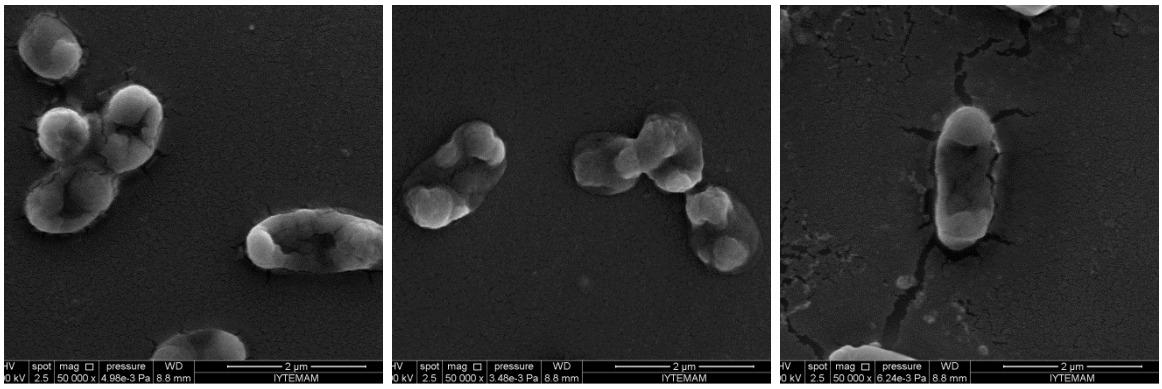
Figure 4.22. SEM micrographs of treated bacterial cells (A) S. Typhimurium ,Eucalyptol; (B) S.aureus, Linalool; (C) S.aureus, α- terpineol

After treatment and observation of bacterial cells with each component, FIC values determined by checkerboard assay were used to treat bacteria and observation with SEM. When SEM images of FIC treated bacteria and component treated ones, it was observed that FIC treated bacteria were highly damaged. Cells treated with components at FIC concentrations revealed severe damaging effect on the cell morphology of the tested pathogens, showing large surface collapse and abnormal cell breaking, as well as complete lysis or dead cell formation Fig. 4.23, Fig. 4.24, Fig. 4.25.



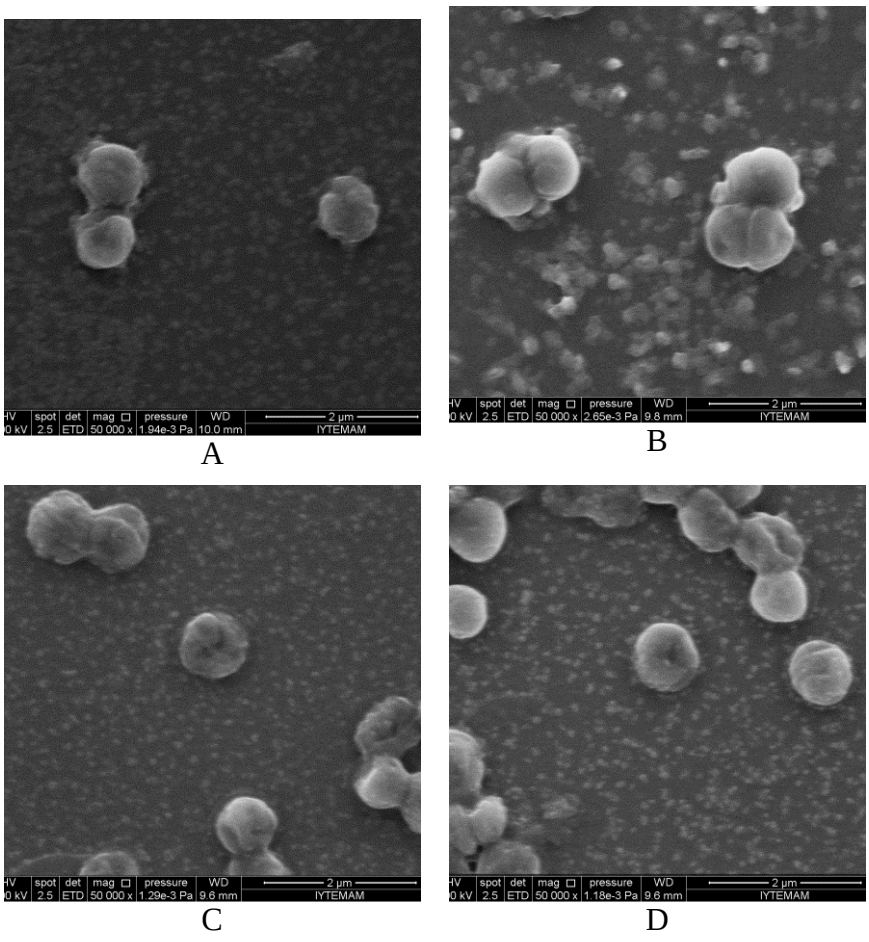
A B C

Figure 4.23. SEM micrographs of treated *E.coli* O157:H7 cells with FIC values (A)T/L; (B) T/E; (C) L/E



A B C

Figure 4.24. SEM micrographs of treated *S.* Typhimurium cells with FIC values (A)T/L; (B) T/E; (C) L/E



A B C D

Figure 4.25. SEM micrographs of treated *S.aureus* cells with FIC values (A) T/L additive; (B) T/L synergistic; (C) L/E; (D) T/E

There are many possible explanations for the observations. Some authors have suggested that the damage to the cell wall and cytoplasmic membrane was the loss of structural integrity (de Billerbeck et al., 2001; Filipowicz et al., 2003; Packiyasothy et al., 2002). The literature suggests that the active components of the plant extracts might

bind to the cell surface and then penetrate to the target sites, possibly the phospholipid bilayer of the cytoplasmic membrane and membrane-bound enzymes (Shan et al., 2007). In addition to interacting with membrane phospholipids, interaction with membrane proteins and intracellular targets is also suggested (Hyldgaard et al., 2012). The effects might include the inhibition of proton motive force, inhibition of the respiratory chain and electron transfer, and inhibition of substrate oxidation. Uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites, and disruption of synthesis of DNA, RNA, protein, lipid, and polysaccharides might follow (Farag et al., 1989; Kim et al., 1995).

These images confirm the loss of shape and integrity which was followed by the cell death. Cell death may have been the result of the extensive loss of cell contents, the exit of critical molecules and ions, or the initiation of autolytic processes (Denyer, 1990).

4.3.2. Structural Observations by Atomic Force Microscope

Bacteria were treated for 2 h with essential oils (bay leaf, thyme and clove) and then examined with AFM to observe the topological changes of the cell walls.

As shown in Fig. 4.26 (A) untreated *E.coli* O157:H7 cells had smooth surfaces with distinct lipopolysaccharide (LPS) bundles typical of Gram-negative bacteria (Cui et al., 2012). When treated with essential oils they displayed collapsing with the LPS bundles no longer visible (Fig. 4.26 B, C, D). After 2 h treatments they get clustered and stick to each other.

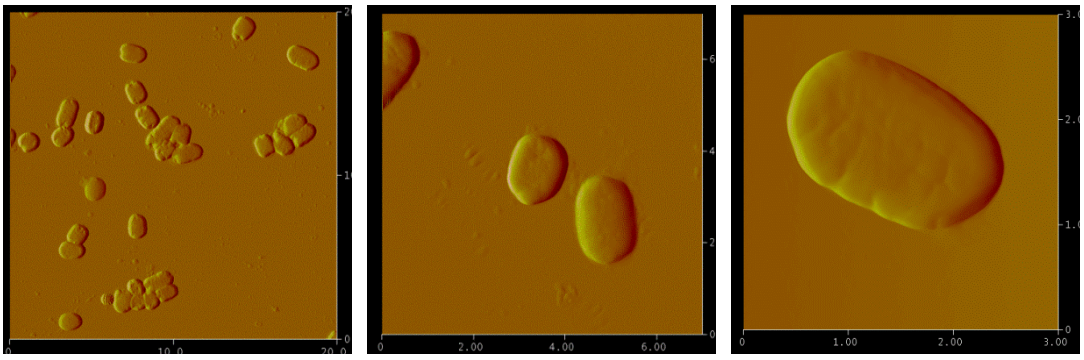
A 

Figure 4.26. AFM images of *E. coli* O157:H7 cells (A) untreated; (B) treated with bay leaf EO; (C) treated by thyme EO; (D) treated by clove EO

(**cont. on next page**)

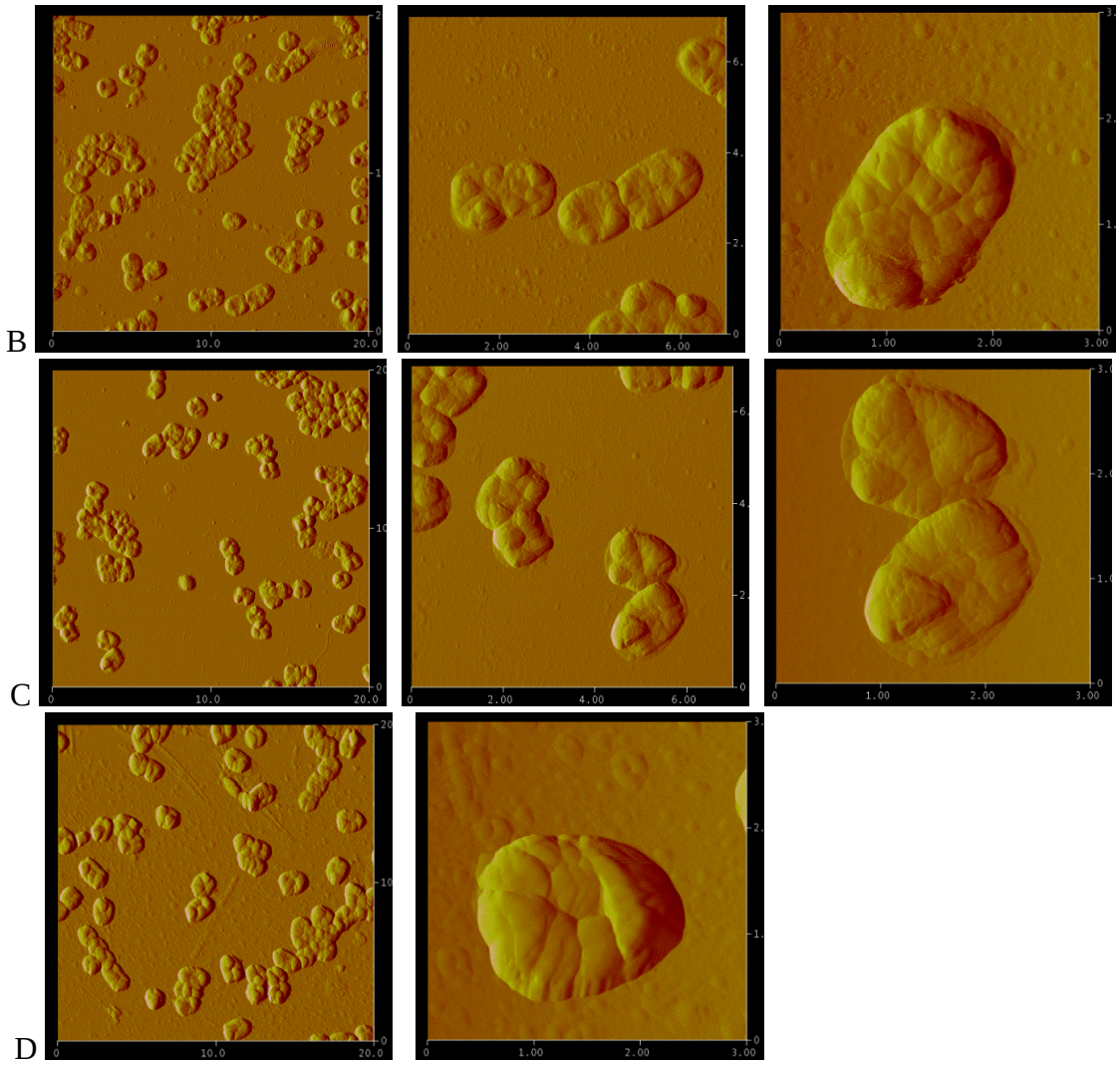


Figure 4.26. (cont.)

Untreated *S.*Typhimurium bacterial cells had also smooth and intact surfaces (Fig. 4.27 A). After treatment with essential oils, severe damages were observed on the surface of cells. Bay leaf and clove EO treated cells (Fig. 4.27 B, D). were obviously collapsed whereas thyme EO treated cells showed abnormal damages and intensive clusters (Fig. 4.27 C).

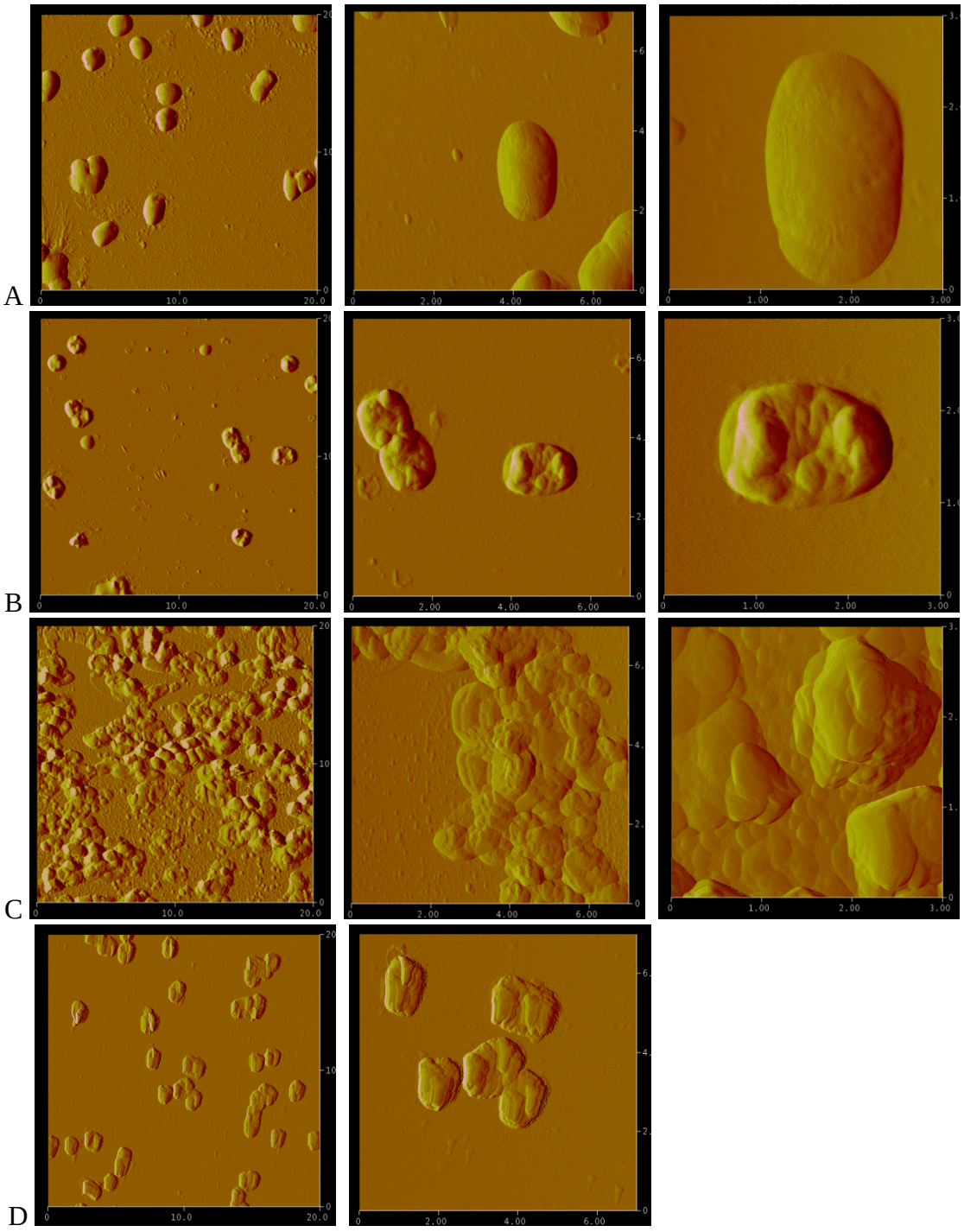


Figure 4.27. AFM images of *S.* Typhimurium cells (A) untreated; (B) treated with bay leaf EO; (C) treated by thyme EO; (D) treated by clove EO

Untreated *S. aureus* cells also showed sleek surface (Fig. 4.28 A). When they were exposed to bay leaf and thyme EOs, the surfaces were severely destructed and cells stuck to each other (Fig. 4.28 B, C). Clove EO induced the formation of grooves on the cell walls of *S. aureus* (Fig. 4.28 D).

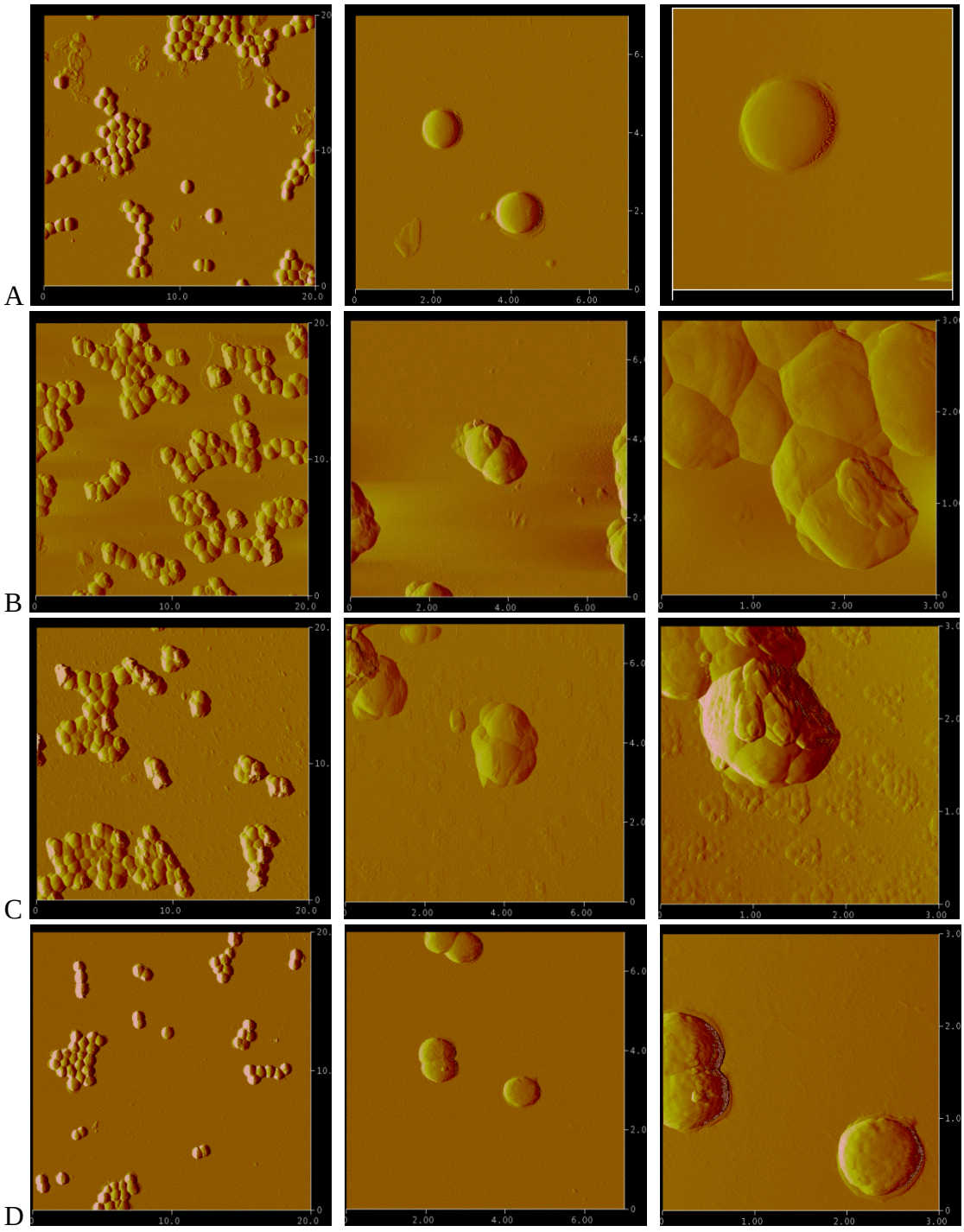


Figure 4.28. AFM images of *S. aureus* cells (A) untreated; (B) treated with bay leaf EO; (C) treated by thyme EO; (D) treated by clove EO

4.3.3. Release of Cellular Material

Treatment with essential oils:

The release of cell constituents was determined by the measurement of the absorbance at 260 nm of the supernatant of three tested strains. Table 4.11 shows the

results when *S. aureus, E. coli* O157:H7 and *S.* Typhimurium were treated with EOs for 1-2 h, respectively. The results indicated that after adding the corresponding EOs to strains, the cell constituents' release increased visibly with the increased concentration of the EOs compared to the control group. The maximum cell constituents' release was observed when bacteria were treated with clove EO at the concentration of 2MIC. The general view is clove EO cause more release of cellular material from the bacteria. An important loss of cell constituents means that irreversible damage to the cytoplasmic membranes occurred, which is in accordance with the results of SEM.

Table 4.11. The effect of essential oils on cell constituents' release of the tested bacteria

Microorganism	**EO Concentration**	**OD260 nm for BL EO**	**OD260 nm for Thyme EO**	**OD260 nm for Clove EO**
Staphylococcus aureus	0	0.033±0.001	0.033±0.001	0.059±0.001
	MIC	0.099±0.010	0.105±0.034	0.309±0.009
	2MIC	0.122±0.017	0.070±0.003	0.383±0.023
E.coli O157:H7	0	0.018±0.01	0.018±0.01	0.046±0.016
	MIC	0.119±0.01	0.072±0.014	0.312±0.007
	2MIC	0.136±0.017	0.126±0.003	0.423±0.010
Salmonella Typhimurium	0	0.030±0.0	0.030±0.0	0.037±0.021
	MIC	0.131±0.006	0.117±0.014	0.307±0.021
	2MIC	0.231±0.015	0.119±0.021	0.434±0.004

Treatment with essential oil components:

Another strategy for determining the mode of action of essential oil components against tested bacteria was performed on the basis of release of 260-nm and 280-nm absorbing materials from the treated cells.

The cell constituents' release was determined by the measurement of the absorbance at 260 nm and 280 nm of the supernatant of three tested strains. The Figure 4.29 shows the results when *S. aureus, E. coli* O157:H7 and *S.* Typhimurium were treated with esential oil components at FIC values for 2 h, respectively. The results indicated that after exposure to the corresponding phenolics, the cell constituents' release increased visibly compared to the control group.

When *E. coli* O157:H7 cells were treated with α-terpineol and eucalyptol combination at FIC value of additive effect, the highest release of 260-nm and 280-nm absorbing materials was observed (Fig. 4.29 A). The treatment with α-terpineol and

linalool at FIC value of additive effect was resulted in higher 280-nm absorbing materials than 260-nm. Whereas, treatment with synergistic effect FIC value caused lower 280-nm absorbing materials than 260-nm. Linalool and eucalyptol combination led to nearly the same release for both 260-nm and 280-nm absorbing materials.

The release of cellular material from *S.* Typhimurium were nearly the same for both 260-nm and 280-nm absorbing materials for the treatments with α-terpineol and linalool at FIC value of additive and synergistic effects (Fig. 4.29 B). α-terpineol and eucalyptol combination caused a higher 280-nm absorbing material than 260-nm.

S. aureus cells showed higher release for 260-nm absorbing material than 280-nm for the treatment of α-terpineol and eucalyptol combination (Fig. 4.29 C). Whereas, 280-nm absorbing material seemed to be higher than 260-nm for α-terpineol and linalool combination. The other combinations led to the nearly the same amount of release for both 260-nm and 280-nm absorbing material. It was also observed that the 280-nm absorbing material releases were nearly the same for all combination pairs.

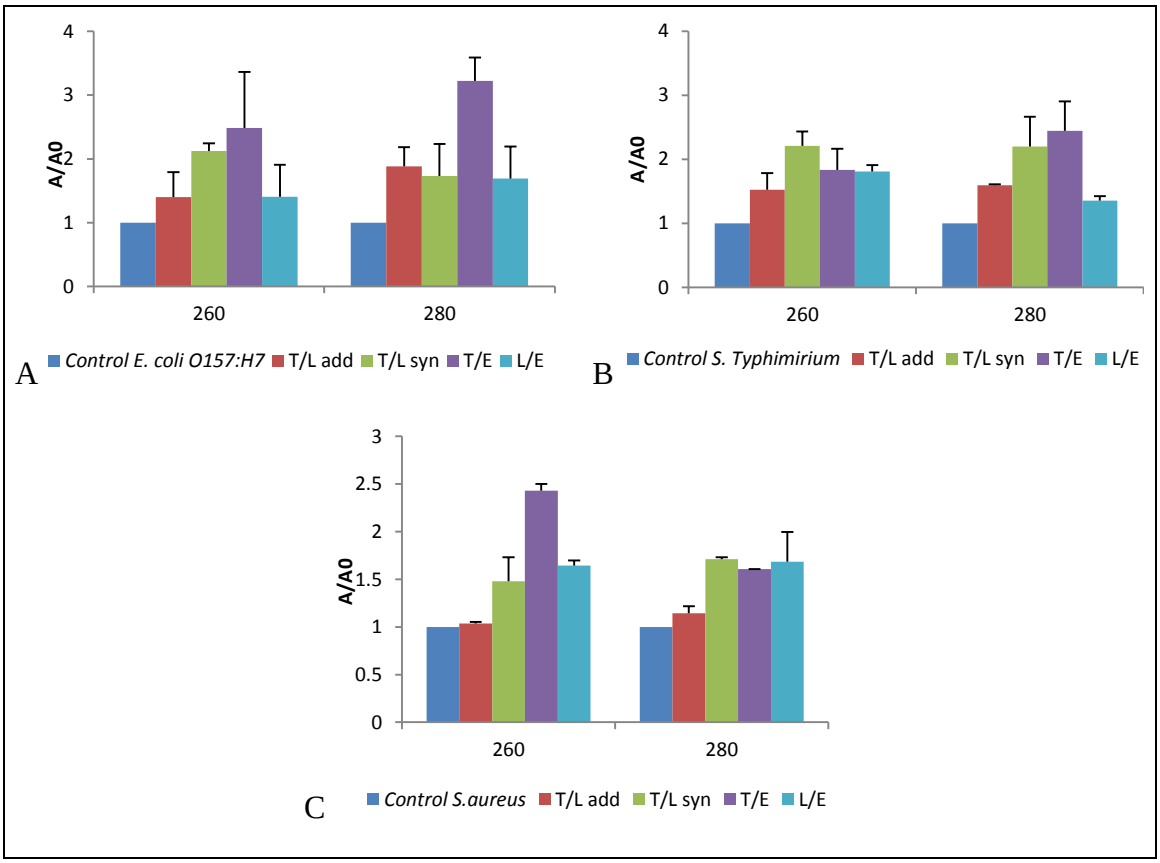


Figure 4.29. Release of cellular material at 260-nm and 280-nm for E. coli O157:H7, S. Typhimurium and S.aureus cells treated with combinations at FIC values

4.3.4. FTIR Observation

Bacterial strains were grown with or without essential oils with 2 MIC values for 4 h and examined by FT-IR spectroscopy. The results presented in Fig. 4.30 shows FTIR spectra of both control untreated and essential oil-treated *S.* Typhimurium, Fig. 4.31 shows FTIR spectra of both control untreated and essential oil-treated *S. aureus* and Fig. 4.32 shows FTIR spectras of *E. coli* O157:H7. For both treated and untreated bacteria, the dominant amide I bands at ~1650 cm^{-1} was attributed to the C=Ostretching vibrations of amide groups of proteins (Huleihel et al., 2009).

S. Typhimurium showed only a different spectrum when it was treated with clove EO for 4h. The band between 1310-1240 cm^{-1} was assigned to the amide III band components of proteins (Al-Qadiri et al., 2008; Alvarez-Ordóñez et al., 2011). There are also differences between clove EO treated and control in the region between 1300-900 cm^{-1} is characterized by vibrational features of cellular proteins, nucleic acids, cell membrane and cell wall components, the main bands in this region include the antisymmetric (~1242 cm^{-1}) and symmetric (~1080 cm^{-1}) P=O stretching modes of the phosphodiester backbone of nucleic acids and the C-O-C stretching vibrations of polysaccharides (1200-900 cm-1) in the cell wall peptidoglycan layer and lipopolysaccharide outer leaflet (Al-Qadiri et al., 2008; Elzinga et al., 2012).

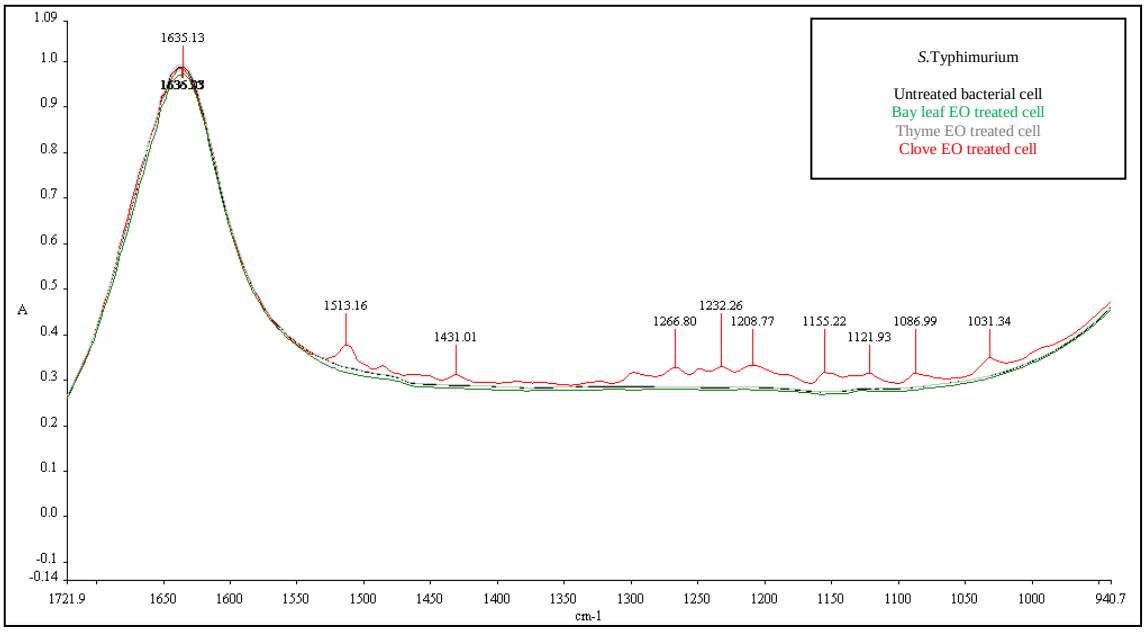


Figure 4.30. FTIR spectra of S. Typhimurium after 4 h growth with or without essential oils

S.aureus was also represented similar FTIR spectral pattern with clove EO treated *S.* Typhimurium cells, when it was treated with 2 MIC clove EO (Fig. 4.31). There are differences between treatments in the region between 1300-900 cm^{-1}, indicating nucleic acid denaturation associated with the P=O antisymmetric stretching mode of the phosphodiester backbone of nucleic acids (~1242 cm^{-1}) and effects on nucleic acid ribose or deoxyribose structure as observed in the difference between treatments in the P=O symmetric stretching mode (~1080 cm^{-1}). Clove EO may also affect the structure of bacterial envelope polysaccharides as observed by differences in C-O-C stretching vibrations (~1100-950 cm^{-1}).

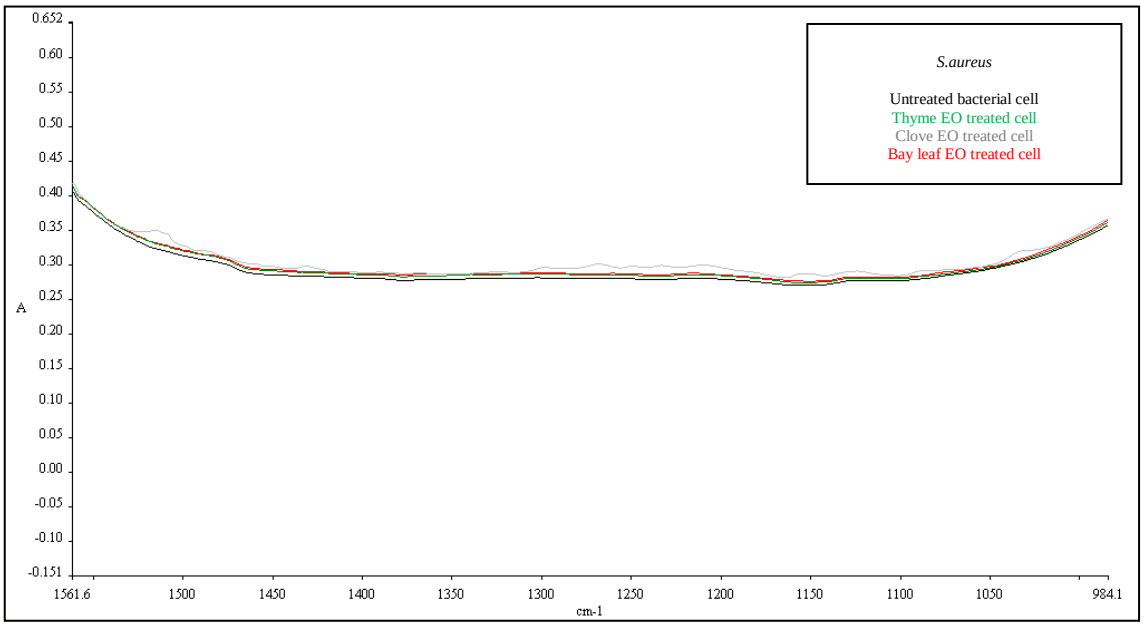


Figure 4.31. FTIR spectra of *S. aureus* after 4 h growth with or without essential oils

As it is indicated in the Figure 4.32, there were no significant differences between the FTIR spectra between the treated and untreated *E.coli* O157:H7.

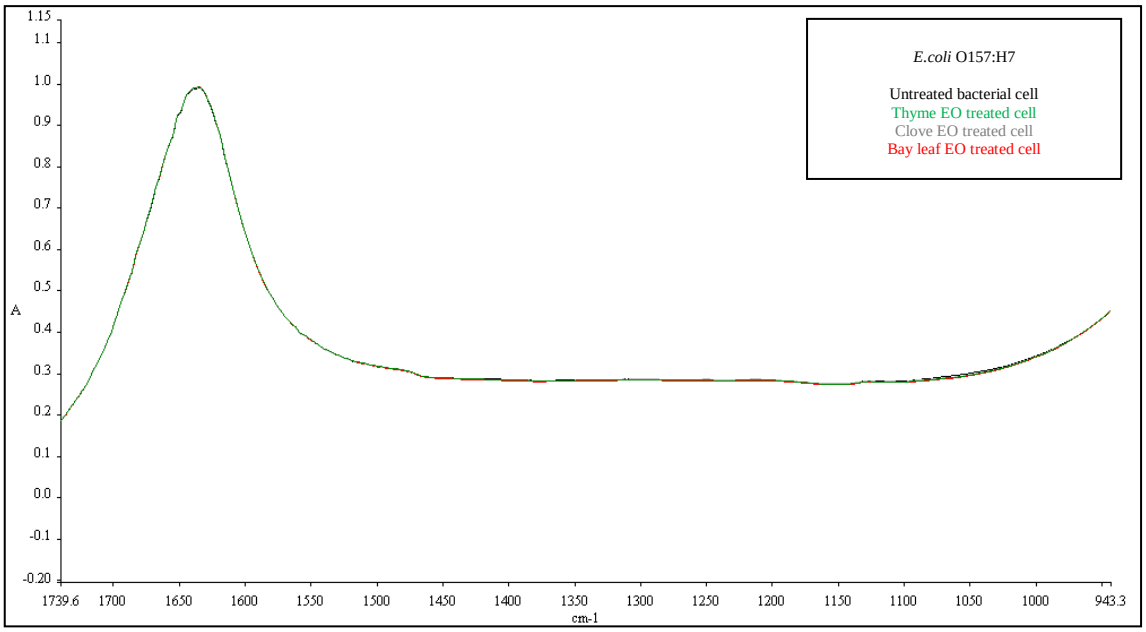


Figure 4.32. FTIR spectra of E.coli O157:H7 after 4 h growth with or without essential oils

4.3.5. Measurement of Bacterial Membrane Potential

Bacterial membrane potential has a critical role in bacterial physiology. Membrane potential of metabolically active bacteria is generated by a difference electrical potential across the membrane, with the interior negative between 100 and 200 mV with respect to the exterior. This electrical potential is intended to resting potential. A reduction in the magnitude of the membrane potential is referred to as electrical depolarization, whereas, an increase in the magnitude of membrane potential is referred to as electrical hyperpolarization. When the membrane integrity is destroyed; membrane potential is reduced and membrane becomes permeable (Cao et al., 2011; Novo et al., 1999).

Figure 4.33 shows the red-versus-green fluorescence dot plots of *S.aureus* for unstained cells, control cells stained with $DiOC_2$, depolarized cells with CCCP and also cells treated with essential oils with MIC and 2MIC vales. The change of the membrane potential was expressed as Mean Fluorescence Intensity (MFI) of the red population divided by the green population. Treatment with all essential oils was resulted in a reduction in fluorescent ratio of red/green similar to depolarized samples as compared with untreated cells. Even essential oils had almost the same effect, bay leaf EO

showed a lower ratio as 1.182. The flow cytometric analysis results indicated that the permeability of bacterial membrane increased with the treatments with essential oils.

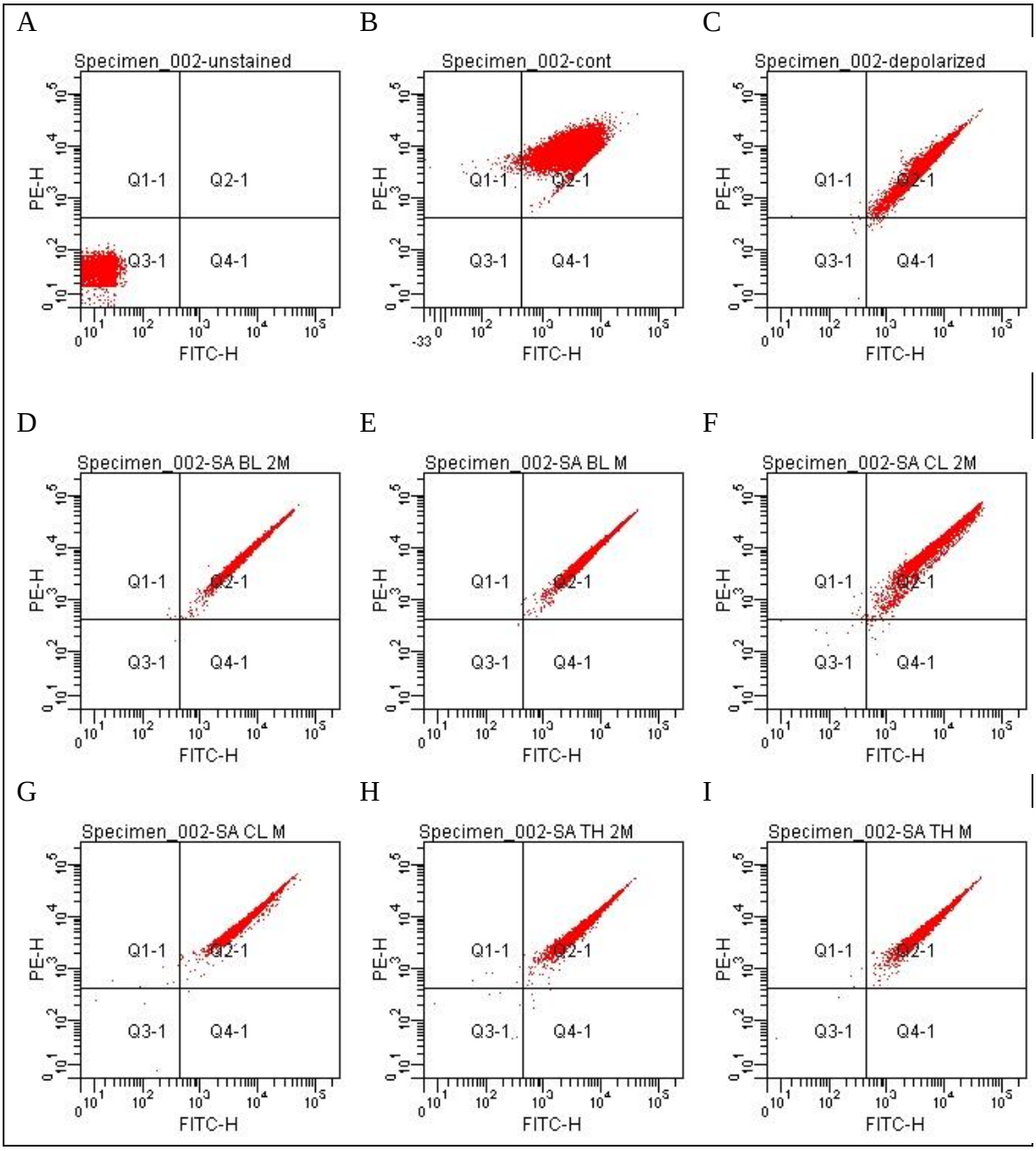


Figure 4.33. The red-versus-green fluorescence dot plots of S.aureus A, unstained; B, stained-control; C, depolarized-CCCP; D, treated with 2MIC bay leaf EO; E, treated with MIC bay leaf EO; F, treated with 2MIC clove EO; G, treated with MIC clove EO; H, treated with 2MIC thyme EO; I, treated with MIC thyme EO

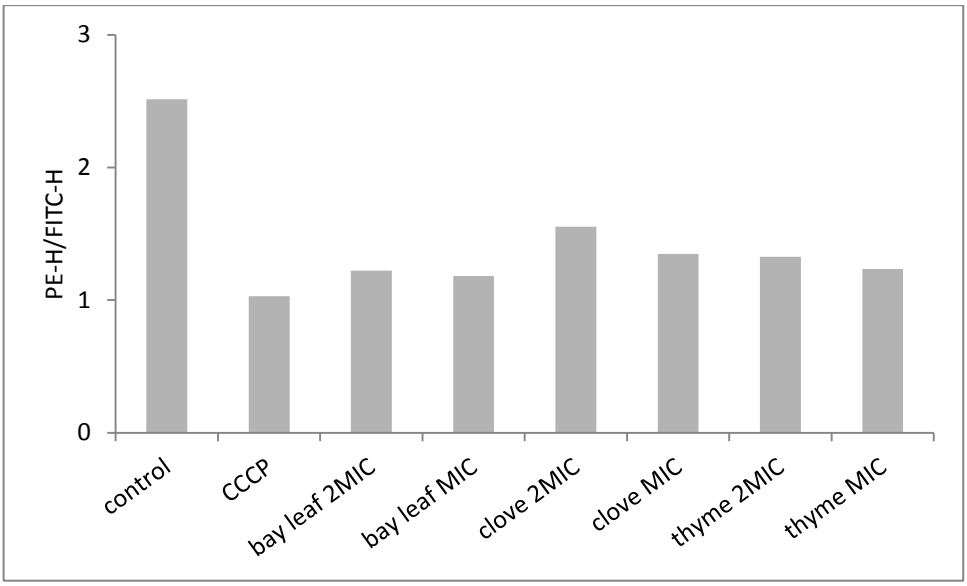


Figure 4.34. Detection of membrane potential of essential oils against *S.aureus* cells. The ratio of red to green (PE-H/FTC-H) fluorescence intensity of $DiOC_2$

4.4. Antioxidant Activity

4.4.1. Total Phenol Content

Total phenolic contents of essential oils and extracts were determined by Folin-Ciocalteu assay. Results were calculated by using the equation obtained from the gallic acid calibration curve (Fig. B.1) and expressed as GAE (mg/ml). Total phenolic content (TPC) of essential oils and extracts are presented in Table 4.12. As it was seen from the table, clove essential oil had the highest content of TPC with 635.327 mg GAE/ml. Thyme and cumin EOs were seen to be a less rich source of total phenols, while bay leaf EO showed the lowest amount of phenolic. The results also indicated that plant extracts had lower TPC when compared with essential oils.

Table 4.12. The total phenolic content (TPC) of essential oils and extracts

Sample	**Total phenolic (mg GAE /ml)**
Bay leaf essential oil	13.522±1.128
Thyme essential oil	30.5576±8.62
Cumin essential oil	35.862±5.54

(**cont. on next page**)

Table 4.12 (cont.)

Clove essential oil	635.327±11.71
Grape seed extract	24.004±0.915
Olive leaf extract	20.547±0.637

Viuda-Martos et al. (2010), Wang et al. (2008) and also Gulcin et al. (2004) also demonstrated that clove bud EO had high phenolic content comparing with other EOs like thyme EO (Gülçin et al., 2004; Viuda-Martos et al., 2010; Wang et al., 2008). Thyme essential oil also contained more total phenols than bay leaf essential oil (Ozcan et al., 2009).

In a study that compared the total concentrations of phenolic compounds of grape and olive based natural extracts, it was demonstrated that grape extract had a higher phenolic contents than olive leaf extract as it is in correlation with our findings (Serra et al., 2008). The amounts of total phenolic contents in the present study were similar to reported findings with slight differences. These differences are not surprising considering the distinctions in extraction methods or solvents, the variety of samples, in terms of both geographic provenance and year of collection (Chidambara Murthy et al., 2002; Göktürk Baydar et al., 2007; Jayaprakasha et al., 2003).

4.4.2. Antioxidant acitivity by FRAP Method

Total antioxidant activities of essential oils, constituents and extracts were determined with both FRAP and DPPH methods.

Antioxidant activity measured by FRAP method were expressed as milimol Trolox equivalent per ml, calculated by using Trolox calibration curve (Fig. B.2), were shown in Table 4.13. Clove EO was showed the highest antioxidant activity among the essential oils, while the lowest antioxidant activity was observed in bay leaf EO. Grape seed extract was more active between the extracts. Essential oil constituents seemed to be had very less antioxidant activity when they were compared with essential oils and extracts.

Table 4.13. Antioxidant activity of EOs, components and extracts measured by FRAP method

Sample	FRAP (mmol Trolox/ml)
Bay leaf essential oil	63.03±1.19
Thyme essential oil	2150.72±47.03
Cumin essential oil	145.09±6.50
Clove essential oil	4357.45±28.83
Grape seed extract	390.30±8.93
Olive leaf extract	259.18±5.64
Eucalyptol	0.58±0.22
α- terpineol	1.23±0.56
Linalool	1.20±0.27

4.4.3. Antioxidant Acitivity by DPPH Method

The ability of essential oils, extracts and the major constituents of essential oils were investigated by using DPPH assay to observe the ability to act as donors of hydrogen atoms or electrons for the transformation of DPPH into its reduced form DPPH (Ojeda-Sana et al., 2013). Results were expressed as 50% of inhibition of DPPH and represented in Table 4.14. Essential oils were able to change the stable violet color of DPPH into yellow-colored DPPH, reaching 50% of reduction with IC_{50} value changing from 0.14 µl/ml to 66.53 µl/ml. Major components of essential oils were also evaluated, but there was almost no significant activity comparing with the essential oils. Extracts also showed antioxidant activity with 1.31 µl/ml for olive leaf extract and 0.14 µl/ml for grape seed extract.

Table 4.14. Antioxidant activity of EOs, components and extracts measured by DPPH method

Sample	DPPH (IC_{50} µl/ml)
Bay leaf essential oil	18.68±1.58
Thyme essential oil	9.88±0.75
Cumin essential oil	66.53±11.91

(**cont. on next page**)

Table 4.14 (cont.)

Clove essential oil	0.14±0.02
Grape seed extract	1.31±0.05
Olive leaf extract	3.30±0.09
Eucalyptol	NA
α- terpineol	433.97±13.69
Linalool	325.05±20.19

NA: Not Active

Both results obtained from FRAP and DPPH assays have nearly the same outcome. Clove essential oil showed the highest antioxidant activity following by thyme essential oil. However, bay leaf and cumin essential oil showed different activity for both assays. Some authors also reported that there was differences in the results obtained from these two assays (Gourine et al., 2010; Politeo et al., 2010). It is difficult to assess the antioxidant activity of a sample referring to a single method. The interpretation of activity may require a combination of different methods. But, it is obvious that it is difficult to compare the results of many different methods as well as it was experienced in this study.

Findings for the major constituents of essential oils exhibited that there were very weak, almost no antioxidant activity. The oxygenated monoterpenes especially thymol and carvacrol have high antioxidant activity. Although, monoterpene hyrocarbons may be considered as active for antioxidative effect, none has stronger than oxygenated monoterpenes. Moreover, sesquiterpene hydrocarbons and their oxygenated derivatives have very low antioxidant activity (Ruberto and Baratta, 2000). In the study, where antioxidant activity of the components of essential oils examined, terpinene-4-ol, 1,8-cineole , camphor, borneol, *p*-cymene, α-pinene and β-pinene showed no activity (Tepe et al., 2005). The reason that essential oils showed much more activity than constituents alone can be attributed to the high percentage of the main components, synergy among the different oil constituents or to micro components acting as pro-oxidants (Viuda-Martos et al., 2010).

When the relation between total phenolic content (TPC) and antioxidant activities were compared, the higher TPC gives the higher antioxidant activity to clove bud EO, whereas the lowest TPC of bay leaf EO resulted in the lowest antioxidant

activity. However there is an exception for cumin essential oil. In the literature**;** some studies obtained good positive linear correlation, but others got poor linear correlation or even could not explain the relationship between total antioxidant activity and phenolic content. Recently, it has been shown that the antioxidant activity of extracts is roughly connected to their phenolic composition and strongly depends upon their phenolic structures (Chaillou and Nazareno, 2006). The relationship and antioxidant activity is also highly influenced by different assay methods (Shan et al., 2005).

4.5. Antimicrobial Effect of Essential Oils in Minced Beef

4.5.1. Microbiological Analyses

4.5.1.1. Effects of Essential Oils on the growth of *Salmonella* Typhimurium

The effect of thyme and clove essential oils with 2MIC and 4MIC values on the growth of *Salmonella* Typhimurium in minced meat samples stored at 4 °C for 9 days were investigated (Figure 4.35). 4 log cfu/g inoculations were performed for each sample. The number of *S.* Typhimurium did not change significantly during 3 days of storage except a slight increase in control and thyme (2MIC) treated samples. At the end of the 9 days storage period, there was a significant difference between control and thyme (4MIC), clove (2MIC and 4MIC) treated samples. When *S.* Typhimurium increased to 6log cfu/g for control sample, thyme (4MIC) and clove (2MIC) treated samples showed ~5 log cfu/g counts respectively (Table 4.15). Clove (4MIC) treated sample represented only 0.77 log increase after 9 days. The results indicated that clove (4MIC) essential oil had more antimicrobial effect ($p<0.05$) on *S.* Typhimurium than use of thyme essential oil even with 4MIC value.

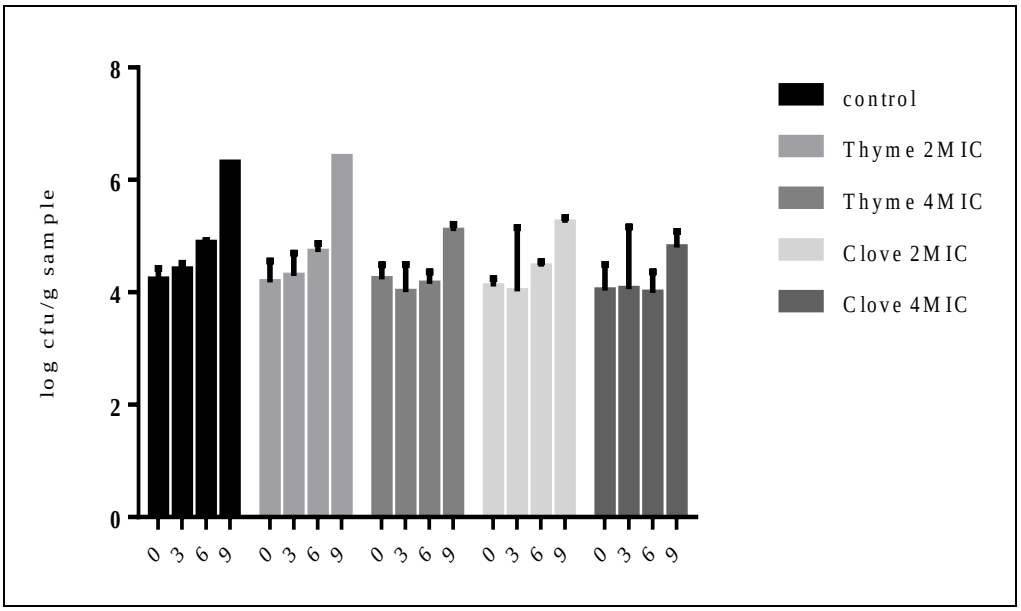


Figure 4.35. The growth of *S.* Typhimurium during storage

Table 4.15. The growth of *S.* Typhimurium during storage

Samples	Storage time (days)			
	0	3	6	9
Control (with inoculum)	$4.22\pm0.20^{a,C}$	$4.40\pm 0.11^{a,BC}$	$4.87\pm 0.04^{a,B}$	$6.30\pm 0.00^{a,A}$
Thyme-2MIC	$4.18 \pm0.38^{a,B}$	$4.29\pm 0.41^{a,B}$	$4.72\pm 0.15^{a,B}$	$6.40 \pm0.00^{a,A}$
Thyme-4MIC	$4.23 \pm0.26^{a,A}$	$4.00 \pm0.49^{a,A}$	$4.15\pm 0.21^{a,A}$	$5.09\pm 0.12^{b,A}$
Clove-2MIC	$4.10 \pm0.14^{a,A}$	$4.02\pm 1.13^{a,A}$	$4.46\pm 0.09^{a,A}$	$5.24\pm 0.09^{b,A}$
Clove-4MIC	$4.03 \pm0.47^{a,A}$	$4.06 \pm1.10^{a,A}$	$3.99\pm 0.38^{a,A}$	$4.80\pm 0.29^{b,A}$

$^{a-b}$: Means having different letters indicate significant difference at $p<0.05$ within each storage time
$^{A-B}$: Means having different letters indicate significant difference at $p<0.05$ within each treatment

4.5.1.2. Effect of Essential Oils on Aerobic Mesophilic Microorganisms

The effect of thyme and clove essential oils with 2MIC and 4MIC values the number of aerobic mesophilic microorganisms was also examined during 9 days of storage (Fig. 4.36). The minced meat samples showed a high initial load as seen in Table 4.16. The initial AMM was almost the same for all samples approximately 7 log cfu/g. During the time periods AMM showed an increase up to 11 log cfu/g except the clove EO (4MIC) treated sample. At the end of storage for 9 days, TVC increased up to 10 log cfu/g for clove EO (4MIC) treated sample. There was a 1 decimal difference between control and other treated samples and clove (4MIC) treated sample.

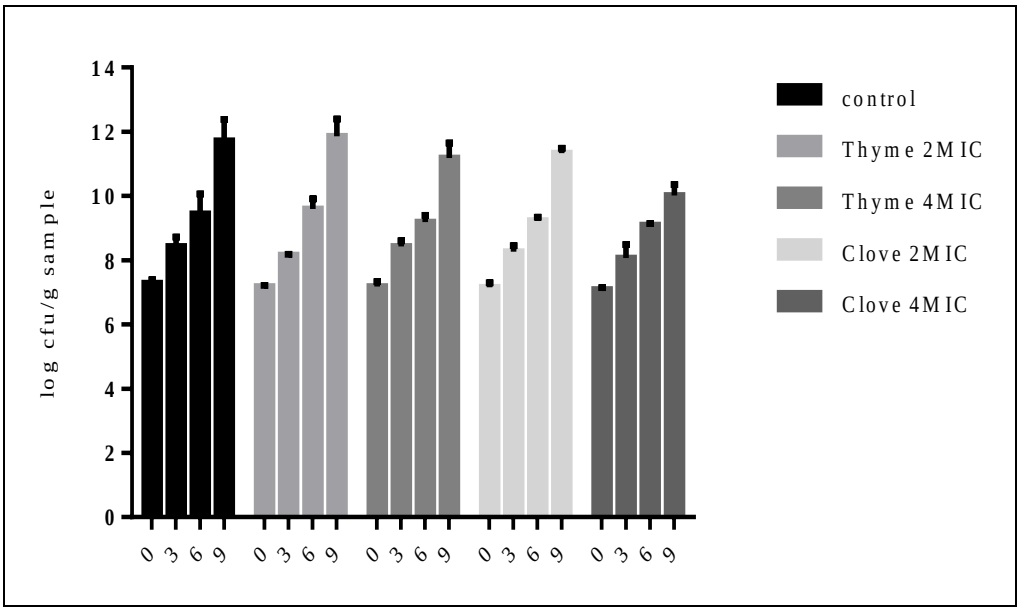


Figure 4.36. The growth of AMM during storage

Table 4.16. The growth of AMM during storage

Samples	Storage time (days)			
	0	3	6	9
Control (with inoculum)	$7.28\pm0.11^{a,B}$	$8.43\pm0.29^{a,B}$	$9.44\pm0.62^{a,AB}$	$11.72\pm0.66^{a,A}$
Thyme-2MIC	$7.19\pm0.03^{a,C}$	$8.16\pm0.03^{a,BC}$	$9.60\pm0.31^{a,B}$	$11.86\pm0.53^{a,A}$
Thyme-4MIC	$7.18\pm0.15^{a,C}$	$8.43\pm0.18^{a,BC}$	$9.19\pm0.21^{a,B}$	$11.18\pm0.47^{a,A}$
Clove-2MIC	$7.16\pm0.14^{a,D}$	$8.26\pm0.19^{a,C}$	$9.24\pm0.11^{a,B}$	$11.34\pm0.14^{a,A}$
Clove-4MIC	$7.09\pm0.06^{a,C}$	$8.07\pm0.41^{a,BC}$	$9.09\pm0.05^{a,AB}$	$10.02\pm0.33^{a,A}$

$^{a-b}$: Means having different letters indicate significant difference at $p<0.05$ within each storage time
$^{A-D}$: Means having different letters indicate significant difference at $p<0.05$ within each treatment

4.5.1.3. Effect of Essential Oils on The Growth of Total Coliform

The effect on the growth of total coliform was also determined for control and essential oil treated samples (Fig. 4.37). The initial load of total coliform was almost the same for all samples (Table 4.17). During the storage period the number of total coliform increased for control, thyme (2MIC and 4MIC) and clove (2MIC) treated samples. However, a 0.26 decimal reduction was observed for the clove (4MIC) treated sample at the end of 9 days. The results of the growth of total coliform indicated that clove essential oil ($p<0.05$) inhibited the growth of coliform bacteria during 9 days period.

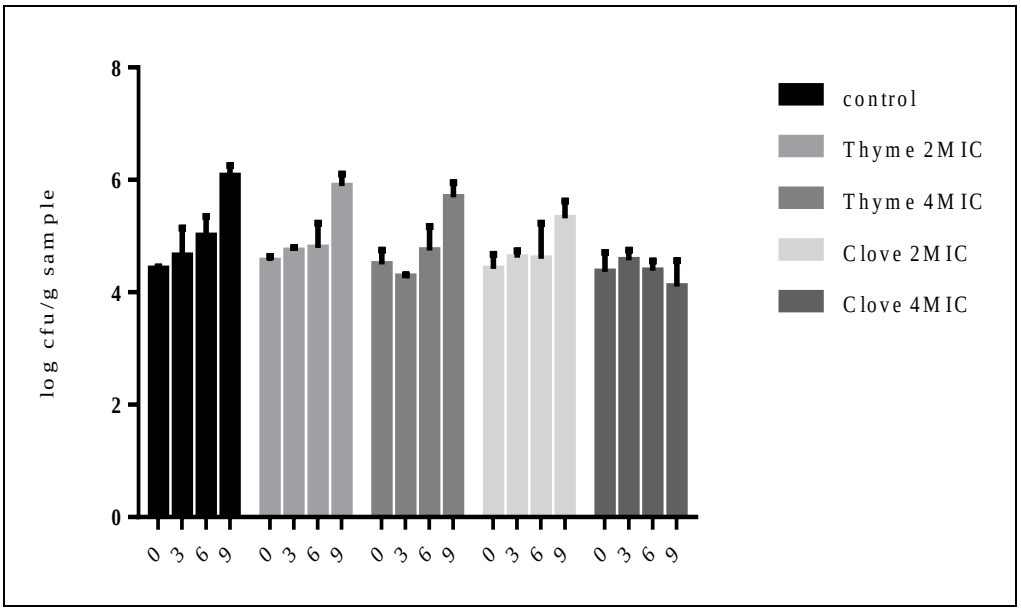


Figure 4.37. The growth of total coliform during storage

Table 4.17. The growth of total coliform during storage

Samples	Storage time (days)			
	0	3	6	9
Control (with inoculum)	$4.41\pm0.04^{a,A}$	$4.65\pm0.49^{a,A}$	$5.01\pm0.34^{a,A}$	$6.07\pm0.19^{a,A}$
Thyme-2MIC	$4.56\pm0.08^{a,A}$	$4.74\pm0.06^{a,A}$	$4.79\pm0.44^{a,A}$	$5.89\pm0.21^{a,A}$
Thyme-4MIC	$4.50\pm0.25^{a,A}$	$4.28\pm0.03^{a,A}$	$4.74\pm0.43^{a,A}$	$5.69\pm0.26^{a,A}$
Clove-2MIC	$4.42\pm0.26^{a,A}$	$4.62\pm0.12^{a,A}$	$4.59\pm0.63^{a,A}$	$5.32\pm0.30^{ab,A}$
Clove-4MIC	$4.36\pm0.35^{a,A}$	$4.57\pm0.18^{a,A}$	$4.39\pm0.17^{a,A}$	$4.10\pm0.46^{b,A}$

$^{a-b}$: Means having different letters indicate significant difference at $p<0.05$ within each storage time

4.5.1.4. Effect of Essential Oils on The Growth of Total Yeasts and Molds

The initial numbers of total yeasts and molds were almost the same with approximately 6 log cfu/g for all samples. The count of yeasts and molds of control and treated samples increased dramatically during 9 days of storage (Fig. 4.38). Although the load of all samples exceeded to 11 log cfu/g at the end of 9 days, clove (4MIC) treated sample increased up to 10.09 log cfu/g (Table 4.18). There was not great difference between samples. However, the differences between control and clove (4MIC) and also thyme (2MIC) and clove (4MIC) were statistically significant (*$p<0.05$*).

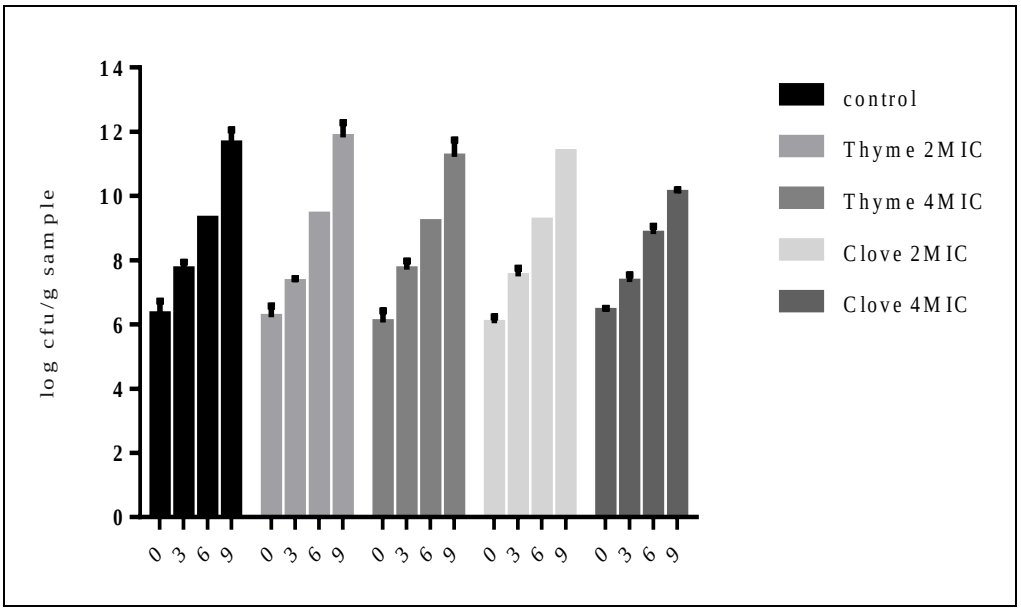


Figure 4.38. The growth of total yeast and molds count during storage

Table 4.18. The growth of total yeast and molds count during storage

Samples	Storage time (days)			
	0	3	6	9
Control (with inoculum)	$6.30\pm0.43^{a,C}$	$7.71\pm0.23^{a,BC}$	$9.29\pm0.00^{ab,B}$	$11.63\pm0.43^{a,A}$
Thyme-2MIC	$6.23\pm0.35^{a,C}$	$7.31\pm0.11^{a,C}$	$9.41\pm0.00^{a,B}$	$11.82\pm0.45^{a,A}$
Thyme-4MIC	$6.06\pm0.36^{a,C}$	$7.70\pm0.27^{a,BC}$	$9.18\pm0.00^{ab,B}$	$11.22\pm0.53^{a,A}$
Clove-2MIC	$6.04\pm0.19^{a,D}$	$7.50\pm0.25^{a,C}$	$9.22\pm0.00^{ab,B}$	$11.35\pm0.00^{a,A}$
Clove-4MIC	$6.42\pm0.09^{a,C}$	$7.33\pm0.21^{a,C}$	$8.81\pm0.25^{b,B}$	$10.09\pm0.11^{a,A}$

$^{a-b}$: Means having different letters indicate significant difference at $p<0.05$ within each storage time
$^{A-D}$: Means having different letters indicate significant difference at $p<0.05$ within each treatment

4.5.1.5. Effect of Essential Oils on Psychrotrophic Organisms

The count of psychrotrophic organisms was determined by incubation at 7 °C for 10 days. A dramatic growth of psychrotrophic organisms were observed during 9 days of storage for all samples (Fig. 4.39). There was no significant difference between the samples (*p>0.05*), as it was observed from theFigure 4.39, there was an identical pattern of growth for all samples. However, control, thyme (2MIC and 4MIC) and clove (2MIC) treated samples showed growth ended up with approximately 11 log cfu/g, whereas clove (4MIC) ended up with a ~ 10 log cfu/g of psychrotrophic organisms (Table 4.19).

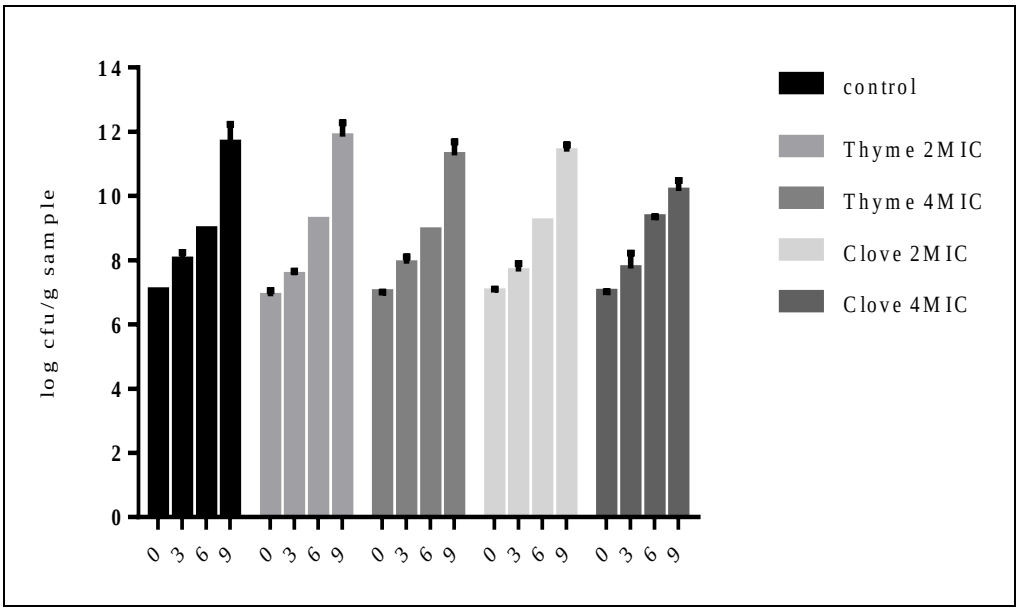


Figure 4.39. The growth of Psychrotrophic microorganisms during storage

Table 4.19. The growth of Psychrotrophic microorganisms during storage

Samples	Storage time (days)			
	0	3	6	9
Control (with inoculum)	$7.05\pm0.01^{a,C}$	$8.01\pm0.23^{a,BC}$	$8.95\pm0.00^{c,B}$	$11.64\pm0.58^{a,A}$
Thyme-2MIC	$6.87\pm0.18^{a,C}$	$7.54\pm0.12^{a,C}$	$9.24\pm0.00^{b,B}$	$11.85\pm0.44^{a,A}$
Thyme-4MIC	$6.99\pm0.02^{a,C}$	$7.89\pm0.21^{a,BC}$	$8.92\pm0.00^{c,B}$	$11.27\pm0.42^{a,A}$
Clove-2MIC	$7.02\pm0.08^{a,C}$	$7.65\pm0.24^{a,C}$	$9.20\pm0.00^{b,B}$	$11.38\pm0.21^{a,A}$
Clove-4MIC	$7.01\pm0.01^{a,B}$	$7.74\pm0.48^{a,B}$	$9.32\pm0.03^{a,A}$	$10.15\pm0.34^{a,A}$

$^{a-c}$: Means having different letters indicate significant difference at $p<0.05$ within each storage time
$^{A-C}$: Means having different letters indicate significant difference at $p<0.05$ within each treatment

4.5.2. Determination of Lipid Oxidation

Lipid oxidation in food products is considered to be one of the important factor limiting product quality and acceptability due to the production of potentially toxic reactive oxygen species and off-flavors from unsaturated fatty acids (Cui et al., 2012). The oxidation conditions of minced meat samples were evaluated by observing the absorbance at 532 nm. TBA values were determined by using the thiobarbituric acid-reactive substances (TBARS) assay (Table 4.20). Measurements were obtained over 9 days storage and TBA values of control showed a rapid increase with increasing storage time. TBA values of all treatments on day 0 were significantly lower than those for the control sample ($p<0.05$). Moreover, treatment samples had significantly lower TBA values than control at each day of testing throughout storage. Although, there was no

significant difference among treated samples, each treatment showed significant reduction comparing with control. The results indicated that thyme (2MIC and 4MIC) and clove (2MIC and 4MIC) essential oils retarded lipid oxidation during 9 days storage.

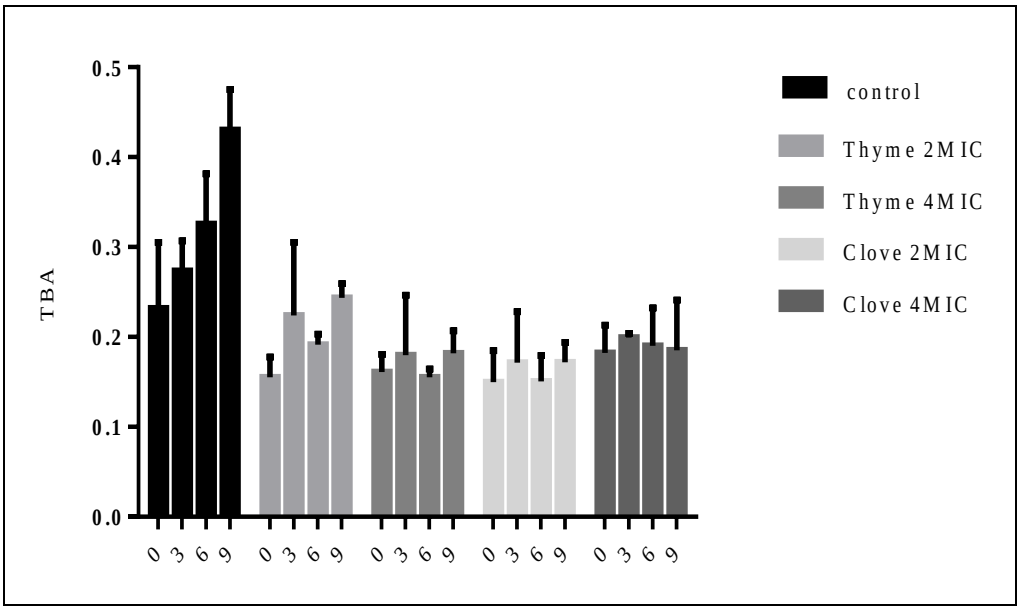


Figure 4.40. TBA values of minced beef samples during storage

Table 4.20. TBA values of minced beef samples during storage

Samples	Storage time (days)			
	0	3	6	9
Control (with inoculum)	$0.23\pm0.07^{a,A}$	$0.27\pm0.03^{a,A}$	$0.33\pm0.06^{a,A}$	$0.43\pm0.04^{a,A}$
Thyme-2MIC	$0.16\pm0.02^{a,A}$	$0.22\pm0.08^{a,A}$	$0.19\pm0.01^{ab,A}$	$0.24\pm0.02^{b,A}$
Thyme-4MIC	$0.16\pm0.02^{a,A}$	$0.18\pm0.07^{a,A}$	$0.16\pm0.01^{ab,A}$	$0.18\pm0.03^{b,A}$
Clove-2MIC	$0.15\pm0.03^{a,A}$	$0.17\pm0.06^{a,A}$	$0.15\pm0.03^{b,A}$	$0.17\pm0.02^{b,A}$
Clove-4MIC	$0.18\pm0.03^{a,A}$	$0.20\pm0.00^{a,A}$	$0.19\pm0.04^{ab,A}$	$0.19\pm0.06^{b,A}$

$^{a-b}$: Means having different letters indicate significant difference at $p<0.05$ within each storage time

4.5.3. Color Evaluation

The parameters of color L*, a*, b* values were shown in Table 4.21. The observation of L* (lightness) values showed that there was no significant darkening or browning in color due to metmyoglobin formation among all meat samples in terms of both treatments and storage time ($p>0.05$) (Fig. 4.41). The initial L* values were maintained during the storage period.

Table 4.21. Color changes of samples during storage

Samples		Storage time (days)			
		0	3	6	9
Control (with inoculum)	L*	49.57±0.28^{a,A}	50.03±0.18^{a,A}	50.89±0.15^{a,A}	50.79±1.64^{a,A}
	a*	19.77±1.53^{a,A}	18.02±0.62^{a,A}	16.70±0.21^{a,A}	14.81±1.21^{a,A}
	b*	10.09±0.09^{a,A}	10.40±0.07^{a,A}	10.49±0.42^{a,A}	9.44±0.57^{a,A}
Thyme-2MIC	L*	49.91±0.45^{a,A}	49.85±0.46^{a,A}	49.23±0.07^{a,A}	51.19±0.62^{a,A}
	a*	19.61±2.26^{a,A}	16.26±5.08^{a,A}	16.76±0.91^{a,A}	16.54±3.65^{a,A}
	b*	9.06±0.85^{a,A}	10.21±1.74^{a,A}	10.64±0.57^{a,A}	10.46±0.43^{a,A}
Thyme-4MIC	L*	49.82±0.22^{a,A}	50.29±0.16^{a,A}	50.24±1.27^{a,A}	49.08±1.79^{a,A}
	a*	19.60±0.74^{a,A}	16.88±0.69^{a,A}	18.83±1.34^{a,A}	18.50±0.96^{a,A}
	b*	9.72±0.18^{a,A}	10.73±0.32^{a,A}	10.70±0.47^{a,A}	9.99±0.68^{a,A}
Clove-2MIC	L*	49.35±0.29^{a,A}	50.96±1.32^{a,A}	50.36±0.64^{a,A}	51.00±0.74^{a,A}
	a*	20.80±0.90^{a,A}	17.56±1.04^{a,A}	18.57±0.99^{a,A}	18.44±0.99^{a,A}
	b*	9.89±0.30^{a,B}	10.69±0.12^{a,AB}	10.95±0.12^{a,A}	11.35±0.12^{a,A}
Clove-4MIC	L*	48.65±1.25^{a,A}	50.60±1.35^{a,A}	49.63±0.82^{a,A}	51.12±0.62^{a,A}
	a*	18.29±0.83^{a,A}	19.38±0.45^{a,A}	19.24±2.32^{a,A}	17.60±4.45^{a,A}
	b*	9.37±0.01^{a,A}	10.48±0.66^{a,A}	11.28±0.17^{a,A}	11.09±0.58^{a,A}

$^{a-b}$: Means having different letters indicate significant difference at $p<0.05$ within each storage time

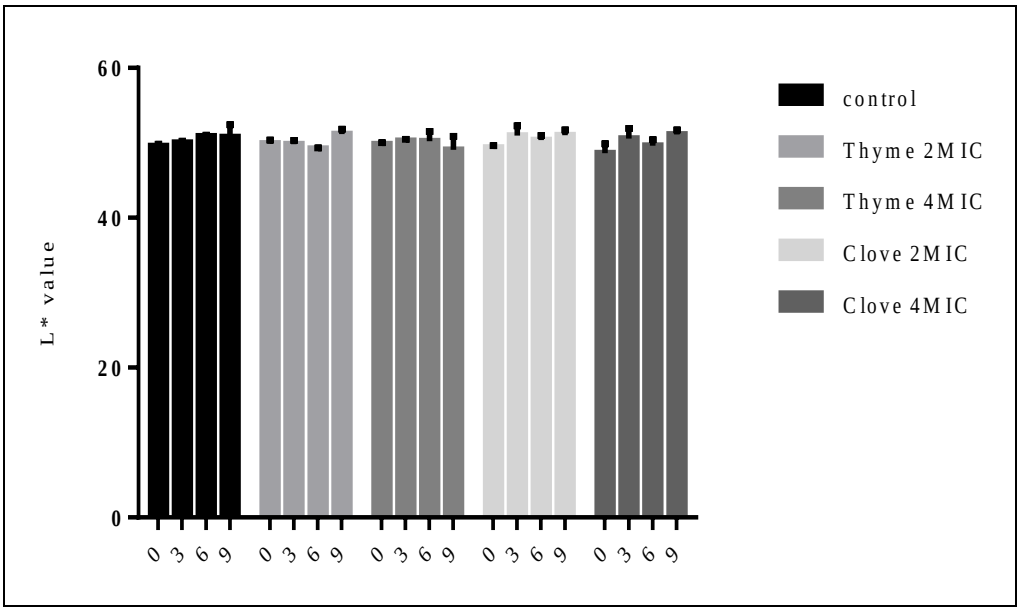


Figure 4.41. L* values of samples during storage

a* value of control sample showed a dramatic reduction following the time period of storage. There was no significant difference among a* values of control and treated samples throughout the storage period (Figure 4.42). However, a* value was

maintained during 9 days storage in clove EO (4MIC) treated samples comparing with control sample.

There was also no significant difference between b* values of treated samples (Table 4.21). But b* values showed a significant alteration among over the time period ($p<0.05$) (Figure 4.43).

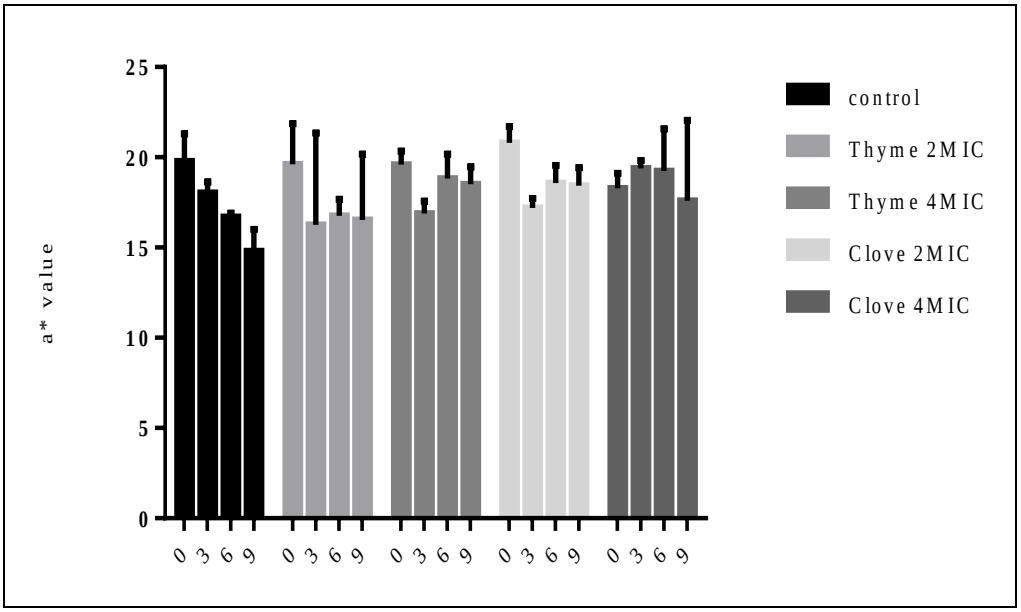


Figure 4.42. a* values of samples during storage

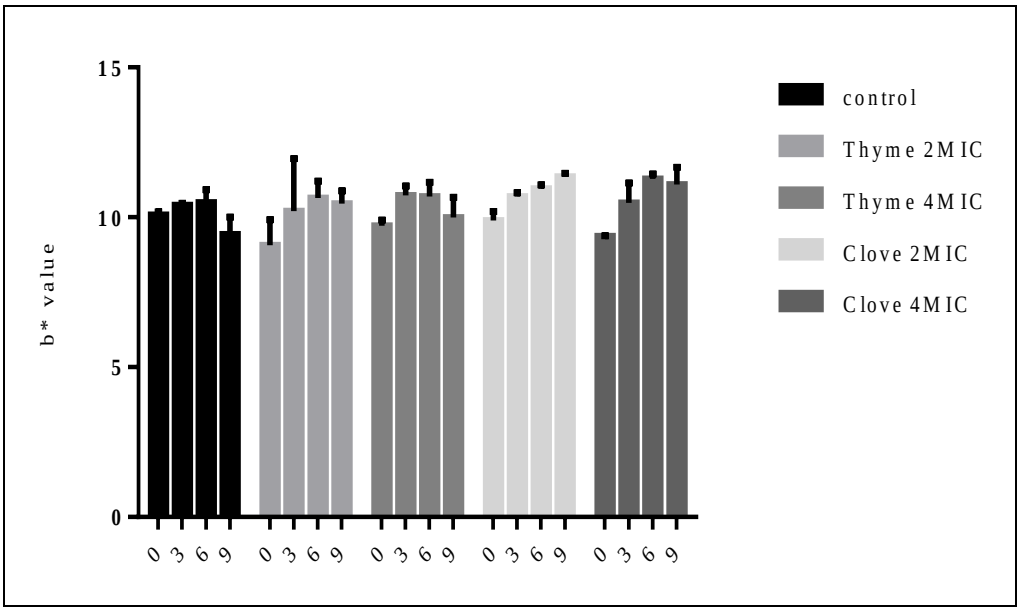


Figure 4.43. b* values of samples during storage

CHAPTER 5

CONCLUSIONS

In recent years, there is an increasing trend in utilization of natural compounds in pharmaceutical, food and cosmetic industries among the consumers. Using plants serve as a "safe" alternative to chemical or synthetic antimicrobials and antioxidants to fight with the food-borne pathogens or food spoilage organisms, to inhibit lipid oxidation and thus to extend shelf life.

The biological activities of natural compounds are highly affected by their chemical composition. Essential oils and extracts are complex natural mixtures. Beside the major components, minor components are also effective on the biological activities, possibly by producing a synergistic effect with other components. For a better understanding of the antimicrobial and antioxidant properties, chemical characterization was performed for all essential oils and extracts. Although some common components were observed among essential oils, the major constituents varied. This variation assists to comprehend the differences among the antimicrobial and antioxidant activities.

At the beginning of the study four different essential oils and two extract were subjected to chemical characterization and they were screened according to their antimicrobial and antioxidant activity. Among the tested compounds thyme, clove and bay leaf essential oils were observed as the most potent ones. These oils showed effective antimicrobial activity against all tested bacteria with changing MIC values. The synergistic antimicrobial effect of selected essential oil constituents was also observed and the interaction effects were determined. Essential oils also represented high antioxidant activity in both DPPH and FRAP assays.

In the proceeding parts of the study, essential oils were evaluated against three most sensitive pathogen bacteria with respect to the antimicrobial mode of action. The exact mechanism of antimicrobial activity of natural compounds is not fully understood yet. Though, it is known that the activity is due to their phenolic substances. The results indicated that essential oils caused physical disturbance and changed the structures of bacteria. The structural changes were observed by microscopic observations (SEM and AFM). These images directly illustrated the destructive effects of the essential oils on

the tested bacteria. Non-treated cells (control) were intact and showed a smooth surface while bacterial cells treated with the essential oils underwent considerable damage. The disruption of cytoplasmic membrane affected the membrane potential and when the membrane integrity was destroyed; membrane potential was reduced and membrane became permeable which was demonstrated by flow cytometry assay. Release of cellular constituents was observed as the permeability of membrane increased. The FTIR observations supported that treatment with essential oils affected especially the cellular proteins and polysaccharides corresponding to the cell membrane and cell wall components.

The biological activities of essential oils could provide alternatives to chemical or synthetic antimicrobials and antioxidants. Thus they are suitable to use in food industry. The activity of two most potent essential oils was investigated in the food application study. The antimicrobial activity on *S.* Typhimurium, a severe problem for meat and meat products, coliform bacteria, total viable count, total yeasts and molds and also psychrotrophic organisms were examined. Beside the antimicrobial activity, the effects on oxidation and the color characteristics was observed during storage period. The findings represented that, although clove essential oil restricted the growth of *S.* Typhimurium and coliform bacteria comparing with thyme essential oil with 4 fold of its MIC value. It did not inhibit the aerobic mesophilic bacteria, total yeasts and molds and also psychrotrophic organisms. However, the growth of these microorganisms was less than the control sample. As it was demonstrated in the results, essential oils were not very effective on color characteristic. Thus, L* and a* values were maintained during the storage period. The featured effect of essential oils was their antioxidant characteristic in meat application study. All treatments showed significant reduction in TBA value comparing with control. But there was no significant difference among treated samples.

REFERENCES

Ahmad-Qasem, M. H., Cánovas, J., Barrajón-Catalán, E., Micol, V., Cárcel, J. A., and García-Pérez, J. V. (2013). Kinetic and compositional study of phenolic extraction from olive leaves (var. Serrana) by using power ultrasound. *Innovative Food Science & Emerging Technologies* 17, 120-129.

Ait-Ouazzou, A., Cherrat, L., Espina, L., Lorán, S., Rota, C., and Pagán, R. (2011a). The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innovative Food Science & Emerging Technologies* 12, 320-329.

Ait-Ouazzou, A., Loran, S., Bakkali, M., Laglaoui, A., Rota, C., Herrera, A., Pagan, R., and Conchello, P. (2011b). Chemical composition and antimicrobial activity of essential oils of Thymus algeriensis, Eucalyptus globulus and Rosmarinus officinalis from Morocco. *J Sci Food Agric* 91, 2643-51.

Aktas, A. B., Ozen, B., Tokatli, F., and Sen, I. (2013). Phenolics profile of a naturally debittering olive in comparison to regular olive varieties. *Journal of the Science of Food and Agriculture*, n/a-n/a.

Al-Qadiri, H. M., Al-Alami, N. I., Al-Holy, M. A., and Rasco, B. A. (2008). Using Fourier Transform Infrared (FT-IR) Absorbance Spectroscopy and Multivariate Analysis To Study the Effect of Chlorine-Induced Bacterial Injury in Water. *Journal of Agricultural and Food Chemistry* 56, 8992-8997.

Al-Reza, S. M., Rahman, A., Lee, J., and Kang, S. C. (2010). Potential roles of essential oil and organic extracts of Zizyphus jujuba in inhibiting food-borne pathogens. *Food Chemistry* 119, 981-986.

Altıok, E., Bayçın, D., Bayraktar, O., and Ülkü, S. (2008). Isolation of polyphenols from the extracts of olive leaves (Olea europaea L.) by adsorption on silk fibroin. *Separation and Purification Technology* 62, 342-348.

Alvarez-Ordóñez, A., Mouwen, D. J. M., López, M., and Prieto, M. (2011). Fourier transform infrared spectroscopy as a tool to characterize molecular composition and stress response in foodborne pathogenic bacteria. *Journal of Microbiological Methods* 84, 369-378.

Angioni, A., Barra, A., Coroneo, V., Dessi, S., and Cabras, P. (2006). Chemical composition, seasonal variability, and antifungal activity of Lavandula stoechas L. ssp. stoechas essential oils from stem/leaves and flowers. *J Agric Food Chem* 54, 4364-70.

Bakkali, F., Averbeck, S., Averbeck, D., and Idaomar, M. (2008). Biological effects of essential oils – A review. *Food and Chemical Toxicology* 46, 446-475.

Bassolé, I. H. N., Lamien-Meda, A., Bayala, B., Tirogo, S., Franz, C., Novak, J., Nebié, R. C., and Dicko, M. H. (2010). Composition and Antimicrobial Activities of Lippia multiflora Moldenke, Mentha x piperita L. and Ocimum basilicum L.

Essential Oils and Their Major Monoterpene Alcohols Alone and in Combination. *Molecules* 15, 7825-7839.

Baydar, H., Sağdiç, O., Özkan, G., and Karadoğan, T. (2004a). Antibacterial activity and composition of essential oils from Origanum, Thymbra and Satureja species with commercial importance in Turkey. *Food Control* 15, 169-172.

Baydar, N. G., Özkan, G., and Sağdiç, O. (2004b). Total phenolic contents and antibacterial activities of grape (Vitis vinifera L.) extracts. *Food Control* 15, 335-339.

Baydar, N. G., Sagdic, O., Ozkan, G., and Cetin, S. (2006). Determination of antibacterial effects and total phenolic contents of grape (Vitis vinifera L.) seed extracts. *International Journal of Food Science & Technology* 41, 799-804.

Behera, S., Nagarajan, S., and Jagan Mohan Rao, L. (2004). Microwave heating and conventional roasting of cumin seeds (Cuminum cyminum L.) and effect on chemical composition of volatiles. *Food Chemistry* 87, 25-29.

Benavente-García, O., Castillo, J., Lorente, J., Ortuño, A., and Del Rio, J. A. (2000). Antioxidant activity of phenolics extracted from Olea europaea L. leaves. *Food Chemistry* 68, 457-462.

Benchaar, C., and Greathead, H. (2011). Essential oils and opportunities to mitigate enteric methane emissions from ruminants. *Animal Feed Science and Technology* 166–167, 338-355.

Bevilacqua, A., Ficelo, S., Corbo, M. R., and Sinigaglia, M. (2010). Bioactivity of grapefruit seed extract against pseudomonas spp. *Journal of Food Processing and Preservation* 34, 495-507.

Bouaziz, M., Chamkha, M., and Sayadi, S. (2004). Comparative Study on Phenolic Content and Antioxidant Activity during Maturation of the Olive Cultivar Chemlali from Tunisia. *Journal of Agricultural and Food Chemistry* 52, 5476-5481.

Bourgou, S., Pichette, A., Marzouk, B., and Legault, J. (2010). Bioactivities of black cumin essential oil and its main terpenes from Tunisia. *South African Journal of Botany* 76, 210-216.

Bozin, B., Mimica-Dukic, N., Simin, N., and Anackov, G. (2006). Characterization of the Volatile Composition of Essential Oils of Some Lamiaceae Spices and the Antimicrobial and Antioxidant Activities of the Entire Oils. *Journal of Agricultural and Food Chemistry* 54, 1822-1828.

Burt (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology* 94, 223-253.

Burt, S. A., and Reinders, R. D. (2003). Antibacterial activity of selected plant essential oils against Escherichia coli O157:H7. *Letters in Applied Microbiology* 36, 162-167.

Caillet, S., Salmiéri, S., and Lacroix, M. (2006). Evaluation of free radical-scavenging properties of commercial grape phenol extracts by a fast colorimetric method. *Food Chemistry* 95, 1-8.

Cao, S., Sun, L. Q., and Wang, M. (2011). Antimicrobial activity and mechanism of action of Nu-3, a protonated modified nucleotide. *Ann Clin Microbiol Antimicrob* 10, 1.

Cardinali, A., Cicco, N., Linsalata, V., Minervini, F., Pati, S., Pieralice, M., Tursi, N., and Lattanzio, V. (2010). Biological Activity of High Molecular Weight Phenolics from Olive Mill Wastewater. *Journal of Agricultural and Food Chemistry* 58, 8585-8590.

Carpenter, R., O'Grady, M. N., O'Callaghan, Y. C., O'Brien, N. M., and Kerry, J. P. (2007). Evaluation of the antioxidant potential of grape seed and bearberry extracts in raw and cooked pork. *Meat Science* 76, 604-610.

Carson, C. F., and Riley, T. V. (1995). Antimicrobial activity of the major components of the essential oil of Melaleuca alternifolia. *J Appl Bacteriol* 78, 264-9.

Chaillou, L. L., and Nazareno, M. A. (2006). New method to determine antioxidant activity of polyphenols. *J Agric Food Chem* 54, 8397-402.

Chen, Q., Gan, Z., Zhao, J., Wang, Y., Zhang, S., Li, J., and Ni, Y. (2014). In vitro comparison of antioxidant capacity of cumin (Cuminum cyminum L.) oils and their main components. *LWT - Food Science and Technology*.

Chidambara Murthy, K. N., Singh, R. P., and Jayaprakasha, G. K. (2002). Antioxidant activities of grape (Vitis vinifera) pomace extracts. *J Agric Food Chem* 50, 5909-14.

Chouliara, E., Karatapanis, A., Savvaidis, I. N., and Kontominas, M. G. (2007). Combined effect of oregano essential oil and modified atmosphere packaging on shelf-life extension of fresh chicken breast meat, stored at 4 degrees C. *Food Microbiol* 24, 607-17.

Cimanga, K., Kambu, K., Tona, L., Apers, S., De Bruyne, T., Hermans, N., Totté, J., Pieters, L., and Vlietinck, A. J. (2002). Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. *Journal of Ethnopharmacology* 79, 213-220.

Cox, Gustafson, Mann, Markham, Liew, Hartland, Bell, Warmington, and Wyllie (1998). Tea tree oil causes K+ leakage and inhibits respiration in Escherichia coli. *Letters in Applied Microbiology* 26, 355-358.

Cui, Y., Oh, Y. J., Lim, J., Youn, M., Lee, I., Pak, H. K., Park, W., Jo, W., and Park, S. (2012). AFM study of the differential inhibitory effects of the green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) against Gram-positive and Gram-negative bacteria. *Food Microbiology* 29, 80-87.

Dadalioğlu, I., and Evrendilek, G. A. (2004). Chemical Compositions and Antibacterial Effects of Essential Oils of Turkish Oregano (Origanum minutiflorum), Bay Laurel (Laurus nobilis), Spanish Lavender (Lavandula stoechas L.), and Fennel

(Foeniculum vulgare) on Common Foodborne Pathogens. *Journal of Agricultural and Food Chemistry* 52, 8255-8260.

de Azeredo, G. A., Stamford, T. L. M., Nunes, P. C., Gomes Neto, N. J., de Oliveira, M. E. G., and de Souza, E. L. (2011). Combined application of essential oils from Origanum vulgare L. and Rosmarinus officinalis L. to inhibit bacteria and autochthonous microflora associated with minimally processed vegetables. *Food Research International* 44, 1541-1548.

de Billerbeck, V. G., Roques, C. G., Bessi, egrave, re, J. M., Fonvieille, J. L., and Dargent, R. (2001). Effects of Cymbopogon nardus (L.) W. Watson essential oil on the growth and morphogenesis of Aspergillus niger. *Canadian Journal of Microbiology* 47, 9-17.

de Oliveira, C. E. V., Stamford, T. L. M., Neto, N. J. G., and de Souza, E. L. (2010). Inhibition of Staphylococcus aureus in broth and meat broth using synergies of phenolics and organic acids. *International Journal of Food Microbiology* 137, 312-316.

de Sousa, J. P., de Azeredo, G. A., de Araujo Torres, R., da Silva Vasconcelos, M. A., da Conceicao, M. L., and de Souza, E. L. (2012a). Synergies of carvacrol and 1,8-cineole to inhibit bacteria associated with minimally processed vegetables. *Int J Food Microbiol* 154, 145-51.

de Sousa, J. P., Torres Rde, A., de Azeredo, G. A., Figueiredo, R. C., Vasconcelos, M. A., and de Souza, E. L. (2012b). Carvacrol and 1,8-cineole alone or in combination at sublethal concentrations induce changes in the cell morphology and membrane permeability of Pseudomonas fluorescens in a vegetable-based broth. *Int J Food Microbiol* 158, 9-13.

Delaquis, P. J., Stanich, K., Girard, B., and Mazza, G. (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology* 74, 101-109.

Delgado Adámez, J., Gamero Samino, E., Valdés Sánchez, E., and González-Gómez, D. (2012). In vitro estimation of the antibacterial activity and antioxidant capacity of aqueous extracts from grape-seeds (Vitis vinifera L.). *Food Control* 24, 136-141.

Denyer, S. P. (1990). Mechanisms of action of biocides. *International Biodeterioration* 26, 89-100.

Devi, K. P., Nisha, S. A., Sakthivel, R., and Pandian, S. K. (2010). Eugenol (an essential oil of clove) acts as an antibacterial agent against Salmonella typhi by disrupting the cellular membrane. *Journal of Ethnopharmacology* 130, 107-115.

Devlieghere, F., Vermeiren, L., and Debevere, J. (2004). New preservation technologies: Possibilities and limitations. *International Dairy Journal* 14, 273-285.

Dickert, H., Machka, K., and Braveny, I. (1981). The uses and limitations of disc diffusion in the antibiotic sensitivity testing of bacteria. *Infection* 9, 18-24.

Djenane, D., Aïder, M., Yangüela, J., Idir, L., Gómez, D., and Roncalés, P. (2012). Antioxidant and antibacterial effects of Lavandula and Mentha essential oils in minced beef inoculated with E. coli O157:H7 and S. aureus during storage at abuse refrigeration temperature. *Meat Science* 92, 667-674.

Dorman, H. J. D., Figueiredo, A. C., Barroso, J. G., and Deans, S. G. (2000). In vitro evaluation of antioxidant activity of essential oils and their components. *Flavour and Fragrance Journal* 15, 12-16.

Economou, G., Panagopoulos, G., Tarantilis, P., Kalivas, D., Kotoulas, V., Travlos, I. S., Polysiou, M., and Karamanos, A. (2011). Variability in essential oil content and composition of Origanum hirtum L., Origanum onites L., Coridothymus capitatus (L.) and Satureja thymbra L. populations from the Greek island Ikaria. *Industrial Crops and Products* 33, 236-241.

El-Abbassi, A., Kiai, H., and Hafidi, A. (2012). Phenolic profile and antioxidant activities of olive mill wastewater. *Food Chemistry* 132, 406-412.

El-Sawi, S. A., and Mohamed, M. A. (2002). Cumin herb as a new source of essential oils and its response to foliar spray with some micro-elements. *Food Chemistry* 77, 75-80.

Elzinga, E. J., Huang, J.-H., Chorover, J., and Kretzschmar, R. (2012). ATR-FTIR Spectroscopy Study of the Influence of pH and Contact Time on the Adhesion of Shewanella putrefaciens Bacterial Cells to the Surface of Hematite. *Environmental Science & Technology* 46, 12848-12855.

Farag, R. S., Daw, Z. Y., Hewedi, F. M., and El-Baroty, G. S. A. (1989). Antimicrobial activity of some Egyptian spice essential oils. *Journal of food protection* 52, 665-667.

Filipowicz, N., Kamiński, M., Kurlenda, J., Asztemborska, M., and Ochocka, J. R. (2003). Antibacterial and antifungal activity of juniper berry oil and its selected components. *Phytotherapy Research* 17, 227-231.

Friedman, M., Henika, P. R., and Mandrell, R. E. (2002). Bactericidal Activities of Plant Essential Oils and Some of Their Isolated Constituents against Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, and Salmonella enterica. *Journal of Food Protection* 65, 1545-1560.

Gallucci, M. N., Oliva, M., Casero, C., Dambolena, J., Luna, A., Zygadlo, J., and Demo, M. (2009). Antimicrobial combined action of terpenes against the food-borne microorganisms Escherichia coli, Staphylococcus aureus and Bacillus cereus. *Flavour and Fragrance Journal* 24, 348-354.

Gomes Neto, N. J., da Silva Luz, I., Honório, V. G., da Conceição, M. L., and de Souza, E. L. (2012). Pseudomonas aeruginosa cells adapted to Rosmarinus officinalis L. essential oil and 1,8-cineole acquire no direct and cross protection in a meat-based broth. *Food Research International* 49, 143-146.

Gomez, M., and Lorenzo, J. M. (2012). Effect of packaging conditions on shelf-life of fresh foal meat. *Meat Sci* 91, 513-20.

Goñi, P., López, P., Sánchez, C., Gómez-Lus, R., Becerril, R., and Nerín, C. (2009). Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. *Food Chemistry* 116, 982-989.

Gourine, N., Yousfi, M., Bombarda, I., Nadjemi, B., Stocker, P., and Gaydou, E. M. (2010). Antioxidant activities and chemical composition of essential oil of Pistacia atlantica from Algeria. *Industrial Crops and Products* 31, 203-208.

Göktürk Baydar, N., Özkan, G., and Yaşar, S. (2007). Evaluation of the antiradical and antioxidant potential of grape extracts. *Food Control* 18, 1131-1136.

Guan, W., Li, S., Yan, R., Tang, S., and Quan, C. (2007). Comparison of essential oils of clove buds extracted with supercritical carbon dioxide and other three traditional extraction methods. *Food Chemistry* 101, 1558-1564.

Gutierrez, J., Barry-Ryan, C., and Bourke, P. (2008). The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology* 124, 91-97.

Gutierrez, J., Barry-Ryan, C., and Bourke, P. (2009). Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interactions with food components. *Food Microbiol* 26, 142-50.

Gülçin, İ., Güngör Şat, İ., Beydemir, Ş., Elmastaş, M., and İrfan Küfrevioğlu, Ö. (2004). Comparison of antioxidant activity of clove (Eugenia caryophylata Thunb) buds and lavender (Lavandula stoechas L.). *Food Chemistry* 87, 393-400.

Hammer, K. A., Carson, C. F., and Riley, T. V. (1999). Antimicrobial activity of essential oils and other plant extracts. *J Appl Microbiol* 86, 985-90.

Hao, Y. Y., Brackett, R. E., and Doyle, M. P. (1998). Efficacy of plant extracts in inhibiting Aeromonas hydrophila and Listeria monocytogenes in refrigerated, cooked poultry. *Food microbiology* 15, 367-378.

Hayes, J. E., Allen, P., Brunton, N., O'Grady, M. N., and Kerry, J. P. (2011). Phenolic composition and in vitro antioxidant capacity of four commercial phytochemical products: Olive leaf extract (Olea europaea L.), lutein, sesamol and ellagic acid. *Food Chemistry* 126, 948-955.

Hazzit, M., Baaliouamer, A., Veríssimo, A. R., Faleiro, M. L., and Miguel, M. G. (2009). Chemical composition and biological activities of Algerian Thymus oils. *Food Chemistry* 116, 714-721.

Helander, I. M., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E. J., Gorris, L. G. M., and von Wright, A. (1998). Characterization of the Action of Selected Essential Oil Components on Gram-Negative Bacteria. *Journal of Agricultural and Food Chemistry* 46, 3590-3595.

Hernández-Jiménez, A., Gómez-Plaza, E., Martínez-Cutillas, A. n., and Kennedy, J. A. (2009). Grape Skin and Seed Proanthocyanidins from Monastrell × Syrah Grapes. *Journal of Agricultural and Food Chemistry* 57, 10798-10803.

Hinneburg, I., Damien Dorman, H. J., and Hiltunen, R. (2006). Antioxidant activities of extracts from selected culinary herbs and spices. *Food Chemistry* 97, 122-129.

Hosni, K., Hassen, I., Chaâbane, H., Jemli, M., Dallali, S., Sebei, H., and Casabianca, H. (2013). Enzyme-assisted extraction of essential oils from thyme (Thymus capitatus L.) and rosemary (Rosmarinus officinalis L.): Impact on yield, chemical composition and antimicrobial activity. *Industrial Crops and Products* 47, 291-299.

Huleihel, M., Pavlov, V., and Erukhimovitch, V. (2009). The use of FTIR microscopy for the evaluation of anti-bacterial agents activity. *Journal of Photochemistry and Photobiology B: Biology* 96, 17-23.

Hyldgaard, M., Mygind, T., and Meyer, R. L. (2012). Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components. *Front Microbiol* 3, 12.

Iacopini, P., Baldi, M., Storchi, P., and Sebastiani, L. (2008). Catechin, epicatechin, quercetin, rutin and resveratrol in red grape: Content, in vitro antioxidant activity and interactions. *Journal of Food Composition and Analysis* 21, 589-598.

Jacob, J. K., Hakimuddin, F., Paliyath, G., and Fisher, H. (2008). Antioxidant and antiproliferative activity of polyphenols in novel high-polyphenol grape lines. *Food Research International* 41, 419-428.

Jayaprakasha, G. K., Selvi, T., and Sakariah, K. K. (2003). Antibacterial and antioxidant activities of grape (Vitis vinifera) seed extracts. *Food Research International* 36, 117-122.

Jayasena, D. D., and Jo, C. (2013). Essential oils as potential antimicrobial agents in meat and meat products: A review. *Trends in Food Science & Technology*.

Jirovetz, L., Buchbauer, G., Stoilova, I., Stoyanova, A., Krastanov, A., and Schmidt, E. (2006). Chemical Composition and Antioxidant Properties of Clove Leaf Essential Oil. *Journal of Agricultural and Food Chemistry* 54, 6303-6307.

Joshi, S. C., Verma, A. R., and Mathela, C. S. (2010). Antioxidant and antibacterial activities of the leaf essential oils of Himalayan Lauraceae species. *Food and Chemical Toxicology* 48, 37-40.

Juven, B. J., Kanner, J., Schved, F., and Weisslowicz, H. (1994). Factors that interact with the antibacterial action of thyme essential oil and its active constituents. *J Appl Bacteriol* 76, 626-31.

Kao, T.-T., Tu, H.-C., Chang, W.-N., Chen, B.-H., Shi, Y.-Y., Chang, T.-C., and Fu, T.-F. (2010). Grape seed extract inhibits the growth and pathogenicity of Staphylococcus aureus by interfering with dihydrofolate reductase activity and folate-mediated one-carbon metabolism. *International Journal of Food Microbiology* 141, 17-27.

Karadag, A., Ozcelik, B., and Saner, S. (2009). Review of Methods to Determine Antioxidant Capacities. *Food Analytical Methods* 2, 41-60.

Karaosmanoglu, H., Soyer, F., Ozen, B., and Tokatli, F. (2010). Antimicrobial and Antioxidant Activities of Turkish Extra Virgin Olive Oils. *Journal of Agricultural and Food Chemistry* 58, 8238-8245.

Karatzas, A. K., Kets, E. P., Smid, E. J., and Bennik, M. H. (2001). The combined action of carvacrol and high hydrostatic pressure on Listeria monocytogenes Scott A. *J Appl Microbiol* 90, 463-9.

Katalinić, V., Možina, S. S., Skroza, D., Generalić, I., Abramovič, H., Miloš, M., Ljubenkov, I., Piskernik, S., Pezo, I., Terpinc, P., and Boban, M. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 Vitis vinifera varieties grown in Dalmatia (Croatia). *Food Chemistry* 119, 715-723.

Kedia, A., Prakash, B., Mishra, P. K., and Dubey, N. K. (2014). Antifungal and antiaflatoxigenic properties of Cuminum cyminum (L.) seed essential oil and its efficacy as a preservative in stored commodities. *International Journal of Food Microbiology* 168–169, 1-7.

Kilic, A., Hafizoglu, H., Kollmannsberger, H., and Nitz, S. (2004). Volatile constituents and key odorants in leaves, buds, flowers, and fruits of Laurus nobilis L. *J Agric Food Chem* 52, 1601-6.

Kim, J., Marshall, M. R., and Wei, C.-i. (1995). Antibacterial activity of some essential oil components against five foodborne pathogens. *Journal of Agricultural and Food Chemistry* 43, 2839-2845.

Klančnik, A., Piskernik, S., Jeršek, B., and Možina, S. S. (2010). Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. *Journal of Microbiological Methods* 81, 121-126.

Lambert, R. J., Skandamis, P. N., Coote, P. J., and Nychas, G. J. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol* 91, 453-62.

Lau, D. W., and King, A. J. (2003). Pre- and Post-Mortem Use of Grape Seed Extract in Dark Poultry Meat To Inhibit Development of Thiobarbituric Acid Reactive Substances. *Journal of Agricultural and Food Chemistry* 51, 1602-1607.

Lee, K.-G., and Shibamoto, T. (2001). Antioxidant property of aroma extract isolated from clove buds [Syzygium aromaticum (L.) Merr. et Perry]. *Food Chemistry* 74, 443-448.

Lee, O.-H., and Lee, B.-Y. (2010). Antioxidant and antimicrobial activities of individual and combined phenolics in Olea europaea leaf extract. *Bioresource Technology* 101, 3751-3754.

Lee, O.-H., Lee, B.-Y., Lee, J., Lee, H.-B., Son, J.-Y., Park, C.-S., Shetty, K., and Kim, Y.-C. (2009). Assessment of phenolics-enriched extract and fractions of olive leaves and their antioxidant activities. *Bioresource Technology* 100, 6107-6113.

Lutterodt, H., Luther, M., Slavin, M., Yin, J.-J., Parry, J., Gao, J.-M., and Yu, L. (2010). Fatty acid profile, thymoquinone content, oxidative stability, and antioxidant properties of cold-pressed black cumin seed oils. *LWT - Food Science and Technology* 43, 1409-1413.

Lv, F., Liang, H., Yuan, Q., and Li, C. (2011). In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. *Food Research International* 44, 3057-3064.

Mackay, M. L., Milne, K., and Gould, I. M. (2000). Comparison of methods for assessing synergic antibiotic interactions. *International Journal of Antimicrobial Agents* 15, 125-129.

Maier, T., Schieber, A., Kammerer, D. R., and Carle, R. (2009). Residues of grape (Vitis vinifera L.) seed oil production as a valuable source of phenolic antioxidants. *Food Chemistry* 112, 551-559.

Marino, M., Bersani, C., and Comi, G. (1999). Antimicrobial activity of the essential oils of Thymus vulgaris L. measured using a bioimpedometric method. *J Food Prot* 62, 1017-23.

Markin, D., Duek, L., and Berdicevsky, I. (2003). In vitro antimicrobial activity of olive leaves. *Mycoses* 46, 132-136.

Martini, H., Weidenborner, M., Adams, S., and Kunz, B. (1996). Eugenol and carvacrol: the main fungicidal compounds in clove and savory. *Italian journal of food science.* 8, 63-67.

Masotti, V., Juteau, F., Bessiere, J. M., and Viano, J. (2003). Seasonal and phenological variations of the essential oil from the narrow endemic species Artemisia molinieri and its biological activities. *J Agric Food Chem* 51, 7115-21.

Miguel, M. G. (2010). Antioxidant activity of medicinal and aromatic plants. A review. *Flavour and Fragrance Journal* 25, 291-312.

Moosavy, M.-H., Basti, A. A., Misaghi, A., Salehi, T. Z., Abbasifar, R., Mousavi, H. A. E., Alipour, M., Razavi, N. E., Gandomi, H., and Noori, N. (2008). Effect of Zataria multiflora Boiss. essential oil and nisin on Salmonella typhimurium and Staphylococcus aureus in a food model system and on the bacterial cell membranes. *Food Research International* 41, 1050-1057.

Moreno, S., Scheyer, T., Romano, C. S., and Vojnov, A. A. (2006). Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. *Free Radic Res* 40, 223-31.

Mourey, A., and Canillac, N. (2002). Anti-Listeria monocytogenes activity of essential oils components of conifers. *Food Control* 13, 289-292.

Mulinacci, N., Romani, A., Galardi, C., Pinelli, P., Giaccherini, C., and Vincieri, F. F. (2001). Polyphenolic Content in Olive Oil Waste Waters and Related Olive Samples. *Journal of Agricultural and Food Chemistry* 49, 3509-3514.

Mulyaningsih, S., Sporer, F., Zimmermann, S., Reichling, J., and Wink, M. (2010). Synergistic properties of the terpenoids aromadendrene and 1,8-cineole from the essential oil of Eucalyptus globulus against antibiotic-susceptible and antibiotic-resistant pathogens. *Phytomedicine* 17, 1061-6.

Mylonaki, S., Kiassos, E., Makris, D. P., and Kefalas, P. (2008). Optimisation of the extraction of olive (Olea europaea) leaf phenolics using water/ethanol-based

solvent systems and response surface methodology. *Anal Bioanal Chem* 392, 977-85.

Napoli, E. M., Curcuruto, G., and Ruberto, G. (2010). Screening of the essential oil composition of wild Sicilian thyme. *Biochemical Systematics and Ecology* 38, 816-822.

Naveena, B. M., Muthukumar, M., Sen, A. R., Babji, Y., and Murthy, T. R. (2006). Improvement of shelf-life of buffalo meat using lactic acid, clove oil and vitamin C during retail display. *Meat Sci* 74, 409-15.

Negi, P. S. (2012). Plant extracts for the control of bacterial growth: efficacy, stability and safety issues for food application. *Int J Food Microbiol* 156, 7-17.

Novo, D., Perlmutter, N. G., Hunt, R. H., and Shapiro, H. M. (1999). Accurate flow cytometric membrane potential measurement in bacteria using diethyloxacarbocyanine and a ratiometric technique. *Cytometry* 35, 55-63.

Nychas, G.-J. E., Skandamis, P. N., Tassou, C. C., and Koutsoumanis, K. P. (2008). Meat spoilage during distribution. *Meat Science* 78, 77-89.

Obied, H. K., Allen, M. S., Bedgood, D. R., Prenzler, P. D., Robards, K., and Stockmann, R. (2005). Bioactivity and Analysis of Biophenols Recovered from Olive Mill Waste. *Journal of Agricultural and Food Chemistry* 53, 823-837.

Obied, H. K., Bedgood Jr, D. R., Prenzler, P. D., and Robards, K. (2007). Bioscreening of Australian olive mill waste extracts: Biophenol content, antioxidant, antimicrobial and molluscicidal activities. *Food and Chemical Toxicology* 45, 1238-1248.

Ojeda-Sana, A. M., van Baren, C. M., Elechosa, M. A., Juárez, M. A., and Moreno, S. (2013). New insights into antibacterial and antioxidant activities of rosemary essential oils and their main components. *Food Control* 31, 189-195.

Oussalah, M., Caillet, S., Saucier, L., and Lacroix, M. (2006). Antimicrobial effects of selected plant essential oils on the growth of a Pseudomonas putida strain isolated from meat. *Meat Sci* 73, 236-44.

Ozcan, M. M., Erel, O., and Herken, E. E. (2009). Antioxidant activity, phenolic content, and peroxide value of essential oil and extracts of some medicinal and aromatic plants used as condiments and herbal teas in Turkey. *J Med Food* 12, 198-202.

Özcan, M., and Boyraz, N. (2000). Antifungal properties of some herb decoctions. *European Food Research and Technology* 212, 86-88.

Özcan, M. Ö., and Erkmen, O. (2001). Antimicrobial activity of the essential oils of Turkish plant spices. *European Food Research and Technology* 212, 658-660.

Packiyasothy, V., E., Kyle, and S. (2002). "Antimicrobial properties of some herb essential oils," Australian Institute of Food Science and Technology, North Sydney, AUSTRALIE.

Park, S. N., Lim, Y. K., Freire, M. O., Cho, E., Jin, D., and Kook, J. K. (2012). Antimicrobial effect of linalool and alpha-terpineol against periodontopathic and cariogenic bacteria. *Anaerobe* 18, 369-72.

Pastrana-Bonilla, E., Akoh, C. C., Sellappan, S., and Krewer, G. (2003). Phenolic Content and Antioxidant Capacity of Muscadine Grapes. *Journal of Agricultural and Food Chemistry* 51, 5497-5503.

Pei, R. S., Zhou, F., Ji, B. P., and Xu, J. (2009). Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against E. coli with an improved method. *J Food Sci* 74, M379-83.

Pereira, A., Ferreira, I., Marcelino, F., Valentão, P., Andrade, P., Seabra, R., Estevinho, L., Bento, A., and Pereira, J. (2007a). Phenolic Compounds and Antimicrobial Activity of Olive (Olea europaea L. Cv. Cobrançosa) Leaves. *Molecules* 12, 1153-1162.

Perumalla, A. V. S., and Hettiarachchy, N. S. (2011). Green tea and grape seed extracts - Potential applications in food safety and quality. *Food Research International* 44, 827-839.

Pichersky, E., Noel, J. P., and Dudareva, N. (2006). Biosynthesis of Plant Volatiles: Nature's Diversity and Ingenuity. *Science* 311, 808-811.

Politeo, O., Jukic, M., and Milos, M. (2010). Comparison of chemical composition and antioxidant activity of glycosidically bound and free volatiles from clove (eugenia caryophyllata thunb.). *Journal of Food Biochemistry* 34, 129-141.

Rahman, A., and Kang, S. C. (2009). In vitro control of food-borne and food spoilage bacteria by essential oil and ethanol extracts of Lonicera japonica Thunb. *Food Chemistry* 116, 670-675.

Ramos, C., Teixeira, B., Batista, I., Matos, O., Serrano, C., Neng, N. R., Nogueira, J. M. F., Nunes, M. L., and Marques, A. (2011). Antioxidant and antibacterial activity of essential oil and extracts of bay laurel Laurus nobilis Linnaeus (Lauraceae) from Portugal. *Natural Product Research*, 1-12.

Revilla, E., and Ryan, J. M. (2000). Analysis of several phenolic compounds with potential antioxidant properties in grape extracts and wines by high-performance liquid chromatography-photodiode array detection without sample preparation. *J Chromatogr A* 881, 461-9.

Rhodes, P. L., Mitchell, J. W., Wilson, M. W., and Melton, L. D. (2006). Antilisterial activity of grape juice and grape extracts derived from Vitis vinifera variety Ribier. *International Journal of Food Microbiology* 107, 281-286.

Rockenbach, I. I., Gonzaga, L. V., Rizelio, V. M., Gonçalves, A. E. d. S. S., Genovese, M. I., and Fett, R. (2011). Phenolic compounds and antioxidant activity of seed and skin extracts of red grape (Vitis vinifera and Vitis labrusca) pomace from Brazilian winemaking. *Food Research International* 44, 897-901.

Rodríguez Montealegre, R., Romero Peces, R., Chacón Vozmediano, J. L., Martínez Gascueña, J., and García Romero, E. (2006). Phenolic compounds in skins and

seeds of ten grape Vitis vinifera varieties grown in a warm climate. *Journal of Food Composition and Analysis* 19, 687-693.

Romano, C. S., Abadi, K., Repetto, V., Vojnov, A. A., and Moreno, S. (2009). Synergistic antioxidant and antibacterial activity of rosemary plus butylated derivatives. *Food Chemistry* 115, 456-461.

Rosato, A., Vitali, C., De Laurentis, N., Armenise, D., and Antonietta Milillo, M. (2007). Antibacterial effect of some essential oils administered alone or in combination with Norfloxacin. *Phytomedicine* 14, 727-32.

Ruberto, G., and Baratta, M. T. (2000). Antioxidant activity of selected essential oil components in two lipid model systems. *Food Chemistry* 69, 167-174.

Ruiz-Navajas, Y., Viuda-Martos, M., Sendra, E., Perez-Alvarez, J. A., and Fernández-López, J. (2012). Chemical characterization and antibacterial activity of Thymus moroderi and Thymus piperella essential oils, two Thymus endemic species from southeast of Spain. *Food Control* 27, 294-299.

Safaei-Ghomi, J., Ebrahimabadi, A. H., Djafari-Bidgoli, Z., and Batooli, H. (2009). GC/MS analysis and in vitro antioxidant activity of essential oil and methanol extracts of Thymus caramanicus Jalas and its main constituent carvacrol. *Food Chemistry* 115, 1524-1528.

Sağdıç, O., Kuşçu, A., Özcan, M., and Özçelik, S. (2002). Effects of Turkish spice extracts at various concentrations on the growth of Escherichia coli O157:H7. *Food Microbiology* 19, 473-480.

Sangun, M. K., Aydin, E., Timur, M., Karadeniz, H., Caliskan, M., and Ozkan, A. (2007). Comparison of chemical composition of the essential oil of Laurus nobilis L. leaves and fruits from different regions of Hatay, Turkey. *J Environ Biol* 28, 731-3.

Scherer, R., and Godoy, H. T. (2009). Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method. *Food Chemistry* 112, 654-658.

Schwarz, K., Bertelsen, G., Nissen, L. R., Gardner, P. T., Heinonen, M. I., Hopia, A., Huynh-Ba, T., Lambelet, P., McPhail, D., Skibsted, L. H., and Tijburg, L. (2001). Investigation of plant extracts for the protection of processed foods against lipid oxidation. Comparison of antioxidant assays based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds. *European Food Research and Technology* 212, 319-328.

Serra, A. T., Matias, A. A., Nunes, A. V. M., Leitão, M. C., Brito, D., Bronze, R., Silva, S., Pires, A., Crespo, M. T., San Romão, M. V., and Duarte, C. M. (2008). In vitro evaluation of olive- and grape-based natural extracts as potential preservatives for food. *Innovative Food Science & Emerging Technologies* 9, 311-319.

Shan, B., Cai, Y.-Z., Brooks, J. D., and Corke, H. (2007). Antibacterial Properties and Major Bioactive Components of Cinnamon Stick (Cinnamomum burmannii): Activity against Foodborne Pathogenic Bacteria. *Journal of Agricultural and Food Chemistry* 55, 5484-5490.

Shan, B., Cai, Y. Z., Sun, M., and Corke, H. (2005). Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J Agric Food Chem* 53, 7749-59.

Shi, J., Yu, J., Pohorly, J. E., and Kakuda, Y. (2003). Polyphenolics in grape seeds-biochemistry and functionality. *J Med Food* 6, 291-9.

Shimamura, T., Zhao, W.-H., and Hu, Z.-Q. (2007). Mechanism of Action and Potential for Use of Tea Catechin as an Antiinfective Agent. *Anti-Infective Agents in Medicinal Chemistry (Formerly `Current Medicinal Chemistry - Anti-Infective Agents)* 6, 57-62.

Singh, S., and Singh, R. P. (2008). In Vitro Methods of Assay of Antioxidants: An Overview. *Food Reviews International* 24, 392-415.

Skandamis, P. N., and Nychas, G. J. (2000). Development and evaluation of a model predicting the survival of Escherichia coli O157:H7 NCTC 12900 in homemade eggplant salad at various temperatures, pHs, and oregano essential oil concentrations. *Appl Environ Microbiol* 66, 1646-53.

Slinkard, K., and Singleton, V. L. (1977). Total Phenol Analysis: Automation and Comparison with Manual Methods. *American Journal of Enology and Viticulture* 28, 49-55.

Smith, P., Stewart, and Fyfe (1998). Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Letters in Applied Microbiology* 26, 118-122.

Sokmen, A., Gulluce, M., Askin Akpulat, H., Daferera, D., Tepe, B., Polissiou, M., Sokmen, M., and Sahin, F. (2004). The in vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius. *Food Control* 15, 627-634.

Solomakos, N., Govaris, A., Koidis, P., and Botsoglou, N. (2008). The antimicrobial effect of thyme essential oil, nisin and their combination against Escherichia coli O157:H7 in minced beef during refrigerated storage. *Meat Sci* 80, 159-66.

Sudan, R., Bhagat, M., Gupta, S., Chitrarakha, and Devi, T. (2013). Comparative analysis of cytotoxic and antioxidant potential of edible Cinnamomum verum (bark) and Cinnamomum tamala (Indian bay leaf). *Free Radicals and Antioxidants*.

Sudjana, A. N., D'Orazio, C., Ryan, V., Rasool, N., Ng, J., Islam, N., Riley, T. V., and Hammer, K. A. (2009). Antimicrobial activity of commercial Olea europaea (olive) leaf extract. *International Journal of Antimicrobial Agents* 33, 461-463.

Teixeira, B., Marques, A., Ramos, C., Neng, N. R., Nogueira, J. M. F., Saraiva, J. A., and Nunes, M. L. (2013). Chemical composition and antibacterial and antioxidant properties of commercial essential oils. *Industrial Crops and Products* 43, 587-595.

Tepe, B., Sokmen, M., Akpulat, H. A., Daferera, D., Polissiou, M., and Sokmen, A. (2005). Antioxidative activity of the essential oils of Thymus sipyleus subsp.

sipyleus var. sipyleus and Thymus sipyleus subsp. sipyleus var. rosulans. *Journal of Food Engineering* 66, 447-454.

Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., and Hawkins Byrne, D. (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis* 19, 669-675.

Tiwari, B. K., Valdramidis, V. P., O' Donnell, C. P., Muthukumarappan, K., Bourke, P., and Cullen, P. J. (2009). Application of Natural Antimicrobials for Food Preservation. *Journal of Agricultural and Food Chemistry* 57, 5987-6000.

Trombetta, D., Castelli, F., Sarpietro, M. G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G., and Bisignano, G. (2005). Mechanisms of antibacterial action of three monoterpenes. *Antimicrob Agents Chemother* 49, 2474-8.

Tserennadmid, R., Tako, M., Galgoczy, L., Papp, T., Pesti, M., Vagvolgyi, C., Almassy, K., and Krisch, J. (2011). Anti yeast activities of some essential oils in growth medium, fruit juices and milk. *Int J Food Microbiol* 144, 480-6.

Turgis, M., Vu, K. D., Dupont, C., and Lacroix, M. (2012). Combined antimicrobial effect of essential oils and bacteriocins against foodborne pathogens and food spoilage bacteria. *Food Research International* 48, 696-702.

Tyagi, A. K., and Malik, A. (2010). In situ SEM, TEM and AFM studies of the antimicrobial activity of lemon grass oil in liquid and vapour phase against Candida albicans. *Micron* 41, 797-805.

Ultee, Gorris, and Smid (1998). Bactericidal activity of carvacrol towards the food-borne pathogen Bacillus cereus. *Journal of Applied Microbiology* 85, 211-218.

Ultee, A., Bennik, M. H. J., and Moezelaar, R. (2002). The Phenolic Hydroxyl Group of Carvacrol Is Essential for Action against the Food-Borne Pathogen Bacillus cereus. *Applied and Environmental Microbiology* 68, 1561-1568.

Verma, R. S., Padalia, R. C., Chanotiya, C. S., and Chauhan, A. (2010). Chemical investigation of the essential oil of Thymus linearis (Benth. ex Benth) from western Himalaya, India. *Nat Prod Res* 24, 1890-6.

Viuda-Martos, M., Ruiz Navajas, Y., Sánchez Zapata, E., Fernández-López, J., and Pérez-Álvarez, J. A. (2010). Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet. *Flavour and Fragrance Journal* 25, 13-19.

Vrinda Menon, K., and Garg, S. R. (2001). Inhibitory effect of clove oil on Listeria monocytogenes in meat and cheese. *Food Microbiology* 18, 647-650.

Vuuren, S. F. v., and Viljoen, A. M. (2007). Antimicrobial activity of limonene enantiomers and 1,8-cineole alone and in combination. *Flavour and Fragrance Journal* 22, 540-544.

Wang, H. F., Wang, Y. K., and Yih, K. H. (2008). DPPH free-radical scavenging ability, total phenolic content, and chemical composition analysis of forty-five kinds of essential oils. *J Cosmet Sci* 59, 509-22.

Wei, A., and Shibamoto, T. (2010). Antioxidant/lipoxygenase inhibitory activities and chemical compositions of selected essential oils. *J Agric Food Chem* 58, 7218-25.

Wilkins, T. D., and Thiel, T. (1973). Modified broth-disk method for testing the antibiotic susceptibility of anaerobic bacteria. *Antimicrob Agents Chemother* 3, 350-6.

Xia, E. Q., Deng, G. F., Guo, Y. J., and Li, H. B. (2010). Biological activities of polyphenols from grapes. *Int J Mol Sci* 11, 622-46.

Xu, J., Zhou, F., Ji, B. P., Pei, R. S., and Xu, N. (2008). The antibacterial mechanism of carvacrol and thymol against Escherichia coli. *Lett Appl Microbiol* 47, 174-9.

Xu, W., Qu, W., Huang, K., Guo, F., Yang, J., Zhao, H., and Luo, Y. (2007). Antibacterial effect of Grapefruit Seed Extract on food-borne pathogens and its application in the preservation of minimally processed vegetables. *Postharvest Biology and Technology* 45, 126-133.

Yilmaz, Y., and Toledo, R. T. (2004). Health aspects of functional grape seed constituents. *Trends in Food Science & Technology* 15, 422-433.

Zhou, F., Ji, B., Zhang, H., Jiang, H. U. I., Yang, Z., Li, J., Li, J., and Yan, W. (2007). The antibacterial effect of cinnamaldehyde, thymol, carvacrol and their combinations against the foodborne pathogen salmonella typhimurium. *Journal of Food Safety* 27, 124-133.

APPENDIX A

GROWTH AND INHIBITION CURVES

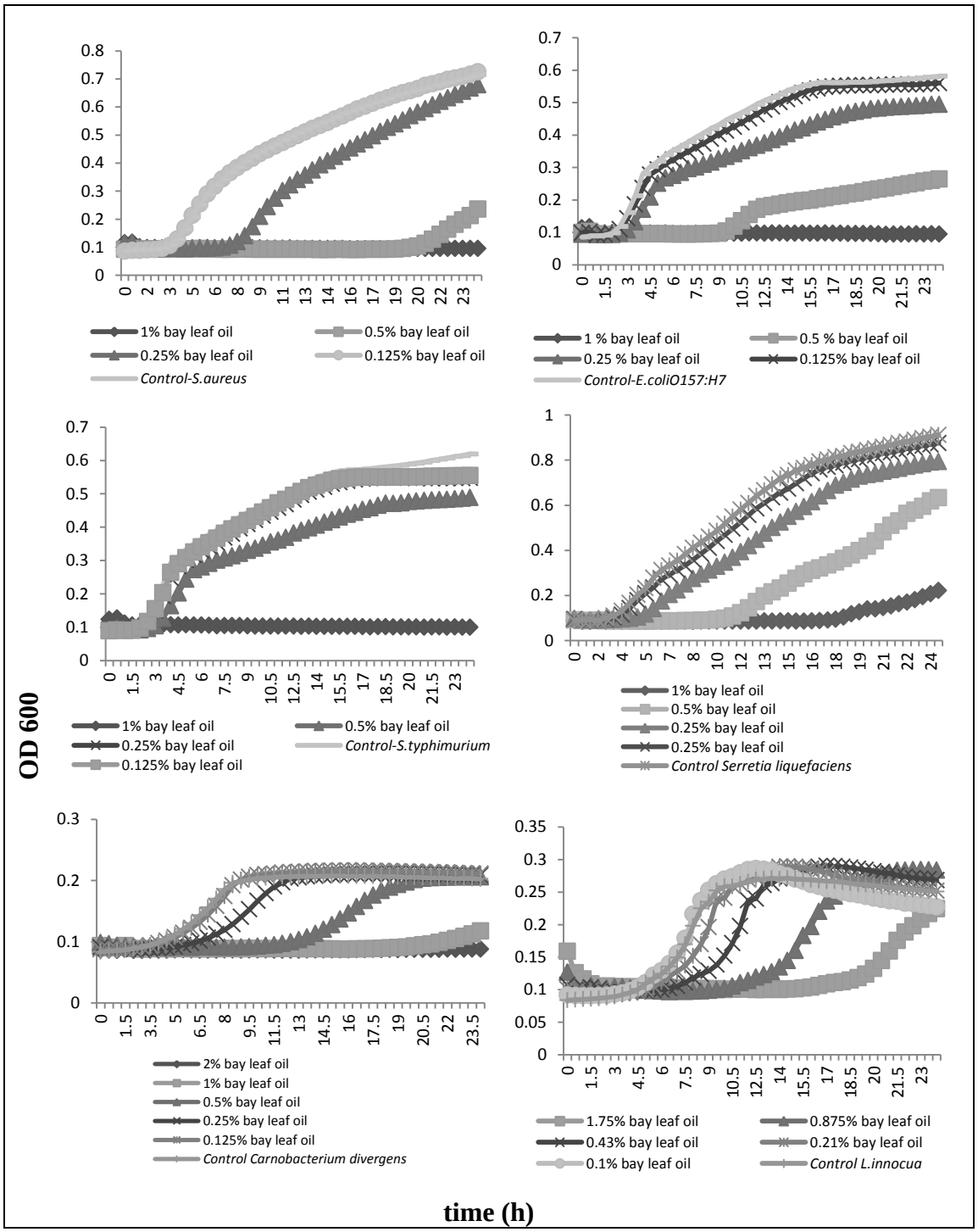


Figure A.1. The growth and inhibition of bacteria treated with bay leaf EO

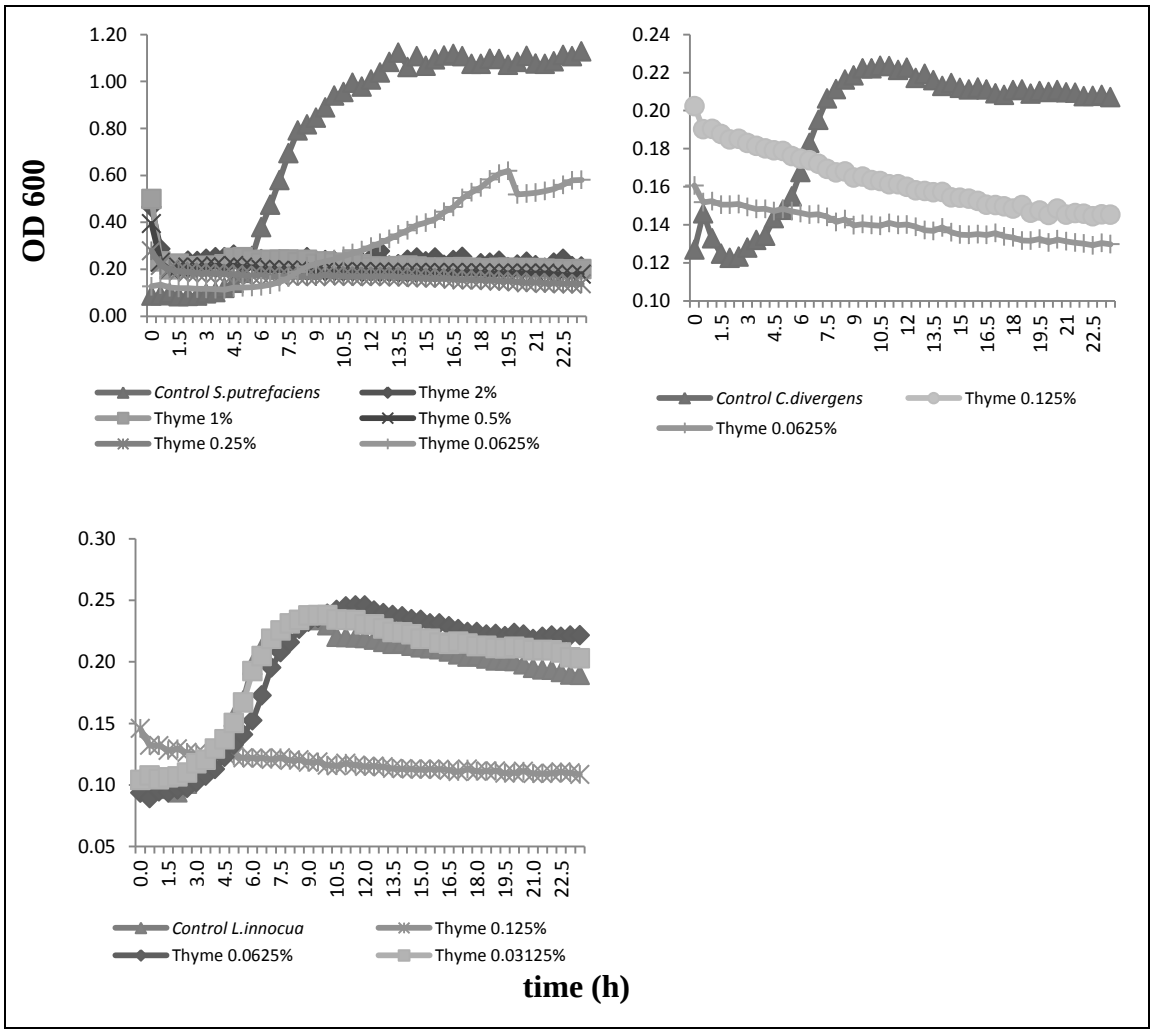


Figure A.2. The growth and inhibition of bacteria treated with thyme EO

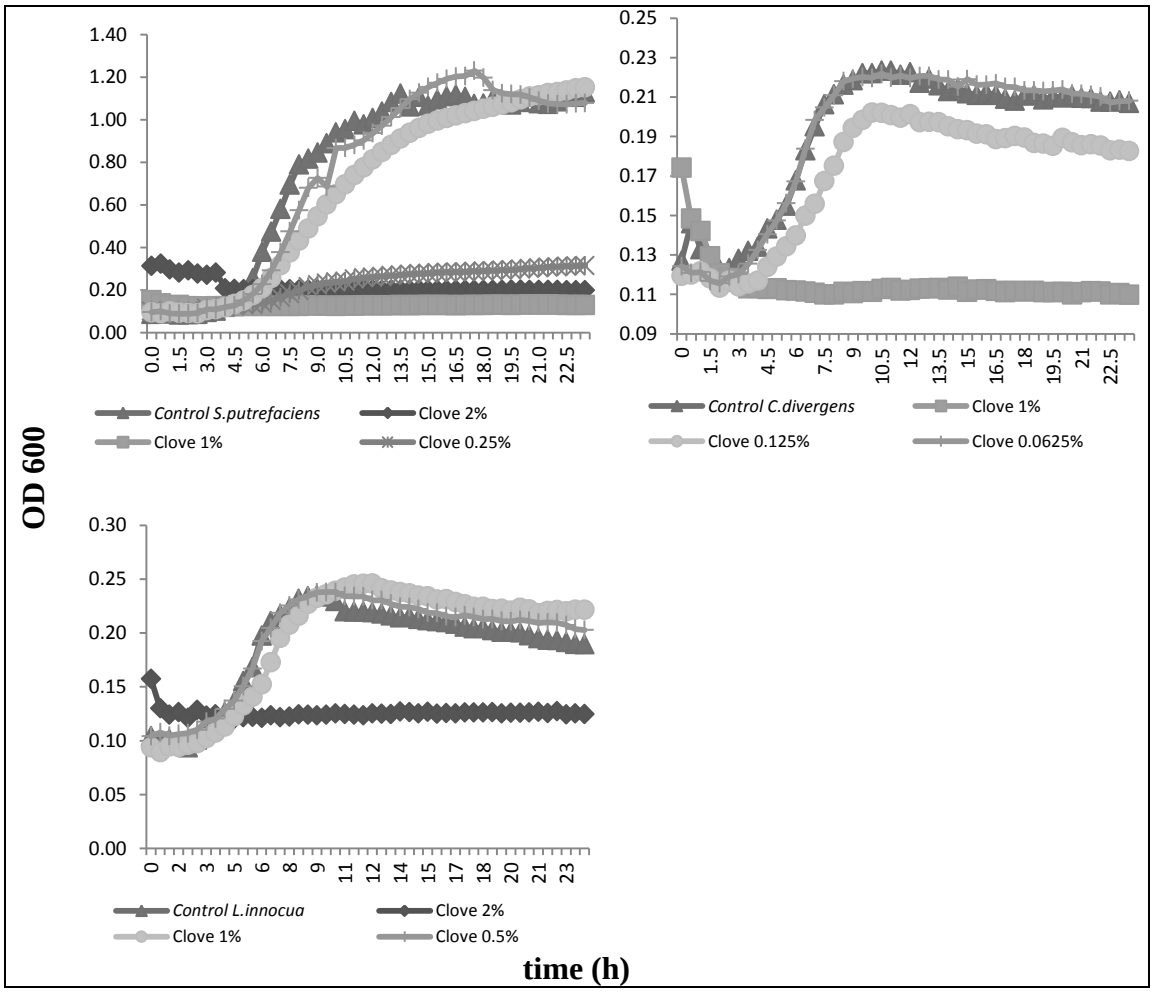


Figure A.3. The growth and inhibition of bacteria treated with clove EO

APPENDIX B

CALIBRATION GRAPHICS

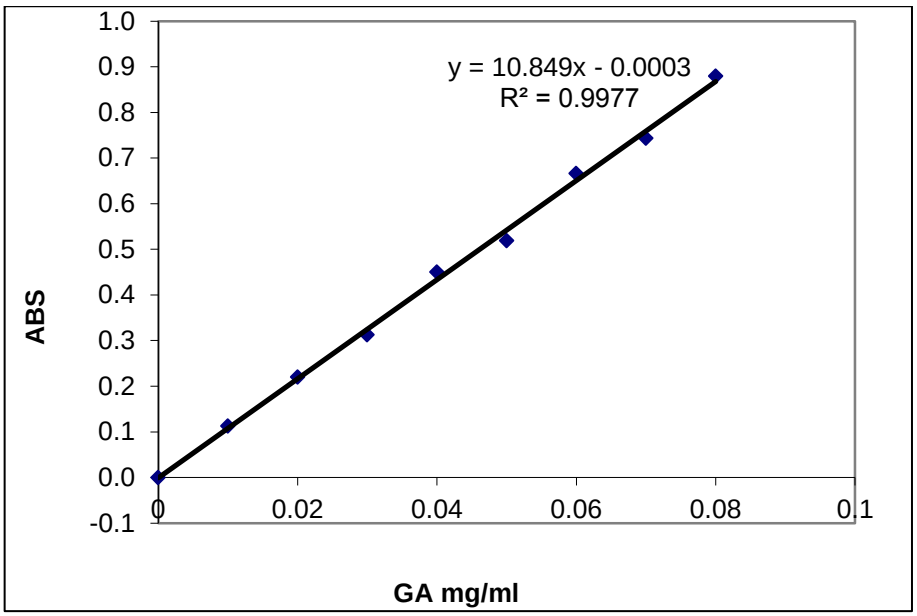


Figure B.1. Calibration curve of Gallic acid for Total Phenol Content

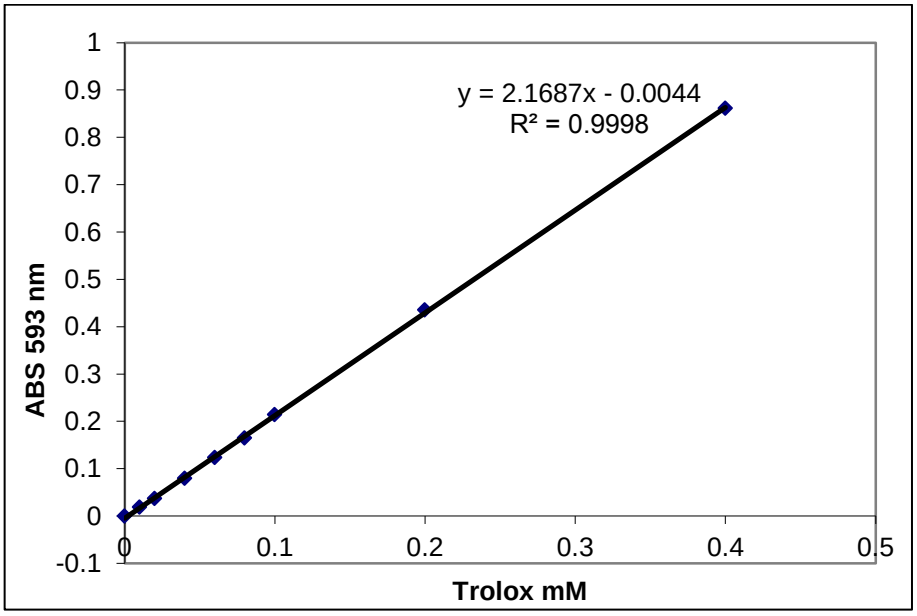


Figure B.2. Calibration curve of Trolox for FRAP assay

VITA

Name, Surname: Hatice YAVUZDURMAZ

Date of Birth : 04.05.1980

Place of Birth: Batman

Education

2007 Master of Science (M.S.) İzmir Institute of Technology, Department of Food Engineering

2003 Bachelor of Science (B.S.) Ankara University, Department of Food Engineering

Academic Work Experience:

Research Assistant, İzmir Institute of Technology, Food Engineering 2005-2014

Scientific Articles Published in International Journals:

- Alkan D., Aydemir LY., Arcan I., Yavuzdurmaz H., Atabay H I., Ceylan C., Yemenicioglu A. Development of Flexible Antimicrobial Packaging Materials against *Campylobacter jejuni* by Incorporation of Gallic Acid into Zein-Based Films. *Journal of Agricultural and Food Chemistry*, 59,20, 11003-11010, 2011.

Presentations:

- Yavuzdurmaz H., Baysal H. Chemical Composition and Antimicrobial Effect of Turkish Bay Laurel. IFT 12 Annual Meeting and Food Expo, June 2012, Las Vegas, Nevada USA.