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Antibacterial And Antioxidative Properties Of Essential Oils From Armoise (Artemisia Herba-Alba), Bay (Laurus Nobilis) And Rose Geranium (Pelargonium Capitatum X Radens) In Vitro

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Biographical Sketch

Ragina B. Rafiq was born at Chapainawabgonj, Bangladesh in 1st November, 1982, to Md. Rafiqul Alam and Begum Rokeya Rafiq. She received her bachelor's degree in Chemistry from University of Chittagong, Bangladesh in the year 2008. From the same institution she received a master's degree in Chemistry focusing on Inorganic Chemistry in the year 2010. During her masters, her research was concentrated on the biological and toxicological investigation of milk and sweetmeats available in Chittagong, Bangladesh. She became interested in food science and decided to continue higher education in this field. She enrolled in North Carolina A&T State University in the Department of Family and Consumer Sciences for her graduate studies. Her graduate research has focused on the evaluation of antibacterial and antioxidative properties of three different essential oils (armoise, bay and rose geranium). She has also served as Graduate Research Assistant in the Department of Family and Consumer Sciences. After graduation, Mrs. Rafiq plans to pursue a doctoral degree in food science.

Dedication

I dedicate my current research work to my parents, Md. Rafiqul Alam and Begum Rokeya Rafiq who always inspired me in achieving higher education.

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List of Symbols and Abbreviations

ABTS	2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
BHI	Brain Heart Infusion
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
ºC	Celsius
CDC	Center for Disease Control and Prevention
CFU	Colony Forming Unit
DPPH	1,1-diphenyl-2-picrylhydrazyl
EO	Essential oil
E. coli	*Escherichia coli*
DAEC	Diffusely adherent *E.coli*
EAEC	Enteroaggregative *E.coli*
EHEC	Enterohemorrhagic *E.coli*
EIEC	Enteroinvasive *E.coli*
EPEC	Enteropathogenic *E.coli*
ETEC	Enterotoxigenic *E.coli*
GC-MS	Gas chromatography-mass spectrometry
HC	Hemorrhagic colitis
HUS	Hemolytic Uremic Syndrome
h	Hour

LB	Luria-Bertani
MDA	Malondialdehyde
µL	Microliters
µM	Micromolar
MIC	Minimum inhibitory concentration
MLC	Minimum lethal concentration
ND	Not detectable
nm	Nanometers
mL	milliliter
O.D	Optical density
PG	Propyl gallet
ppm	Parts per million
*S.*Typhimurium	*Salmonella.* Typhimurium
SD	Standard deviation
STEC	Shiga Toxin Producing *E. coli*
Spp	Species
TBHQ	Tertiary butylhydroquinone
TTP	Thrombatic thrombocytopenic purpura
v/v	Volume/volume

Abstract

The prevalence of food contamination by pathogenic bacteria resulting in foodborne illness has raised concern to the food industry. In addition, oxidation in food, which affects the shelf life and quality of the food through the formation of off odor and off flavor, is another concern to the food industry. Nitrates, sulfates, benzoates of sodium and potassium, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are commonly used as preservatives in the food industry. However, long-term safety issues of synthetic preservatives in human health along with negative perception of consumers have lead researchers to find alternative natural preservatives. The purpose of this study is to find the most effective essential oil (EO) that has high inhibitory effect against pathogenic bacteria as well as high antioxidant activity among three commercially available EOs derived from armoise (*Artemisia herba-alba*), bay (*Laurus nobilis*) and rose geranium (*Pelargonium capitatum x radens*) plants. Antibacterial activity was tested against *E. coli* O157:H7 and *Salmonella* Typhimurium using growth over time assay and agar diffusion spot technique. The antioxidant activity was assessed using DPPH radical scavenging test, reducing power method and total antioxidant activity assay. Results showed that all three EOs showed antibacterial activity with different susceptibility. *S.* Typhimurium growth was completely inhibited by 0.2% of each EO whereas *E. coli* O157:H7 growth was completely prevented by 0.5% of each EO in growth over time assay. Among the three EOs, armoise exhibited the most effective inhibition against these two bacteria. However, rose geranium EO had the most effective inhibition against these bacteria in agar diffusion spot technique confirmed by the lowest minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC).

These EOs also demonstrated antioxidant activity with highest effect in bay EO. Taken together, rose geranium EO efficiently inhibited both *S.* Typhimurium and *E. coli* O157:H7 growth through two different antimicrobial assays with moderate antioxidant activity. This indicates that rose geranium EO may be a potential candidate to be developed as a natural preservative in the food industry.

CHAPTER 1

Introduction

The presence of microbial pathogens like bacteria, parasites, fungi or viruses in food products may cause human illness which in general is known as foodborne illness (Buzby, Frenzen, & Rasco, 2001). These pathogens are not only spread by insects and animals, but can also be spread by human activities like unsanitized handling, packaging and transportation of food. In this regard, usage of untreated sewage or manure and lack of knowledge on hygiene can also play a role in this regard (Brackett, 1999). Foodborne illness, caused by the consumption of food contaminated with pathogenic bacteria or viruses, has been a major safety concern throughout the world. Not only it is an important health risk for our community but it also remains a major burden for all the countries. The major food categories where foodborne pathogens have been detected include dairy, seafood, beef, poultry, seafood, pork, breads and bakery, eggs, beverages and multi-ingredient foods such as sandwiches, salads, etc. (Center for Science in The Public Interest, 2014).

In the United States, around 48 million foodborne illnesses are reported each year, out of which 128,000 result in hospitalization while 3,000 lead to deaths (Scallan et al., 2011). The primary contributor of these huge numbers are pathogenic bacteria which accounts for 3.65 million foodborne illnesses, 35,796 hospitalizations and 861 deaths per year in the United States (Scallan et al., 2011). Even though there has been a decrease in the total amount of outbreaks by 42% between 2002 and 2011 (Center for Science in The Public Interest, 2014), mostly due to the application of Hazard Analysis and Critical Control Points (HACCP), and better application of food safety(Centers for Disease Control and Prevention, 2012), concerns towards foodborne illnesses have not been reduced. It still remains a major concern for public health and thus this

needs attention from food producers. Some of the most common symptoms which are usually observed due to foodborne illness are vomiting, diarrhea, fever and chills. Foodborne illnesses are caused suddenly and, in many cases, the patients recover on their own without any treatment; sometimes complex situations may occur from these conditions.

About thirty one pathogens have been identified to be responsible for the known sources of foodborne illness (Scallan et al., 2011). Eight of these pathogens were responsible for 89% of the total outbreaks with *Salmonella* (26%) being the major contributor among the bacteria family. *Salmonella* was responsible for the major portion of the total number of hospitalizations (44%) while it also resulted in the most number of deaths. The bacteria family alone was responsible for 77% of the total deaths with three of them (*Salmonella, Listeria* and *E. Coli*) comprising of 65% (Gould et al., 2013).

Different chemical compounds like chlorine, chlorine dioxide, trisodium phosphate, sorbates, sulfites, nitrates of potassium or sodium, etc. have been popular and effective in preserving foods from foodborne pathogens (Park, Hung, Doyle, Ezeike, & Kim, 2001) while different physical treatment processes like heating, chilling, drying, etc. have also been available (Zeuthen & Bøgh-Sørensen, 2003). But the gradual increased use of chemicals has prompted research studies which have shown negative impacts that chemicals have when used for food protection. Preservation techniques like irradiation have proven to be safe and efficient in many studies, but still fails to convince the consumers about the safety of the technology (Zeuthen & Bøgh-Sørensen, 2003). Taking into account the consumers demand about a much accepted and safe food preservation technique, a lot of research work is undergoing related to using natural antimicrobials in the food industry.

In addition to the foodborne pathogens, food spoilage due to oxidation, especially lipid oxidation, is also a major concern in the food industry. Foods with fat content, lipids, terpens and branched hydrocarbons are susceptible to heat treatment and lengthy storage. This results in chain oxidation which degrades the acceptability and sensory values of the food. Different types of inhibitors are used to protect the food against contact with oxygen which are also known as antioxidants. Antioxidants are primarily of two types: synthetic and natural (Duthie, Wahle, & James, 1989; Stringer, Görög, Freeman, & Kakkar, 1989; Zeuthen & Bøgh-Sørensen, 2003). Butylated Hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are the two most frequently used synthetic antioxidants in food industry. While BHT is used in chewing gum, potato flakes, shortenings, cereals, meats, etc., BHA is used as an antioxidant agent in beverages, ice creams, deserts, cereals, soup, etc.(Cottone, 2009).

Due to availability and low cost, synthetic antioxidants are very popular in the food industry, but similar to synthetic antimicrobials, the possibility of a toxic consequence in human health has created awareness among people towards their use in foods (Duthie et al., 1989; Stringer et al., 1989; Zeuthen & Bøgh-Sørensen, 2003). The demand for natural antioxidants has thus been increased because of long term safety issues and negative consumer perception of synthetic antioxidants (Namiki, 1990).

Among the available antioxidants of natural origin, research has primarily focused upon essential oil which is a volatile organic extract of herbs and spices. Some common examples of herbs and spices which were found to have antioxidant properties are pepper, sage, mace, ginger, oregano, thyme, rosemary, nutmeg, etc. (Nakatani, 1997). Some of these have been reported to be used for commercial purposes.

In addition to the goal of reducing usage of synthetic compounds, research is ongoing to find natural compounds which can function as both antimicrobials and antioxidants to lessen the amount of preservatives added into the food. Earlier studies that have been reported are not sufficient and need further investigation before this purpose can be achieved. Thus, extensive research on essential oils which can perform both antimicrobial and antioxidant activities is necessary. Considering such prospect, three essential oils have been chosen to investigate both, their antibacterial and antioxidant activities. Thus the objectives of the present study include:

1. To evaluate the antibacterial activity of armoise, bay and rose geranium EOs *in vitro*.
2. To evaluate the antioxidant activities of *Artemisia herba-alba*, *Laurus nobilis* and *Pelargonium capitatum x radens* EOs *in vitro*.

CHAPTER 2

Literature Review

Foodborne outbreaks caused by consumption of contaminated food have raised concerns about food safety. Since the reporting of foodborne diseases initiated in the United States, various research studies have been performed to reduce the prevalence of microbial contaminations (Lynch, Painter, Woodruff, & Braden, 2006). In 2013, a surveillance report revealed 13,405 foodborne outbreaks had occured between 1998 and 2008; 7,998 of those were suspected due to etiologic agents of which around 63% were later confirmed. Both bacteria and viruses were the primary contributor to these outbreaks (Gould et al., 2013). About 31 pathogens have been associated with occurrence of foodborne illness (Scallan et al., 2011). Eight of these 31 pathogens were responsible for 89% of the total outbreaks. Norovirus (39%) was the most responsible for these outbreaks while 48% were due to bacteria. *Salmonella* (26%) was the major contributor among the bacteria family followed by *E. coli* (6%), *Clostridium perfringes (5%)*, *Staphylococcus* enterotoxin (3%) and *Campylobacter jejuni* (2%) (Gould et al., 2013).

Salmonella, *Listeria* and *E. Coli* were responsible for the greatest number of multistate outbreaks (112 out of 128) between 1998 and 2008 which caused 65% of the 200 people reported dead during this period. *Listeria* was the most severe of the pathogens. Almost a quarter (48 out of 216) of the total people hospitalized due to *Listeria* were reported to be dead. This was followed by *E.* coli (1.73%) and *Salmonella* (1.48%). *Salmonella* was also responsible for the major portion of the total number of hospitalizations (44%) while it also resulted in the death of 60 people being dead; which was the largest number of deaths among all pathogens (Gould et al., 2013).

2.1 *Salmonella*

Salmonella spp. is gram-negative, facultatively anaerobic, rod-shaped bacteria which belongs to the *Enterobacteriaceae* family. It is classified into two species: *Salmonella enterica* and *Salmonella bongori*. The former is classified into six subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*) while the later consists of 23 serovars (Humphrey, 2000). Out of the six subspecies of *Salmonella enterica* subspecies *enterica* is the one most responsible for human diseases while the other five subspecies, in addition to *Salmonella bongori*, are less responsible for human diseases (McQuiston et al., 2008).

Salmonella contamination causes bacterial enteric illness in humans, called salmonellosis (Montville , Matthews , & Kniel, 2012). The symptoms of the infection are fever, diarrhea and abdominal cramps, which usually occurs for 4 to 7 days (Centers for Disease Control and Prevention, June 20, 2013). About 1.2 million cases of *Salmonella* infection occur in the United States each year. *Salmonella* being the leading cause of hospitalization and deaths has prompted the U.S. Department of Health and Human Services to set a target and thus implement initiatives to reduce foodborne illnesses due to the *Salmonella* family by 25% (Jackson, Griffin, Cole, Walsh, & Chai, 2013). Health problems, like bacterial endocarditis and osteomyelitis, caused by *Salmonella* results in serious health problems and, if it lacks attention, may result in fatal conditions (Hunter, 2009).

Poultry, complex food (produce with nonproduce ingredients), produce, egg and beef are commonly associated with *Salmonella* outbreaks (Batz, Hoffmann, & Morris Jr, 2012). *Salmonella* outbreak was thought to be linked only to contaminated poultry products. However, a number of fresh produce related outbreaks of *Salmonella* have been reported. From 2002 to 2003 the number of *Salmonella* outbreaks from fresh produce was 31 while that from poultry was 29.

Animal manure as well as contaminated water are considered the primary reasons for fresh produce contamination (Hanning, Nutt, & Ricke, 2009). A wide variety of foods are found to be associated with *Salmonella* outbreak. In 1985, a massive outbreak of *Salmonella* resulting from milk products occurred and resulted in more than 200,000 illnesses. This epidemic resulted in more than 4,500 people being diagnosed with Aseptic Arthritis (Hunter, 2009). Twenty-eight people became ill with *S.* Saintpaul in 2013. This outbreak was linked to the consumption of cucumbers (Centers for Disease Control and Prevention, June 20, 2013). *Salmonella* Heidelberg was responsible for the largest outbreak of 2013 in the United States. In two separate incidents, more than 600 people were reported sick with a hospitalization rate of 38%. In both cases the outbreak was associated with contaminated chicken (Center for Science in The Public Interest, 2014).

2.1.1 Salmonella Typhimurium. *S.* Typhimurium, one of the subspecies of *Salmonella enterica,* is responsible for most of the foodborne diseases in the United State (Hunter, 2009). Even though the total percent of foodborne illness is decreasing, frequency of *S.* Typhimurium related diseases has increased from 1% to 34% of the total salmonellosis between 1980 and 1996 (Hunter, 2009). This strain also accounts for 26% of all *salmonella* isolates resulting in diarrheal disease (Zhang et al., 2003).

In 2008, a large multi-state outbreak occurred when 1,535 people were reported sick across 42 states which resulted in 308 people being hospitalized while two were reported dead. This outbreak occurred through jalapeno and serrano peppers, which were found to be contaminated by *S.* Typhimurium. (Center for Science in The Public Interest, 2014). In 2011, outbreaks of *S.* Agona were linked to the consumption of papaya among 160 individuals. Another outbreak occurred in 2012 which was linked to cantaloupe. Two different *Salmonella*

species were found to cause the illness where 228 people were infected with *S.*Typhimurium and 33 people became sick from *S.* Newport (Centers for Disease Control and Prevention, 2012). In 2013, live poultry, especially those kept in backyards, were responsible for the illness of 356 persons across 39 states. *S.* Typhimurium was the pathogen responsible for that outbreak and it resulted in 62 persons being hospitalized (Centers for Disease Control and Prevention, 2013). In June 2014, *S.* Typhimurium contamination occurred through biology and microbiology laboratories in schools and colleges, where about 41 persons were reported sick (Centers for Disease Control and Prevention, 2014a).

2.2 *Escherichia coli*

Escherichia coli is a gram-negative, facultative anaerobe and rod shaped bacteria which inhabits the intestines of healthy human and animals (Cassin, Lammerding, Todd, Ross, & McColl, 1998; QiongQiong et al., 2013). Despite its beneficial functions in the human body, including synthesizing useful vitamins, it still remains one of the most harmful bacteria due to the severity of the epidemics caused by this bacterium. With some exceptions, despite being considered harmless in most of the cases, its tendency in causing infections in incapacitated or immune-suppressed hosts has made it known as opportunistic pathogens. There are also groups of *E. coli* which are hazardous for human health and are known as pathogenic *E. coli*. These pathogenic *E. coli,* in addition to being transmittable through humans as a medium, can be spread through contaminated drinking water as well as raw milk, vegetables and improperly cooked meat (Brzuszkiewicz et al., 2011). Farm animal manure is one of the major sources of spreading *E. coli* while food crops get contaminated through irrigation water (Duffitt, Reber, Whipple, & Chauret, 2010).

There are six major groups of pathogenic *E. coli*: enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteroaggregative (EAEC) and diffusely adherent *E.coli* (DAEC) (QiongQiong et al., 2013). EPEC are primarily responsible for infantile diarrhea which may only last a few days, in severe cases it may last up to 14 days. In addition to infantile diarrhea, ETEC is also responsible for traveler's diarrhea. The symptoms may include watery diarrhea, diarrhea without blood and mucus with rare cases of fever and vomiting. EIEC contamination may result in chills, fever, headache and abdominal cramps which are accompanied by watery diarrhea. The remaining two groups, EAEC and DAEC, are the least reported infectious groups which are characterized by persistent and watery diarrhea (QiongQiong et al., 2013).

E. coli O157:H7, which is the most fatal and the most widely known serotype of the *E. coli* family, is a part of large Shiga toxin producing group named Shiga toxigenic *E. coli* (STEC) which also consists of the subset of EHEC (QiongQiong et al., 2013). EHEC is the most hazardous of the *E. coli* groups. The primary illness caused with this group is hemorrhagic colitis (HC). The major symptoms of HC are abdominal cramps, bloody diarrhea, vomiting and in a severe case all bloody diarrhea is noticed in every 15~30 minutes. HC might sometimes (3~7%) lead to severe conditions such as hemolytic uremic syndrome (HUS) and thrombatic thrombocytopenic purpura (TTP). Both HUS and TTP patients have a mortality rate of 3~5% with the survivors in many cases are left with permanent disabilities. TTP also affects the central nervous system which sometimes results in fever and other neurological deficiencies (QiongQiong et al., 2013).

2.2.1 Escherichia coli O157:H7. *E. coli* O157:H7 is the most common strain of *E. Coli* with nearly 75,000 infections reported every year(Duffitt et al., 2010). Its ability to adapt to

unfamiliar environments (Duffitt et al., 2010) is also a challenge for scientists and researchers in preventing epidemics. In 1982, *E. coli* O157:H7 was identified as a pathogen because of its association with two food related outbreaks of an unusual gastrointestinal illness (Doyle, 1991). Although the number of outbreaks from *E. coli* O157:H7 is low, as few as ten cells can cause infection in humans thus making *E. coli* O157:H7 one of the main concerns in food safety. Annually, 73,480 people became ill due to *E. coli* O157:H7 infection resulting in 2,168 hospitalizations and 61 deaths (Rahal, Kazzi, Nassar, & Matar, 2012). Beef and beef products are frequently contaminated by *E. coli* O157:H7. In 2009, *E. coli* O157:H7 was the cause of almost 550,000 pounds of beef from Fairbanks Farms being contaminated, resulting in 26 individuals across eight states being affected (Centers for Disease Control and Prevention, 2009a). In the same year, 380,000 pounds of beef from JBS Swift Beef Company were contaminated. The outbreak caused by *E. coli* O157:H7 resulted in 23 people becoming sick (Centers for Disease Control and Prevention, 2009b). In May 2014, approximately 1.8 million pounds of ground beef were found to be contaminated by STEC O157:H7. This resulted in 12 people becoming ill out of which 58% of the people were hospitalized, with no fatality reported (Centers for Disease Control and Prevention, 2014b).

E. coli O157:H7 outbreak is not only limited to beef products, fresh produce can also become contaminated. Fresh produce related outbreaks were first reported in 1991 and still remain a potential vehicle in transmitting disease. Epidemiological studies from 1985 to 2002 found that in produce related outbreak, *E. coli* O157:H7 was associated with 21% of foodborne outbreaks and 34% of foodborne outbreak–related cases. Lettuce accounted for 34% of the outbreaks, salad 16%, coleslaw 11%, melons 11%, sprouts 8%, and grapes 3% (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005). *E. coli* outbreaks are also associated with leafy vegetables.

Between 1998 and 2010, the number of *E. coli* outbreaks linked to leafy vegetables was 27. In 2006, 199 people were affected by *E. coli* O157:H7 from fresh spinach. Three people died in this outbreak (Centers for Disease Control and Prevention, 2006). Romaine lettuce was found to be linked to a multistate outbreak in 2011. Sixty people were infected with *E. coli* O157:H7 and two people developed hemolytic uremic syndrome (HUS) (Centers for Disease Control and Prevention, 2011). In 2012 there was an outbreak associated with shiga toxin-producing *E. coli* O157:H7 (STEC O157:H7). Thirty three people became ill and HUS was developed in two persons. Organic spinach and spring mix blend was the source of this outbreak (Centers for Disease Control and Prevention 2012).

2.3 Antimicrobials

Antimicrobials are various substances or chemicals used in food products to diminish or slow down the growth rate of microbial pathogens like bacteria, parasites, fungi or viruses in order to prevent or reduce foodborne diseases. Antimicrobials are primarily classified into two categories: synthetic and natural. Synthetic antimicrobials usually consist of chemicals such as nitrates, sulphites, benzoates, sorbates, etc.; whereas natural antimicrobials are extracted from nature sources (Montville et al., 2012). Traditional methods like chilling, freezing, fermentation and pasteurization have played their part in keeping down food spoilage due to microbial pathogens. It is still far from adequate to consider traditional methods a risk free issue (Negi, 2012). Novel techniques like controlled atmosphere, non-thermal treatment and irradiation are also available and provide much better results in limiting the epidemic capacity of the pathogens. The inability of these methodologies to maintain and preserve the organoleptic properties of food and food products has reduced customer acceptability towards these products (Negi, 2012; Tiwari et al., 2009). Synthetic antimicrobials are also responsible for severe health conditions

like hypersensitivity, allergy, asthma, hyperactivity, neurological damage, cancer, etc. (Anand & Sati, 2013). In addition to these adverse health effects of the synthetic antimicrobials, natural preservatives which are more safe and acceptable for the consumer have been developed (Dadalioglu & Evrendilek, 2004).

2.3.1 Natural antimicrobials. Natural antimicrobials are a potential alternative to chemical preservatives in terms of consumer acceptability and their health concerns about chemical preservatives. The sources of natural antimicrobials can be animal, plant or microorganism. Although, the mechanism of action, toxicological and sensory effects of natural antimicrobial are not completely evaluated, new methods are being developed to use natural antimicrobials to improve food safety. From prehistoric times herbs and spices have been used for flavoring purposes. Their uses are not only limited to medicinal purposes but in prevention of food spoilage and deterioration (Irkin & Korukluoglu, 2009). Researchers have proven that these natural antimicrobials primarily tend to improve the safety of food products as well as their storage life (Tiwari et al., 2009). The primary source of natural antimicrobials are plant derived essential oils, organic acids, enzymes from animal sources and natural polymers (Lucera, Costa, Conte, & Del Nobile, 2012). Excess usage of natural antimicrobials might have a negative effect on the quality attributes like taste and smell of food. Thus there is a limiting conditions on the amount of natural antimicrobials that can be used for food preservation (Montville et al., 2012).

Application of natural antimicrobials in food products has produced some promising results. Combination of methyl jasmonate with ethanol has been proven to show better qualities in preserving firmness and color of tomatoes while application of grapefruit seed extract in kiwifruit resulted in delaying microbial growth. A study on apples showed that vanillin (0.3% w/w) has the ability to preserve the sensory quality whereas lemongrass and oregano oil inhibits

the growth of psychrophilic aerobes, yeasts and molds (Lucera et al., 2012). Conte et al. (2007) studied three different concentrations of lemon juice which effectively improved the shelf life of mozzarella cheese (Conte, Scrocco, Sinigaglia, & Del Nobile, 2007). Natural antimicrobials from animal extracts like chitosans are proven to be useful in improving the storage time for orange juice while it was also reported to improve the storage time for dairy products (Hafdani & Sadeghinia, 2011). In addition, antimicrobials derived from microbes are also effective in improving the storage time for chicken as well as the preservation qualities of tomato juice, orange juice and dairy products (Tiwari et al., 2009). Packaging industries are also effectively using plant extracts like cinnamon, grapefruit seed, clove, etc. to stop attacks of pathogens thus reducing spoilage (Ahvenainen, 2003).

2.3.1.1 Essential oil. Essential oil (EO) is an aromatic liquid acquired from plant extract. In general, essential oils are extracted from various parts of the aromatic plant— such as leaves, flowers, fruits, seeds, rhizomes, roots, or bark (Burt, 2004). The term "essential oil" was first derived in the 16th century from the term 'Quinta essentia' which was introduced by Swiss reformer of medicine, Paracelsus von Hohenheim (Guenther, 1948). Essential oil can be extracted by water or steam distillation or by the liquid solvent extraction. The main components of essential oil are terpenoids such as mono-, di- and sesquiterpenes, alkaloids and phenolic substances. (Figueiredo, Barroso, Pedro, & Scheffer, 2008).

The first use of EO was documented around 2000 years ago by the Romans and the Greeks. By the seventeenth century, it became a very popular entity in pharmacies for their medicinal usage (Guenther, 1948). It was not until the latter part of the 19th century, when the antimicrobial characteristics of essential oils were first investigated by De La Croix. Since then its usage in preserving the organoleptic properties of food items gradually became dominant

compared to its application in the field of medicine (Burt, 2004). Commercial availability of EO as an antimicrobial agent has been suggested as early as the year 2000 in the United States (Cutter, 2000).

The antibacterial properties of EOs have been recognized for a long time (Guenther, 1948). Deans and Ritchie studied the antibacterial properties of fifty EOs on twenty five different genera of bacteria. Their research found that angelica showed inhibiting properties against all 25 bacteria while bay, cinnamon, clove, thyme, almond, marjoram, pimento and geranium showed the same against more than twenty of the bacteria (Deans & Ritchie, 1987). Dorman and Deans (2000) conducted a similar study on 25 different gram-positive and gram-negative bacteria. They used six different essential oils for their study where thymus showed the most inhibiting properties followed by oregano, clove, nutmeg, black pepper and geranium (Dorman & Deans, 2000). The bactericidal effect of plant EOs has also been studied. In 1998, a study involving five of the most harmful pathogens (*Campylobacter jejuni, E. coli*, *S.* Typhymurium, *Listeria monocytogenes*, and *Staphylococcus aureus)* against twenty different plants EO was reported. Smith-Palmer et al. found that oils of bay, clove, cinnamon and thyme were most effective and had a bacteriostatic concentration of 0.075% or less (Smith-Palmer, Stewart, & Fyfe, 1998).

In two separate studies conducted by Hao, et al. (1998), plant extracts were applied to cooked chicken breast and beef. In both cases, clove and pimento extracts, were able to inhibit the growth of *Aerominas hydrophila* and *L. monocytogenes* (Hao, Brackett, & Doyle, 1998a, 1998b). Cutter (2000) studied the antimicrobial effect of plant EO on beef. Two commercially available antimicrobial EO products reduced pathogens (*E. coli, S.* Typhimurium and *L. monocytogenes*) on the beef surface while no antibacterial activity was seen in case of ground beef (Cutter, 2000). Kotzekidou et al. (2008) also found success in examining the antimicrobial

effect (against *E. coli, S.* Enteritidis, *S.* Typhimurium, *Staphylococcus aureus, L. monocytogenes* and *Bacillus cereus*) of EOs on chocolates containing foodborne pathogens (Kotzekidou, Giannakidis, & Boulamatsis, 2008).

Armoise (*Artemisia herba-alba*), also known as desert wormwood, is a member of Asteraceae. It is a greenish-silver perennial small shrub and is widely distributed in North Africa. Out of the various secondary metabolites extracted from armoise, sesquiterpene lactones have been the most important one due to their extensive application in medicine and pharmacy. Flavonoids and EOs are also important secondary metabolites of armoise (Mighri, Hajlaoui, Akrout, Najjaa, & Neffati, 2010). Numerous variations have been observed in the chemical composition of EOs of armoise collected at different locations (Mighri et al., 2010). The reasons for such variations are attributed to environmental factors (climate conditions, geographic origin and seasonal dissimilarity) and methodological factors (extraction techniques) (Cherrat et al., 2014).

Armoise plant is traditionally used for medicinal purposes such as relief from stomach disorder, hypertension and diabetes (Friedman, Yaniv, Dafni, & Palewitch, 1986; Ziyyat et al., 1997). Herbal tea made from its species has also been used as antibacterial, antispasmodic, analgesic and hemostatic agents (Laid et al., 2008). Armoise was also found to produce hypoglycemic effect in animal model (Iriadam, Musa, Gümüşhan, & Baba, 2006). Akrout et al. (2009) collected three different aromatic plants including armoise from Southern Tunisia and studied the antimicrobial properties using the agar disc diffusion method. The primary constituents of EO extracted from armoise using hydrodistillation method were β-thujone and α-thujone. Armoise showed reasonable antibacterial activity against *Serratia marcescens* and *Staphylococcus aureus* where essential oil from *Thymus capitatus* showed the best antimicrobial

properties (Amouri, Akrout, & Hajer El Jani, 2009). Mighri et al. (2010) also studied antioxidant and antibacterial properties of armoise EO collected from the same region which had its own unique chemical composition compared to armoise described in other literature. β-thujone, α-thujone/β-thujone, α-thujone and 1,8-cineole/camphor/α-thujone/β-thujone were found as the major components. They observed a very strong antimicrobial activity which was measured using agar-well diffusion method (Mighri et al., 2010). Sbayou et al. (2014) studied the antimicrobial properties of armoise against three standard strains (*E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853) and five clinical strains (*Enterobacter cloacae*, *Staphylococcus aureus*, *Pseudomonas pyocyanique*, *Enterococcus faecium,* and *E. coli*.). The EO was extracted from armoise using steam distillation method and its major constituent was α-thujone. Significant antimicrobial activity was reported against most of the strains which was measured using the disk diffusion method (Sbayou et al., 2014).

Bay (*Laurus nobilis*) is an evergreen tree of Luraceae family which is originally from the Mediterranean region. Bay leaves have been used in culinary and food industry as a spice and flavoring agent. It is also popular in the field of herbal medicine for its antibacterial, antifungal, antidiabetes and anti-inflammatory properties. Its traditional use has been in the treatment of diseases like rheumatism, earaches, indigestion, sprains, etc. while it has also been suggested to be used in preventing migraine (Fang et al., 2005). Although the primary constituents of bay oil differed in various studies, 1, 8-cineole is common on each of them (Caredda, Marongiu, Porcedda, & Soro, 2002; Dadalioglu & Evrendilek, 2004; Yalçin, Anik, Sanda, & Çakir, 2007). Its components were described to have anaesthetic, hypothermic, muscle relaxant properties while it has also been used in the treatment of epilepsy (Sayyah, Valizadeh, & Kamalinejad, 2002).

Bay, in addition to the applications mentioned above, also has attracted the interests of scientists and researchers for a possible antimicrobial quality. Dadalioglu et al. (2004) studied the chemical composition of EOs from four different aromatic plants which also included bay. The essential oil from bay extracted using steam distillation method had 1, 8-cineole as its major component. Using four different major foodborne pathogens (*S.* Typhimurium, *Staphylococcus aureus, L. monocytogenes* and *E. coli*.O157:H7), they found strong antibacterial activity in all the cases. A concentration of 15% (v/v) was found to be the most effective while *E. coli* O157:H7 was the bacteria which was the most inhibited (Dadalioglu & Evrendilek, 2004). The antimicrobial activity of bay was also confirmed by using six different pathogens (*Staphylococcus aureus, Bacillus subtilis, Pseudomonus aeruginosa, E. coli, Candida albicans and Aspergillus niger).* These antimicrobial effects resulted from 6-epi-desacetyllaurenobiolide the primary component of bay (Santoyo et al., 2006). Erturk (2006) studied the antibacterial activity of extracts from eleven different spices and herbs against two gram negative (*E. coli* and *Pseudomonas aeruginosa*) and three gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus* and *S. epidermidis*). The antibacterial activity was analyzed using agar disc dilution method. Bay, which was one of the herbs showed better antioxidant results compared to previous studies (Ertürk, 2006).

Ivanovic et al. (2010) conducted a comparative study between supercritical CO_2 extract and essential oil extract from bay. Gas Chromatography with Flame Ionization Detector (GC-FID) and Gas Chromatography –mass spectrometry (GC-MS) methods were applied to determine the chemical composition of both extracts. The results showed that EO extracts contained comparatively higher proportions of monoterpenes and oxygenated derivatives. They also analyzed the antibacterial activity of both extracts against different *Staphylococcus* strains.

The results obtained using broth macrodilution method showed that both extracts had high and similar antibacterial activity against the observed strain (Ivanović, Mišić, Ristić, Pešić, & Žižović, 2010). Abu-Zaid et al. (2013) reported the chemical composition and antimicrobial properties of three different EOs which included bay. The primary component of EOs from bay leaves, which was extracted using steam distillation method, was 1, 8-cineole. The antimicrobial activity was measured using disc diffusion method. Bay showed the greatest antimicrobial activity against *E. coli* and minimal activity against *Bacillus subtilis* (Abu-Zaid, Alopidi, & EL-Sehrawy, 2013).

In a recent study conducted by Cherrat et al. (2014), they introduced hurdle technology where they combined EO extracted from bay with physical methods (mild heat treatment, pulsed electric fields and high hydrostatic pressure) to study the antimicrobial activities (Cherrat et al., 2014). The essential oil from bay leaves, collected from Morocco, was extracted using steam distillation and primarily consisted of 1, 8-cineole and 2-carene determined by GC-MS method. The antimicrobial activity was measured using agar disc diffusion method. The combination showed strong antimicrobial activity compared to previous individual studies (Cherrat et al., 2014). Tuscan sausages, which were reported to be affected by foodborne pathogens, were subjected to studies of antimicrobial activities when EO from bay was applied. The main components present were 1, 8-cineole and linalool followed by a fraction of sabinene and terpinyl ester acetate. The application of EO degraded the sensory characteristics of the sausage, but was able to reduce the amount of pathogens which resulted in an increase of shelf life by two days (Mello da Silveira et al., 2014).

Rose geranium (*Pelargonium capitatum*) is a perennial, aromatic herb which is a member of the Geraniaceae family. Only 10 of the 700 different species are used for production of

geranium oil (Shawl, Kumar, Chishti, & Shabir, 2006). Rose geranium EO is mainly used in the perfume and cosmetic industries while it has also been used as flavoring agent (Shawl et al., 2006). Its traditional use is not only limited to stop bleeding, treat ulcers, it also is used to treat diarrhea, dysentery and colic (Shawl et al., 2006). Its astringent property has also been reported which is extensively used in aromatherapy. The flavonoids present in the *Pelargonium* extracts are reported to be the reason behind the antibacterial properties shown by the geranium oil (Van Vuuren, 2008). Boukhatem et al. (2013) investigated the antimicrobial properties of rose geranium against 23 different pathogens. Their study suggested that rose geranium showed more antimicrobial properties towards gram positive bacteria compared to gram negative bacteria (Boukhatem, Kameli, & Saidi, 2013).

2.4 Antioxidants

Food as well as biological systems can be subjected to oxidation process. Oxidation is defined as the reaction that takes place between oxygen molecules and any other compounds such as lipid, phospholipid and protein. During the oxidation process some chemical species are generally formed with unpaired electron. Molecules, ions or atoms with unpaired electron (s), called free radicals, are unstable and reactive towards other molecules. In the human body oxidation occurs through reactive oxygen or nitrogen species like superoxide anion radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and nitric oxide radical ($NO^{\cdot}$). These reactive species are generally formed in the metabolism process as well as through the environmental factors. These free radicals upon their creation create more free radicals which may lead to a chain reaction. However, antoxidant enzymes like superoxide dismutase plays a key role in neutralizing these oxidative elements and thus keeps a balance within the body. But when the production of free radicals exceeds the amount of free radicals detoxified by the body's

defense system, it results in oxidative stress. Oxidative stress causes not only damage to proteins, deoxyribonucleic acid (DNA) and cell membrane but also results in chronic diseases like atherosclerosis, cancer and diabetes (Abdollahi, Moridani, Aruoma, & Mostafalou, 2014).

Food components like lipids, carbohydrates and proteins are all susceptible to oxidation. But lipid is comparatively more susceptible due to the presence of unsaturated fatty acids. It has been reported that reactive species and free radicals resulting from the oxidation of lipids, initiates oxidation on protein. The primary methods of lipid oxidation in foods are through autoxidation, photoxidation and lipoxygenase. Although it was previously known that oxidation was primarily controlled by catalysts such as enzymes, light, temperature, etc., the involvement of free radicals and reactive oxygen species was first detected during the 1940's. The mechanism of oxidation was named autoxidation (Shahidi & Zhong, 2005). Photoxidation of lipid occurs in the presence of light and sensitizers. The third mechanism of oxidation takes place by the action of lipoxygenase enzyme.

Autoxidation occurs through free radical mechanism consisting of three steps: initiation, propagation and termination. The primary products are lipid hydroperoxides which are colorless and odorless. Extensive research has been done on the degradation products of lipid hydroperoxides and their impact on food products and health. Decomposition of lipid hydroperoxide is catalyzed by temperature and metals resulting in the formation of various compounds and free radicals (Kubow, 1992). During secondary decomposition a variety of products are formed depending on the type of lipid in lipid hydroperoxides. For example, hydrocarbons produced by oxidation of oleate hydroperoxide are heptanes, octane but those of linoleate hydroperoxides are pentane. Carbonyls, hydrocarbons and alcohols produced from hydroperoxides are three primary reasons for off-flavor and odor in foods (Choe & Min, 2006;

Frankel, 1980). Lipid hydroperoxides as well as their degradation products react with other components of food such as enzymes, proteins and amino acids, thereby reducing the sensory quality and nutritive value of a product. It thus results in reduction of consumer acceptability as well as an economic loss to the food industry (Kubow, 1992; Miguel, 2010).

Studies on the effect of lipid hydroperoxides in animal model showed that oral ingestion of hydroperoxides is less toxic than intravenous administration of hydroperoxides. The possible causes for this was assumed to be less absorption and their conversion to less toxic forms in the digestive tract. But the decomposed products of lipid hydroperoxides such as lipid peroxyl radicals and low molecular weight carbonyl compounds are absorbed and metabolized faster. Some naturally occurring carbonyl compounds such as malondialdehyde (MDA), unsaturated aldehydes and 4-hydroxy unsaturated aldehydes initiate the oxidation stress, reacts with proteins, DNA and also retard cell divisions in mammalian cell lines (Kubow, 1992). But MDA in food is not of great concern because after digestion MDA transforms into 2-propenal lysine which is mostly excreted in urine in an unchanged form (Girón-Calle, Alaiz, Millán, Ruiz-Gutiérrez, & Vioque, 2002; Kubow, 1992). Other products like 4-hydroxy-2-hexenal and 4-hydroxy-2-nonenal are accumulated in the blood after intestinal absorption. These chemical species are also responsible for lipid oxidation *in* vivo and tissue injuries (Awada et al., 2012; Kanazawa, Kanazawa, & Natake, 1985).

Antioxidants are any substances that reduce or prevent the oxidation process. Antioxidants can be classified into two groups: primary antioxidants and secondary antioxidants. As primary antioxidants inhibit oxidation by donating hydrogen to free radicals or reacting with free radicals, they are also called chain-breaking antioxidants. Phenols, butyl hydroxytoluene (BHT), butyl hydroxyanisole (BHA), propyl gallate (PG), tocopherols and ascorbic acid,

aromatic amino compounds are examples of chain breaking antioxidants. Secondary antioxidants prevent oxidation through chelation of metals, quenching of excited singlet oxygen or recover primary antioxidants. Carotenoids, ascorbic acid, citric acid etc. are some examples of secondary antioxidants (Burton & Ingold, 1981; Frankel, 1980).

Antioxidants can also be classified as synthetic and natural based on sources. Synthetic antioxidants are manmade e.g. BHT, BHA, PG. Natural antioxidants are primarily from animal or plant source. Ascorbic acid, carotenoids, tocopherols, phenolic compounds, herbs and spices are examples of natural antioxidants obtained from plants.

During the 1940's synthetic antioxidants were first introduced in fats and fat containing foods to increase their shelf life. The four major synthetic antioxidants used in food products are BHA, BHT, tertiary butylhydroquinone (TBHQ) and PG. Although the amount of antioxidants used in food depends on the fat content, the maximum permitted concentration for TBHQ and PG is 100 parts per million (ppm) that for BHA and BHT is 200 ppm (Shahidi & Zhong, 2005). Several studies have addressed the adverse effects of these antioxidants in animal model. Although lower doses do not cause injury to health, larger doses such as 500mg/kg/day BHT and BHA may cause some changes in pathological, enzyme, and lipids in both rodents and monkeys. Also, physiological development problems and carcinogenesis upon rodents might result from BHT. In addition, long term studies on animals showed that BHT and BHA could result in tumors (Barlow, 1990; Branen, 1975). Therefore, the findings of some studies on synthetic antioxidants and the negative consumer perception to synthetic antioxidants led researchers to find antioxidants from natural sources.

2.4.1 Natural antioxidants. Tocopherols, β-carotene and ascorbic acids are examples of natural antioxidants which are commercially available. Among various isomers of tocopherols

(α, β, γ, and δ), δ-tocopherol shows the highest antioxidant activity. Tocopherols act as chain breaking antioxidants by trapping alkyl and peroxy radicals up to certain concentrations. But at higher concentrations, tocopherols accelerate oxidation reaction. In purified soybean oil, highest antioxidant activity of α, γ and δ was found at 100, 250 and 500 ppm concentration (Jung & Min, 1990; Ohkatsu, Kajiyama, & Arai, 2001). Ascorbic acid is another potential natural antioxidant with multiple functions such as metal chelator, singlet oxygen quencher and reducer of free radicals. It can also regenerate primary antioxidant. Ascorbic acid donates hydrogen to tocopheroxyl radical produced in free radical neutralization step and converts it to tocopherols. Although it is a good antioxidant, it acts as prooxidant in the presence of metal and it is also heat sensitive (Frankel, 1996). β-carotene is a good singlet oxygen quencher and free radical neutralizer. Some studies showed that β-carotene accelerate oxidation reaction when the oxygen pressure is higher than 150 mmHg and concentration of β-carotene is high. Under this condition β-carotene loses antioxidant activity and helps to make other radicals which participate in propagation step of autoxidation.

Herbs and spices have been used for years in food preservation without having any knowledge of their functions (Shahidi & Zhong, 2005). In the last two decades studies on essential oil or extract of herbs and spices have attracted the focus of researchers in order to evaluate their antioxidant properties. Essentially, the interest in the antioxidant activity of these species started after the works of Chipault et al. who examined over thirty herbs and spices during the 1950's (as cited in Madsen & Bertelsen, 1995). They found that sage, rosemary and clove show strong antioxidant activity (Madsen & Bertelsen, 1995; Yanishlieva, Marinova, & Pokorný, 2006). They are recognized as natural preservatives because of their antimicrobial and

antioxidant activity. The presence of terpenoids, phenolic compounds are found to be the primary reason behind their activities (Oussalah, Caillet, Saucier, & Lacroix, 2007).

Shahidi et al. (1995) studied the antioxidant activity of four different ground spices in comminuted pork. Their results showed that antioxidant activity varied depending upon the concentration and clove showed the strongest antioxidant activity whereas ginger and thyme were the weakest (Shahidi, Pegg, & Saleemi, 1995). Tsimidou et al. (1995) assessed the antioxidant activity of oregano on mackerel oil and reported that applying 1% oregano would be similar to 200ppm of BHA (Tsimidou, Papavergou, & Boskou, 1995). In separate studies rosemary and sage showed good antioxidant properties against lard and sausages (Yanishlieva et al., 2006). Lee and Shibamoto investigated the antioxidant properties of volatile extracts of six different herbs and spices. They used aldehyde/carboxylic acid assay and conjugated diene assay to evaluate the antioxidant activities. They found basil and thyme extracts had antioxidant properties to be in the same order as BHT and α-tocopherol (Yanishlieva et al., 2006).

2.4.1.1 Essential oil. Many researches have reported the antioxidant activity of EOs. Researchers have found *Rosmarinus officinalis* L., *Salvia fruticos* Mill. and *Foeniculum dulce* Mill. to show better antioxidant activity than commercially available popular synthetic antioxidant BHT. Previous studies have shown the presence of phenolic compounds to be the primary reasons for antioxidant activity shown by EOs. But some researchers found that essential oil from three *Salvia* species showed antioxidant activity even though they didn't contain any phenolic substance (Miguel, 2010). Milos et al. (2000) determined the chemical composition of oregano essential oil and found fourteen different aglycones in dominated by thymoquinone. They also studied the antioxidant activity of the EO and found it to be comparable with α-tocopherol, which is a widely used natural antioxidant (Milos, Mastelic, & Jerkovic, 2000).

Gachkar et al. (2007) conducted a comparative study of antioxidant properties between rosemary and cumin. They used DPPH radical scavenging and β-carotene method for their study and found that rosemary was a better option to be used as an antioxidant (Gachkar et al., 2007). In 2008, a study on seasonal dependency of chemical composition and antioxidant activity of basil EO was reported by Hussain et al. (2008) the chemical composition measured by GC and GC/MS method showed that the composition primarily dominated by linalool varied depending on the season which it was collected. The antioxidant activity was determined by three different methods: DPPH free radical scavenging, β-carotene in linoleic acid and inhibition of linoleic acid oxidation methods. Essential oil extracted from basil showed good antioxidant activity although varied significantly depending on the seasons they were collected (Hussain, Anwar, Hussain Sherazi, & Przybylski, 2008).

Armoise, which is traditionally used for medicinal purposes, has been subjected to research about its antioxidant activities. Amouri et al. in addition to the antimicrobial properties also studied the antioxidant properties of armoise. EO from armoise showed reasonable amount of antioxidant properties which was measured using DPPH free radical scavenging method (Amouri et al., 2009). Mighri et al. (2010) also examined the antioxidant properties of armoise EO collected from the same region. The antioxidant activity was measured using three different methods: DPPH radical scavenging, β-carotene bleaching assay method and ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging method. Their results suggested very weak antioxidant property (Mighri et al., 2010). In 2013, Kadri et al. studied the antioxidant properties of armoise EO. They used DPPH radical scavenging assay method, β-carotene bleaching assay method and reducing power oxidant method for their research. Their results suggested the potential for using armoise as a natural antioxidant as well as a potential substance

for the pharmaceutical industries (Kadri et al., 2013). In the same year, Khlifi et al. (2013) also worked on evaluating the antioxidant properties of armoise collected from central Tunisia. They used DPPH, ABTS and linoleic acid methods for their study. They found encouraging antioxidant properties in armoise, while it was suggested to be active against diseases like leukemia, bladder and laraynx carcinoma cell lines (Khlifi et al., 2013). Aouadi et al. (2014) tested the antioxidant activity of lamb meat when an EO is applied as a supplementary diet to lambs. They used Folin-Ciocalteu method, ferric reducing antioxidant power (FRAP) method and trolox equivalent antioxidant capacity (TEAC) method for measuring the antioxidant activity of armoise. Even though promising results were obtained in terms of increasing the antioxidant capacity of the meat, the dietary supplement was not able to control lipid oxidation and color deterioration of the meat (Aouadi et al., 2014).

Bay (*Laurus nobilis*) leaves, in addition to its antimicrobial activities, has also been subjected to tests regarding its antioxidant properties. Santoyo et al. (2006) studied the antioxidant activity of two different extracts collected from bay. The antioxidant activity measured by β-carotene and DPPH scavenging methods were found to be similar for both the extracts (Santoyo et al., 2006). In a study reported by Conforti et al. (2006) presented results of antioxidant activity of wild bay and compared their results with cultivated plants. Using GC-MS method, they detected high amount of terpene content that included linool, α-terpenol, α-terpinyl acetate, thymol,caryophyllene, aromandrene, selinene, farnesene and cadinene. They used three different methods for determining the antioxidant activity (DPPH method, β-carotene method and Bovine Brain Peroxidation assay method) which showed good results for use as a natural antioxidant. The reason for such high antioxidant activity was reported as the presence of high level of soluble phenolics in the extracts(Conforti, Statti, Uzunov, & Menichini, 2006). Plitro et

al. (2007) reported a comparative study of chemical composition and antioxidant activities between free volatile aglycones and EO from bay. Benzyl alcohol was the dominant component of aglycones while for EO it was 1,8-cineole. They used BHT as a reference to compare the antioxidant properties which was measured using DPPH and ferric reducing/antioxidant assay (FRAP). The antioxidant activities of EO were comparable to BHT whereas the same for aglycones were much less (Politeo, Jukić, & Miloš, 2007).

Ouchikh et al. (2011) reported antioxidant properties of bay. The β-carotene method to determine the antioxidant properties which showed good antioxidant activity and thus it was suggested to be used effectively in food, cosmetic and pharmaceutical industries (Ouchikh et al., 2011). In the same year, Huang et al. (2010) reported antioxidant properties of twenty five different EOs which also included bay. They used four different methods for measuring the antioxidant properties: DPPH free-radical scavenging assay, total phenolic contents (TPC), trolox equivalent antioxidant capacity (TEAC) and ferric thiocyanate (FTC). Bay showed reasonable amount of antioxidant activity where EOs from cinnamon bark extra, ajowan and oregano showed the best result (Huang, Wang, Chen, Chen, & Yih, 2010). Abu-Zaid et al. (2013) in addition to the antimicrobial properties also assessed the antioxidant properties of bay leaves EO using DPPH free-radical scavenging assay method. They reported that EO from bay showed antioxidant activity due to the presence of methyl eugenol (Abu-Zaid et al., 2013).

Traditional methods like Soxhlet, infusion, maceration supercritical fluid extraction method are commonly used for extracting EO. But the high temperature and long period needed for these procedures results in loss of their desired bioactive components. Thus to improve the chemical characteristics of the EOs to be extracted, Muniz-Marquez et al. (2013) used Ultrasonic-assisted extraction (UAE) for extracting EO from bay leaves and tested their

antioxidant properties. This method not only solves the problems which were part of the previous techniques but also proved to be a cheaper and space-adaptable alternative. After the extraction was completed, they used DPPH method and linolenic acid method to measure the antioxidant activity of the extracted EO. The results were promising and showed strong antioxidant activity of bay (Muñiz-Márquez et al., 2013). Cherrat et al. (2014) in their introduction of "hurdle technology", also studied the antioxidant properties of bay EO using DPPH free-radical scavenging assay, reducing power method and β-carotene method (Cherrat et al., 2014). Despite the low content of phenolic compounds in bay leaves, the presence of which is considered the primary reason for antioxidant properties of EOs, the combination showed strong antioxidant activity (Cherrat et al., 2014).

Guerrini et al. (2011) tested the antioxidant characteristics of rose geranium EO. They used DPPH method and β-carotene method which showed that rose geranium has very poor antioxidant activity (Guerrini, Muzzoli, Romagnolid, & Antognoni, 2011).

The current study has been designed to assess the antibacterial and antioxidant activities of three different essential oils. These three essential oils are chosen based on their popularity in three different fields: Bay in culinary purposes, Armoise in medicinal purposes and Rose Geranium in the field of cosmetics and aromatherapy. Although a good amount of research has been conducted on bay EOs, lack of sufficient research information on the other two EOs and the variation in chemical properties due to variance in environment as well as chemical composition of EOs has also prompted to select these three EOs for the current study. The findings of this study could provide more options for developing natural preservatives and thus contribute to the decrease of the adverse effects of chemicals as well as towards improvement of the everyday life.

CHAPTER 3

Materials and Methods

3.1 Materials

E.coli O157:H7 (ATCC 700728) and *Salmonella* Typhimurium (ATCC 13311) were purchased from American Type Culture Collection (Virginia, USA). Bacterial media were purchased from BD Bioscience (California, USA). Armoise (*Artemisia herba-alba*), bay (*Laurus nobilis*) and rose geranium (*Pelargonium capitatum x radens*) essential oils were obtained from New Directions Aromatics (Mississauga, Canada). All chemicals were obtained from Fischer Scientific (Pennsylvania, USA) and Sigma-Aldrich (Missouri, USA) unless otherwise stated.

3.2 Methods

Objective 1: To evaluate the antibacterial activity of armoise, bay and rose geranium EOs *in vitro*.

The antibacterial activity of EOs was investigated using growth over time assay and agar diffusion spot technique.

3.2.1 Preparation of brain heart infusion (BHI) broth and agar. To activate freeze-dried *E. coli* O157:H7 and *Salmonella* Typhimurium obtained from ATCC, BHI media mixed with beef extract was prepared. 18.5 g of BHI and 2g of beef extract were dissolved in 500 milliliter (mL) of water at 70˚C. The broth was divided into 9 mL each of aliquots and sterilized at 121˚C for 15 minutes. The broth was stored in refrigerator until used.

BHI agar was prepared by dissolving 18.5 g BHI, 7.5 g agar and 2g beef extract in 500 mL of water at 70˚C. The agar broth was sterilized at 121˚C for 15 minutes and then cooled to 48 ˚C in a water bath. About 10-15 mL of the agar media was poured in petri dishes and allowed to solidify. The plates were stored in a refrigerator until needed.

3.2.2 Preparation of Luria-Bertani (LB) broth and agar. LB broth was prepared by dissolving 12.5 g of LB in 500 mL of water. The broth was divided into 9 mL each of aliquots and sterilized at 121˚C for 15 minutes. The broth was stored in refrigerator until used.

LB agar was prepared by dissolving 12.5 g of LB and 7.5 g of agar in a 500 mL of water. The mixture was completely dissolved with stirring at 70 ˚C. After the agar broth was sterilized at 121˚C for 15 minutes, it was allowed to be cooled to 48˚C in a water bath. The agar was poured into petri dishes and allowed to solidify. The plates were stored in a refrigerator until needed.

3.2.3 Preparation of Peptone water. Peptone water was made by dissolving 0.5 g of peptone into 500 mL of water. Peptone water was taken into each 9 mL of aliquots and then sterilized as the same as before. This was stored in a refrigerator until used.

3.2.4 Preparation of different concentrated essential oils. One percent of Tween 80 was prepared in peptone water and autoclaved. Different amount of essential oil was added to peptone water with 1% Tween 80 to obtain 0.1%, 0.15%, 0.25%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 3%, 6% and 8% (v/v) essential oil. The solution was mixed on a vortex for 3 minutes.

3.2.5 Bacteria activation and preparation. Freeze-dried *E. coli* O157:H7 and *Salmonella* Typhimurium obtained from ATCC were activated by adding one pellet or small amount of these freeze dried cells in 1 mL of BHI broth mixed with beef extract. The bacterial suspension was streaked with a sterilized inoculum on BHI-beef extract agar plate. The plate was incubated at 37˚C for 24 hours. A single colony from the plate was picked and then inoculated into LB broth. The cells in LB broth were incubated at 37˚C for 24 hours.

3.2.6 Bacterial enumeration. Bacterial populations were determined by plating the cells onto LB agar plate. In this procedure, samples were serially diluted by transferring 1 mL sample into 9 mL of 0.1% peptone water solution. Then 100 microliter (µL) suspensions of proper

dilutions depending on the growth condition of bacteria were streaked on duplicate LB agar plates using sterilized glass beads. The colonies were counted by hand held colony counter after overnight incubation at 37˚C. When each plate has approximately 25-250 colonies of the cells, the plates were considered for counting colonies (Hayek & Ibrahim, 2012). All experiments were performed in triplicate.

3.2.7 Growth over time assay. Growth over time assay is designed to test the inhibitory or lethal effect of antimicrobial products. The method is based on the growth of microorganism in broth medium in the presence of antimicrobial product (Davidson, Palou, Parish, & Vigil, 2005). This assay was employed, according to the procedure previously described, with some modifications (Hayek & Ibrahim, 2012).

Different amount of EO was dissolved in 1% Tween-80 and then mixed with LB broth in final volume 4.5 mL to make 0%, 0.1%, 0.15%, 0.25%, 0.5% and 1% (v/v) EO contained LB broth. The fresh culture of two bacteria was serially diluted by transferring 1 mL suspension into 9 mL peptone water solution. Samples were inoculated with 0.5 mL bacterial culture (about 4-3 log CFU/mL). Initial bacterial population was determined using bacterial enumeration method at this point to ensure the low inoculum level of bacteria at 2-3 log CFU/mL. The mixtures were incubated with shaking (2 rpm) at 37˚C for 8 hours. Blank samples without bacterial inoculation for each treatment were prepared. Negative control was LB broth containing bacterial suspension. The bacterial growth was monitored by measuring the optical density (O.D. 610 nm) at 2 h interval using Thermo Scientific Genesys 10S UV-Vis spectrophotometer.

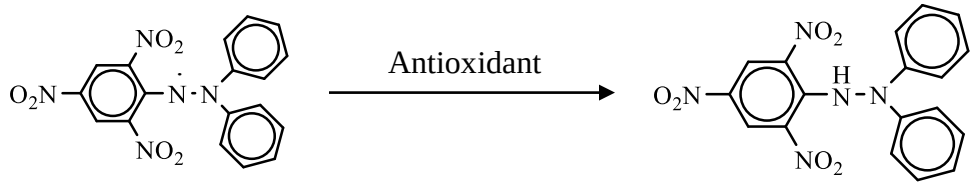
3.2.8 Agar diffusion spot assay. Agar diffusion spot assay was developed to determine the lowest concentration of an antimicrobial that is required to inhibit the growth of bacteria and the minimum concentration required to kill the bacterial cells. In this method, EO is added to an

agar plate previously seeded with the bacterial culture. The EO will form a round spot on the agar and will diffuse. Degree of inhibition is determined by no growth (-), some growth (-/+), or growth (+) (Awaisheh, Al-Nabulsi, Osaili, Ibrahim, & Holley, 2013).

Fresh culture of bacteria was serially diluted by transferring 1 mL culture into 9 mL peptone water solution (four dilutions). The LB agar plates were inoculated with 0.5 mL of fourth dilution of bacterial culture and allowed to dry. Spots were formed by adding 20 µL of essential oils at different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 1, 3 and 8%, v/v). The plates were kept under the biohazard cabinet uncovered and allowed to dry for 30 min at room temperature. Plates were then incubated at 37˚C and the growth of bacteria was visually checked after 8 h and 48 h of incubation.

Objective 2: To evaluate the antioxidant activities of armoise, bay and rose geranium EOs *in vitro*.

3.2.9 DPPH Free Radical Scavenging Activity. DPPH• scavenging activity assay is to test the capacity of a substance to neutralize the free radical either by donating electron or hydrogen. This is based on the reduction of DPPH radical to non radical form by an antioxidant. DPPH• is a stable free radical because the unpaired electron spreads out over the whole molecule. The solution of this radical is characterized by purple color with maximum absorption at 517nm. When DPPH• radical is reduced by accepting hydrogen or electron from an antioxidant, the color of the solution becomes yellow which is determined by spectrophotometer (Molyneux, 2004).



1, 1-diphenyl-2 picrylhydrazyl (free radical) 1, 1-diphenyl-2 picrylhydrazine (nonradical)

DPPH free radical scavenging activity assay was performed using a method described by Yu et al. (Yu et al., 2002) with some modification. Different concentrations (%, v/v) of EO were prepared. Armoise and rose geranium EO was prepared in 10, 5, 3, 2, 1, 0.1 and 0.05% (v/v) whereas bay EO was made in 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.5 and 1% (v/v). 0.25, 0.5, 1, 25, 100, 250, 500 and 1000 micromolar (µM) of BHT was prepared as a positive control. All EOs and BHT were dissolved in methanol. One hundred µL of essential oil and 900 µL of 70 µM DPPH• solution were mixed in a tube. Methanol was used as a negative control. After mixing this solution, a dark environment was created by wrapping tubes with aluminium foil, where they were kept for 30 min at room temperature. The reaction was measured by a spectrophotometer at 517 nm. The experiment was performed in quadruplicate. The absorbance readings were used to calculate % free radical scavenging activity using the following equation:

% scavenging activity = $[1 – (Abs_{sample}/Abs_{control})] \times 100$ %

$Abs_{control}$ = Absorbance of DPPH solution

Abs_{sample} = Absorbance of essential oil/BHT

In addition, the antioxidant activity was expressed as an EC_{50} value. The EC_{50} is defined as the concentration of an antioxidant required to reduce the DPPH radical by 50% which is determined according to the method of Alexander et al. (1999). Based on a principle of right-angled triangle, two assumptions are suggested to calculate EC_{50} value. The first assumption is that the maximum response of the sample is reached from which 50% maximal response can be calculated. The second assumption is that to consider the sigmoid curve as a straight line at least one response of experimental concentrations on both sides of the 50% maximal response should be as close as possible. In that case, the ratio of y^1 to y will be equal to the ratio of x^1 to x (Figure

3.1) (Alexander, Browse, Reading, & Benjamin, 1999). EC_{50} can be calculated from the following equation in the Microsoft Excel 2007 spreadsheet program:

EC_{50} = D - (A-50% max response).x/y

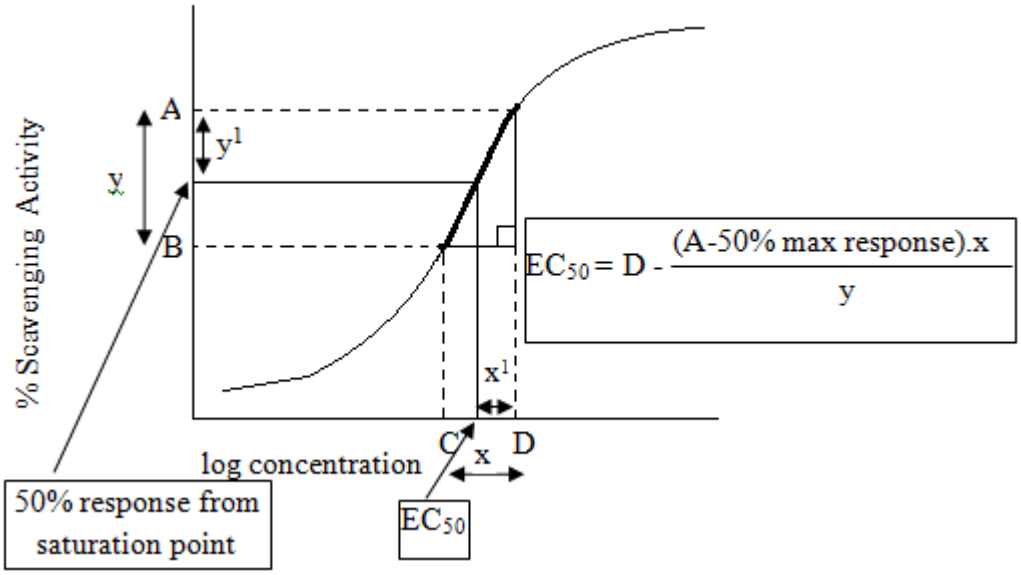


Figure 3.1 EC_{50} derivation from the concentration-response curve

(Alexander et al., 1999)

3.2.10 Reducing Power Activity. Reducing power activity assay is to determine the electron donating capacity of a substance. This method is based on the reduction of potassium ferricyanide (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}). This ferrocyanide reacts with ferric chloride and yield green color ferric ferrous complex which shows maximum absorption at 700 nm (Jayanthi & Lalitha, 2011).

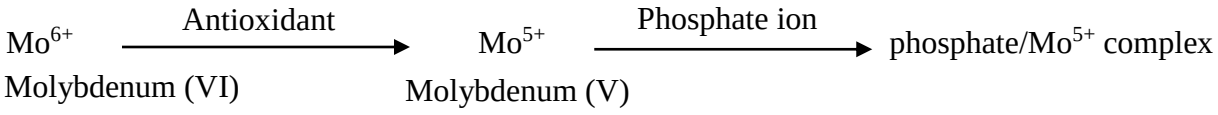
$K_3Fe(CN)_6$ —Antioxidant→ $K_4Fe(CN)_6$ —$FeCl_3$→ $Fe_4[Fe(CN)_6]_3$

Potassium ferricyanide — Potassium ferrocyanide — Ferric ferrous complex

This experiment was performed according to the method described by Pan et al. (Pan et al., 2010). Five mM of ascorbic acid was used as a positive control. One hundred µL of 10 or

100% EO (v/v) was added into reaction mixtures containing 2.5 mL of 0.2 M phosphate buffer (pH 6.6), 2.5 mL of 1% (w/v) potassium ferricyanide and 9% of tween-20. The mixture was incubated for 20 minutes at 50˚C. After incubation, the tubes were kept in water at room temperature for 2 minutes to cool. Then 2.5 mL of 10% (w/v) TCA was added to the reaction mixture and the solution was centrifuged for 10 minutes. After centrifugation, 2.5 mL of upper layer solution was transferred in a new tube and then mixed with 2.5 mL of water and 0.5 mL of 0.10% (w/v) ferric chloride. , The absorbance was measured using spectrophotometer at 700 nm.

3.2.11 Total Antioxidant Activity. Total antioxidant activity test is to assess the electron donating capacity of a substance. This method is based on the reduction of molybdenum (VI) to molybdenum (V) by an antioxidant. At acidic condition the reduced Mo (V) forms green color phosphate/Mo (V) complex having absorption maximum at 695 nm.



Total antioxidant activity test was performed by the method of Pan et al. (2008) with slight modification (Pan et al., 2008). 100 µl of 0.0001% (v/v) EO was mixed with 1 mL of reagent solution (0.6 M of sulphuric acid, 28 mM of sodium phosphate, and 4 mM of ammonium molybdate) and then incubated in water bath at 95˚C for 90 minutes. The reaction was stopped by putting the tubes in water at room temperature for five minutes. Total antioxidant activity was determined by measuring absorbance using spectrophotometer at 695 nm. EO was dissolved in methanol. Five mM ascorbic acid was used as positive control and methanol without EOs or ascorbic acid was used as blank.

3.3 Statistical Analysis

The data are expressed as means ± standard deviation (SD). The data were analyzed using the Tukey's test in R program to compare antibacterial and antioxidant activities of the studied EOs.

CHAPTER 4

Results and Analysis

4.1 Objective 1

4.1.1 Growth over time assay. The growth over time assay was used to test the inhibitory effects of the EOs on the growth of bacteria. The growth of *Salmonella* Typhimurium was checked with different concentrations of armoise, bay and rose geranium EOs at 37˚C for 8 h by measuring O.D. at 610 nm (Figure 4.1-4.3). Figure 4.1 illustrates that control sample without treatment of essential oil showed O.D. of 0.88±0.05 after 8 h incubation which indicates the maximum growth of bacteria. Increasing concentrations of armoise EO (0.1%, 0.15%, 0.2%, 0.25%, 0.5% and 1%, (v/v)) caused a significant reduction (p<0.05) in the growth of *S.* Typhimurium, confirmed by decreased O.D. In the presence of 0.1% armoise EO, there was a slight increase in the O.D. value (0.03±0.02) but was significantly low as compared to no armoise EO treatment sample (p<0.05). It has been reported that no visual growth can be considered with and O.D. reading of less than 0.1 (Hayek & Ibrahim, 2012). Salmonella did not grow at armoise EO concentrations of 0.15% and higher, confirmed by their zero O.D. reading.

Figure 4.2 shows the effect of bay EO on the growth of *S.* Typhimurium. In control, O.D. reached an average of 0.79±0.02 after 8 h of incubation. The addition of bay EO significantly reduced the growth of *S.* Typhimurium (p<0.05). At a concentration of 0.1%, bay EO indicated lower bacterial growth, confirmed by an O.D. of 0.32±0.02 compared to control (p<0.05). Whereas, 0.15%, 0.2% and 0.25% bay EO treatment showed O.D. 0.06±0.05, 0.01±0.01 and 0.01±0.01 respectively (p>0.05) which indicates no visual growth. 0.5% and 1% bay EO exhibited no bacterial growth.

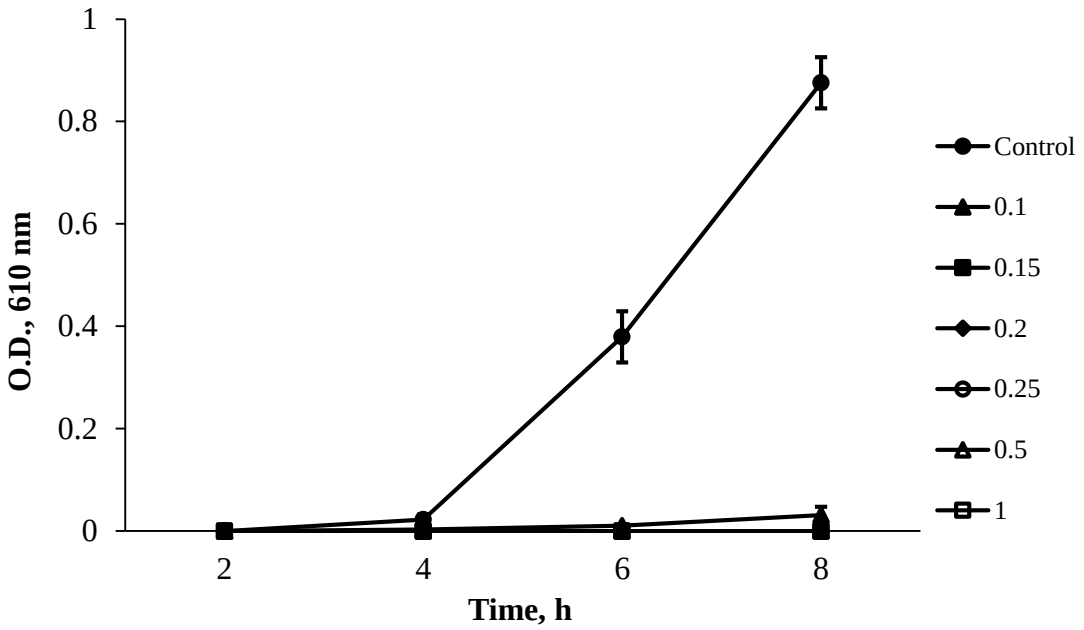


Figure 4.1 Effect of armoise essential oil in the growth of Salmonella Typhimurium.

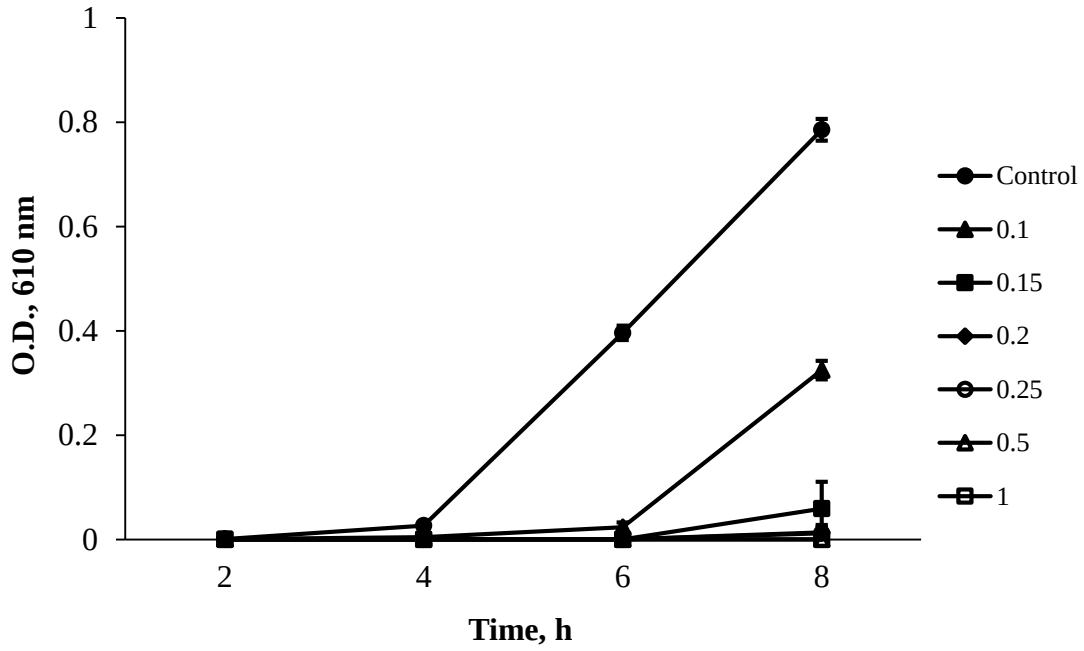


Figure 4.2. Effect of bay essential oil in the growth of Salmonella Typhimurium.

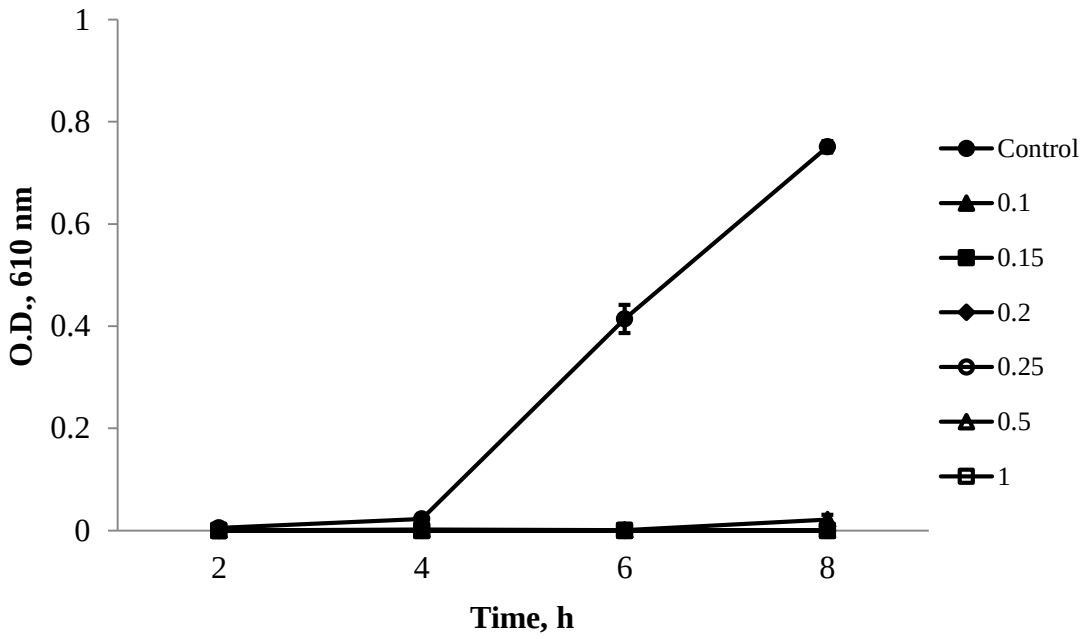


Figure 4.3 Effect of rose geranium essential oil in the growth of Salmonella Typhimurium.

The effect of rose geranium EO on the growth of *S.* Typhimurium is shown in Figure 4.3. The control sample without the treatment of EO, showed O.D. 0.751±0.01 after 8 h of incubation (37˚C). The treatment of 0.01%, 0.15%, 0.2%, 0.25%, 0.5% and 1% of rose geranium EO resulted in a decrease (p<0.05) of *Salmonella* growth. The sample with 0.1% rose geranium EO showed O.D. 0.02±0.01, which indicates no visible growth. The growth of *S.* Typhimurium was completely inhibited with 0.15% and higher concentration of rose geranium EO as indicated by the zero O.D. value.

The final *S.* Typhimurium populations were determined at the end of the incubation period using bacterial enumeration method. Table 4.1 shows the final population of *Salmonella* grown in LB broth treated with different concentrations of armoise, bay and rose geranium EOs after 8 h of incubation at 37˚C. The initial number of *S.* Typhimurium was 2.25 log CFU/mL. After 8 h,

Table 4.1

Bacterial population (Log CFU/mL) of S. Typhimurium in LB broth with different concentration (%, v/v) of EOs after 8 hour incubation (37 ˚C)

S. Typhimurium populations (log CFU/mL broth) in presence of EOs							
Essential oils	Control	0.1	0.15	0.2	0.25	0.5	1
Armoise	9.68^{Aa}±0.14	2.87^{Ab}±0.43	BDL	BDL	BDL	BDL	BDL
Bay	9.34^{Ba}±0.18	7.01^{Bb}±0.42	3.38^{c}±0.33	BDL	BDL	BDL	BDL
Rose geranium	9.15^{Ca}±0.12	1.72^{Cb}±0.22	BDL	BDL	BDL	BDL	BDL

Averages with different upper case letters (A, B and C) in the same column are significantly different (p<0.05)

Averages with different lower case letters (a, b, c and d) in the same row are significantly different (p<0.05)

BDL – Below detectable level

the population of the control sample reached an average of 9.68±0.14 log CFU/mL. Armoise EO inhibited (p<0.05) the growth of *S.* Typhimurium up to undetectable level. Treatment of 0.1% of armoise EO showed 2.87±0.43 log CFU/mL. A log reduction of 6.81 CFU/mL was achieved at this concentration. For concentrations at 0.15% and higher, armoise EO completely inhibited the growth of *S.* Typhimurium as indicated by no cells on agar plates.

A low inhibitory effect of bay EO was observed against *S.* Typhimurium. The final population in the control sample was 9.34±0.18. At 0.1% of bay EO, the survival count of *S.* Typhimurium was 7.01±0.42 (p<0.05) which corresponds to a log reduction of 2.33 CFU/mL. Survival cells were further decreased by increasing concentrations of bay EO. The presence of 0.15% bay EO reduced *S.* Typhimurium up to 5.96 log CFU/mL. No growth was observed at 0.2% and higher concentrations of bay EO.

Rose geranium EO decreased numbers of *S.* Typhimurium in LB growth media during culture. In the control sample, the final population was 9.15±0.12 log CFU/mL. When 0.1% rose geranium EO was added, the survival count in LB broth reached 1.72±0.22 log CFU/mL, thus causing a log reduction of 7.43 CFU/mL. In addition, no cell was detected on the agar plate with 0.15%, 0.20%, 0.25%, 0.5% and 1% rose geranium EO treated sample.

Figure 4.4 shows the growth of *E. coli* O157:H7 in LB broth in the presence of different concentrations of armoise EO at 37˚C. After the 8 h incubation period, the mean O.D. (610 nm) value of the control sample reached 0.84±0.01. Treatment of the sample with armoise EO caused a significant reduction in the growth of *E. coli* O157:H7 as measured by O.D. value. Armoise EO when added at a concentration of 0.1% caused a reduction in the O.D. (p<0.05) value, which

indicates a delay in the growth of *E. coli* O157:H7. An increase of the concentration of armoise

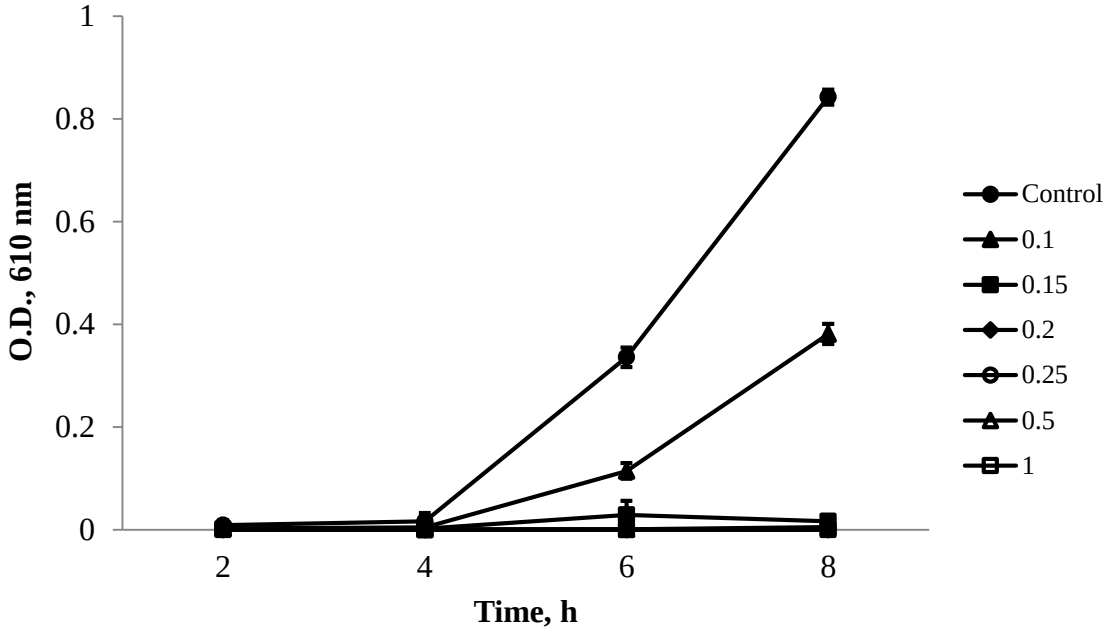


Figure 4.4 Effect of armoise essential oil in the growth of E.coli O157:H7.

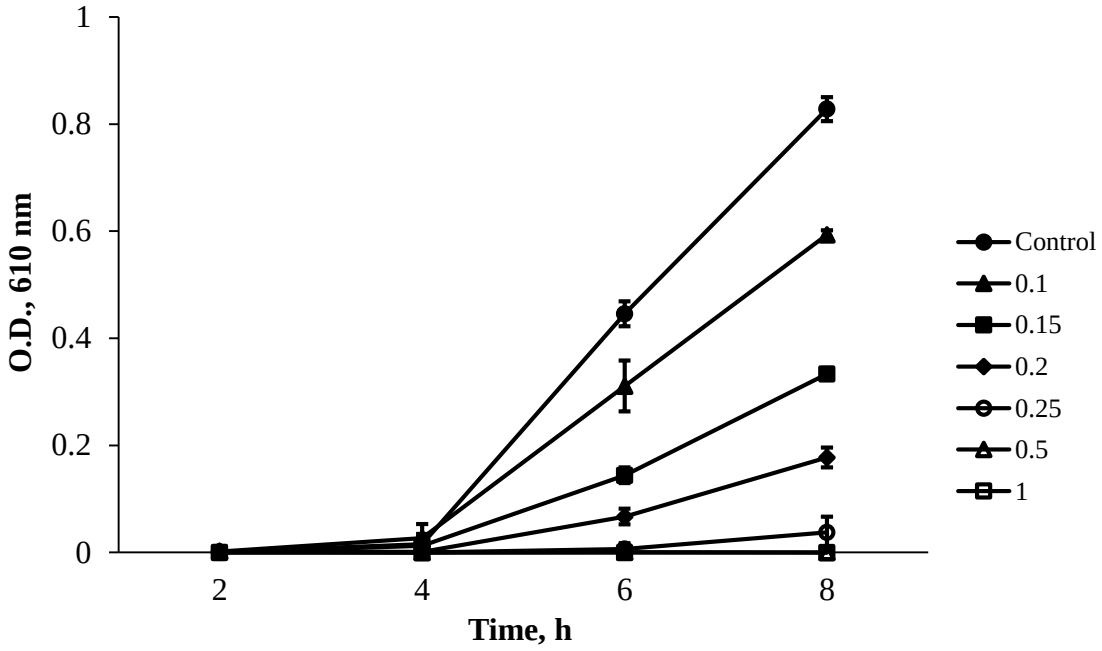


Figure 4.5 Effect of bay essential oil in the growth of E. coli O157:H7.

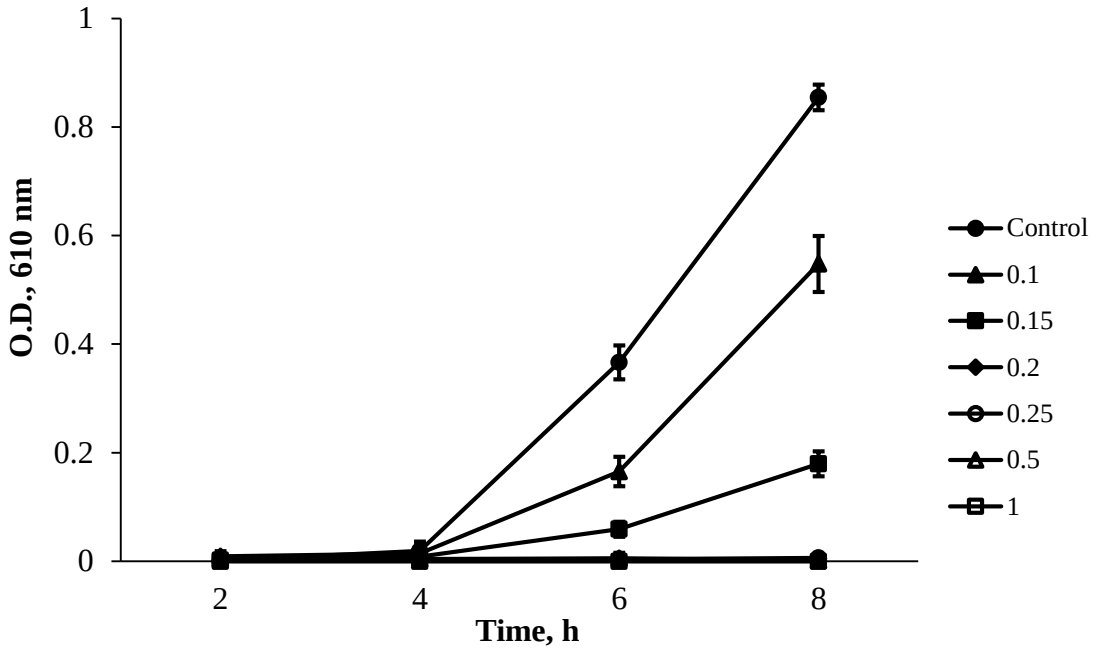


Figure 4.6 Effect of rose geranium essential oil in the growth of E. coli O157:H7.

EO resulted in the further decrease of O.D. value. Samples with 0.15% of armoise EO had an O.D. value of 0.02±0.01 indicating significant inhibition of *E. coli* O157:H7 growth. Complete inhibition of *E. coli* O157:H7 was obtained at the concentration of 0.2%, 0.25%, 0.5% and 1% armoise EO with the O.D. values being 0, 0.0003±0.001, 0.005±0.01 and 0 respectively.

Similar results for bay EO are presented in Figure 4.5. Bay EO showed inhibitory effect at higher concentrations. After an 8 h period of incubation, O.D. of the control sample reached 0.83±0.02. There was a significant difference ($p<0.05$) in the O.D. value of the samples treated with different concentrations of bay EO. When bay EO was added at 0.1%, the growth was slightly retarded as indicated by the O.D. value (0.59±0.01). The growth of *E. coli* O157:H7 was further slowed down by the treatment of 0.2% bay EO (O.D. 0.33±0.01). Although 0.2% bay EO caused a significant reduction in the O.D. value (0.18±0.02) as compared to the control sample, still the growth was visible (O.D.>0.1). Low value in the O.D. (0.04±0.03) of 0.25% bay EO treated sample indicates a significant inhibition in the growth of *E. coli* O157:H7. At 0.5% and

1% concentration of bay EO, the O.D. value of zero indicates the total inhibition of *E. coli* O157:H7 growth.

The growth of *E. coli* O157:H7 in LB broth in the presence of rose geranium EO during an 8 h period of incubation at 37˚C as measured by O.D. is shown in Figure 4.6. The maximum average O.D. of the control sample reached 0.85±0.02. The O.D. value was reduced with the increase of concentration of rose geranium EO (0.1%, 0.15%, 0.2%, 0.25%, 0.5% and 1% (v/v)) applied in the LB broth (p<0.05). The mean O.D. of 0.55±0.05 at 0.1% concentration also, indicates the slow growth of *E. coli* O157:H7. Treatment with 0.15% rose geranium EO caused a significant inhibition in the growth of *E. coli* O157:H7 (O.D. 0.18±0.02), while O.D. of 0.005±0.006, 0.006±0.007, 0.002±0.002 and 0 at 0.2%, 0.25%, 0.5% and 1% (v/v) rose geranium EO indicates complete inhibition.

The final population of *E. coli* O157:H7 was determined at the end of the incubation period using bacterial enumeration method. Table 4.2 shows the final population of *E. coli* O157:H7 grown in LB broth treated with different concentrations of armoise, bay and rose geranium EOs after 8 h of incubation at 37˚C. Since there was no significant difference in the final population of the three control samples, the average was taken. In the control sample, the initial number of *E. coli* O157:H7 was 2.21 log CFU/mL and after 8 h, the population reached an average of 8.99±0.10. When armoise EO was added, it retarded the growth of *E. coli* O157:H7 in LB broth. At 0.1% and 0.15% of armoise EO, the survival count of *E. coli* O157:H7 was 7.02±0.23 log CFU/mL and 3.04±0.14 log CFU/mL respectively, which are significantly

Table 4.2

Bacterial population (log CFU/mL) of E. coli O157:H7 in LB broth with different concentration (%, v/v) of essential oils after 8 hour incubation (37 ˚C)

E. coli O157:H7 populations (log CFU/mL broth) in presence of EOs							
Essential oils	Control	0.1	0.15	0.2	0.25	0.5	1
Armoise	8.89^{Aa}±0.28	7.02^{Ab}±0.23	3.04^{Ac}±0.14	BDL	BDL	BDL	BDL
Bay	8.99^{Aa}±0.20	8.06^{Bb}±0.28	6.30^{Bc}±0.21	5.17^{Ad}±0.19	4.17^{e}±0.31	BDL	BDL
Rose geranium	9.08^{Aa}±0.27	7.81^{ABb}±0.42	5.05^{Cc}±0.30	3.38^{Bd}±0.25	BDL	BDL	BDL

Averages with different upper case letters (A, B and C) in the same column are significantly different (p<0.05)

Averages with different lower case letters (a, b, c and d) in the same row are significantly different (p<0.05)

BDL – Below detectable level

different from the control sample ($p<0.05$). Armoise EO at 0.1% caused a log reduction of 1.89 CFU/mL while 5.85 log CFU/mL reduction was achieved at 0.15% ($p<0.05$). The results indicate that 0.15% has significant inhibitory effect on the growth of *E. coli* O157:H7. No colony was observed on the agar plate at 0.2% and higher concentrations of armoise EO.

Bay EO was observed to have a low inhibitory effect. At 0.1% bay EO, the *E. coli* O157:H7 population was 8.06±0.28 log CFU/mL that corresponds to a less than 1 log reduction of *E. coli* O157:H7 population (0.93 log CFU/mL) compared to the control sample (8.99±0.10 log CFU/mL). The survival count was further decreased in increasing concentrations of bay EO. At 0.15%, 0.20% and 0.25% (v/v) the *E. coli* O157:H7 population was found 6.30±0.21, 5.17±0.19 and 4.17±0.31 log CFU/mL, respectively. The highest log reduction of 4.81 log CFU/mL was achieved at 0.25% ($p<0.05$). The presence of 0.15% and 0.20% bay EO resulted in a log reduction of 2.69 and 3.81 CFU/mL ($p<0.05$). No growth was detected on agar plates from the samples with 0.5% bay EO.

An addition of rose geranium EO caused a reduction in the survival of *E. coli* O157:H7 in LB broth (Table 4.2). In the presence of 0.1%, 0.15% and 0.2% rose geranium EO, the survival count of *E. coli* O157:H7 in LB broth after 8 h of incubation (37˚C) were 7.81±0.23, 5.05±0.30 and 3.38±0.25 log CFU/mL respectively. A 1.27 log CFU/mL reduction of *E. coli* O157:H7 population was obtained at 0.10% of rose geranium EO. A further log reduction was observed with increasing concentrations of rose geranium EO. At 0.15% and 0.2%, the resulting log reduction was 4.03 and 5.70 CFU/mL ($p<0.05$) respectively. No cell was detected on agar plates at 0.25% and higher concentrations of rose geranium EO.

4.1.2 Agar Diffusion Spot Technique. Agar diffusion spot technique was used to determine the minimum inhibitory concentrations (MIC) and minimum lethal concentrations

Table 4.3

Inhibitory effect of EOs against Salmonella Typhimurium after 8 h incubation on LB agar plate

Concentrations (%, v/v)	Armoise	Bay	Rose geranium
0.1	+	+	+
0.2	+	+	+
0.3	+	+	+/-
0.4	+	+	-
0.5	+	+	-
0.6	+	+	-
0.8	+/-	+	-
0.9	-	+/-	-
1	-	+/-	-
3	-	-	-
6	-	-	-
8	-	-	-

(-) no growth; (±) some growth; (+) growth of *Salmonella* Typhimurium

Table 4.4

Inhibitory effect of EOs against Salmonella Typhimurium after 48 h incubation on LB agar plat

Concentrations (%, v/v)	Armoise	Bay	Rose geranium
0.1	+	+	+
0.2	+	+	+
0.3	+	+	+/-
0.4	+	+	-
0.5	+	+	-
0.6	+	+	-
0.8	+/-	+	-
0.9	+/-	+	-
1	+/-	+/-	-
3	-	-	-
6	-	-	-
8	-	-	-

(-) no growth; (±) some growth; (+) growth of *Salmonella* Typhimurium

(MLC) of the EOs. The growth of *Salmonella* Typhimurium on agar plates on the spotted area at different concentrations (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 3%, 6% and 8%) of armoise, bay and rose geranium EOs are presented at Table 4.3 and Table 4.4. After an 8 h incubation period, it was observed that armoise EO at 0.1%, 0.2%, 0.3%, 0.4%, 0.5% and 0.6% did not have any inhibitory effect on the growth of *S.* Typhimurium (Table 4.3). Some colonies were observed on a spotted area with 0.8% armoise EO while no bacterial growth was observed at 0.9% and higher concentrations. The minimum inhibition concentration (MIC) that inhibits the growth of bacteria was found at 0.9% for armoise EO. Longer incubation period caused the growth of *S.* Typhimurium at higher concentrations of armoise EO. After 48 h, some colonies were observed at 0.8%, 0.9% and 1% but the spotted area was clear at 3%, 6% and 8% (Table 4.4). The minimum lethal concentration (MLC) that kills off the bacteria was found at 3% for armoise EO. The inhibitory effect of bay EO on the growth of *S.* Typhimurium was observed for high concentrations. After 8 h of incubation, no inhibition was observed at a concentration of 0.1%, 0.2%, 0.3%, 0.4%, 5%, 0.6% and 0.8% (Table 4.3), while some bacterial growth was detected at 0.9% and 1% of bay EO. Concentrations of 3% and higher, caused complete inhibition on the growth of bacteria in the spotted area. The MIC for bay EO was found to be around 3%. When the incubation period was increased to forty eight hours, the growth of *S.* Typhimurium was observed at 0.9% of bay EO (Table 4.4), while 1% showed slight inhibition. Concentration at 3% still completely retards the growth of *S.* Typhimurium on the spotted area which indicates the MLC of bay EO around 3%.

Table 4.5

Inhibitory effect of EOs against E. coli O157:H7 after 8 h incubation on LB agar plat

Concentration (%, v/v)	Armoise	Bay	Rose geranium
0.1	+	+	+
0.2	+	+	+
0.3	+	+	+/-
0.4	+/-	+	-
0.5	-	+	-
0.7	-	+/-	-
0.8	-	-	-
0.9	-	-	-
1	-	-	-
3	-	-	-
6	-	-	-
8	-	-	-

(-) no growth; (±) some growth; (+) growth of *E. coli* O157:H7

Rose geranium EO showed strong inhibitory effects on the growth of *S.* Typhimurium compared to other EOs. No inhibitory effect was found at 0.1% and 0.2% concentration of rose geranium EO after 8 h of incubation (Table 4.3). The growth of *S.* Typhimurium was slightly inhibited at a concentration of 0.3%, whereas no bacterial colony was observed for concentrations of 0.4% and higher. The results indicated that the MIC of rose geranium EO was approximately 0.4%. Longer incubation (48 h) of the plates caused some growth of *S.* Typhimurium on the spotted area at 0.3% but complete inhibition was observed at 0.4% and higher concentrations of rose geranium EO (Table 4.4). Thus, in this case, the MLC of rose geranium EO was around 0.4%.

Similar results for *E. coli* O157:H7 at different concentrations of armoise, bay and rose geranium EOs are presented in Table 4.5 and Table 4.6. After an 8 h incubation period, it was found that at 0.1%, 0.2% and 0.3% concentrations, armoise EO did not have any inhibitory effect on the growth of *E. coli* O157:H7. At a concentration of 0.4%, some colonies were observed inside the spot, while concentrations of 0.5% and higher, bacterial growth were not observed on the spotted area. Thus the MIC of armoise EO was found to be 0.5%. After 48 h of incubation, *E. coli* O157:H7 was found to grow inside the spot at 0.5% of armoise EO (Table 4.6). No bacterial growth was observed at a concentration of 0.7% and higher, which was the MLC (0.7%) due to the elongated incubation period.

Bay EO required a higher concentration to inhibit the growth of *E. coli* O157:H7. After 8 h of incubation, concentrations of 0.1%, 0.2%, 0.3%, 0.4% and 0.5% showed no inhibitory effect (Table 4.5), whereas some colonies were observed at 0.7% of bay EO. Concentrations of 0.8% and higher caused no growth of bacteria on the spotted EO. The MIC for bay EO was thus found to be 0.8%. A forty-eight hour incubation of the plates caused some growth of *E. coli* O157:H7

at 0.8% and 0.9% of bay EO (Table 4.6). No bacterial growth was observed at 1% and higher concentrations of bay EO. The results suggested that the MLC of bay EO was 1%.

Table 4.6

Inhibitory effect of EOs against E. coli O157:H7 after 48 h incubation on LB agar plate

Concentration (%, v/v)	Armoise	Bay	Rose geranium
0.1	+	+	+
0.2	+	+	+
0.3	+	+	+/-
0.4	+	+	+/-
0.5	+/-	+	-
0.7	-	+	-
0.8	-	+/-	-
0.9	-	+/-	-
1	-	-	-
3	-	-	-
6	-	-	-
8	-	-	-

(-) no growth; (±) some growth; (+) growth of *E. coli* O157:H7

Rose geranium EO also showed good inhibitory effect on the growth of *E. coli* O157:H7. After an 8 h incubation, bacteria were found to grow on the spotted area at a concentration of 0.1% and 0.2% (Table 4.5). The growth was slightly inhibited at a concentration of 0.3% and no bacterial growth was observed at 0.4% and higher concentrations. The results indicated that the MIC of rose geranium EO was 0.4%. Longer incubation (48 h) of the plates caused the slight growth of *E. coli* O157:H7 on the spotted area at 0.3% and 0.4% of rose geranium EO (Table 4.6). Concentrations of 0.5% and higher showed complete inhibition on the growth of *E. coli* O157:H7 which indicates that the MLC of rose geranium EO was 0.5%.

4.2 Objective 2

4.2.1 DPPH Radical Scavenging Activity. Free radicals are part of the propagation of autoxidation and are considered the main reactants. To test three EOs' capacity to neutralize the free radicals, DPPH radical scavenging activity was measured.

Figure 4.7 shows the DPPH radical scavenging activity of armoise EO. Moderate scavenging activity was found for armoise EO. At 0.05% and 0.10% concentrations, the activity was very low (1.52±1.17% and 6.42±8.87% respectively). Higher concentrations caused an increase in scavenging activity. At 1% (v/v) concentrations, armoise EO showed scavenging activity of 22.06±6.02% which is not significantly different from scavenging activity of 0.1% (p>0.05). The scavenging activity was significantly increased for 2% (41.47±8.90%) and 3% (44.47±6.45%) armoise EO compared to 1%. Further increase in concentration showed higher scavenging activity. In the presence of 5% armoise EO, the scavenging activity was found out to be 61.40±9.45%. The concentration at 10% showed the highest activity of 77.63±5.15% which is not significantly different from scavenging activity of 5% armoise EO.

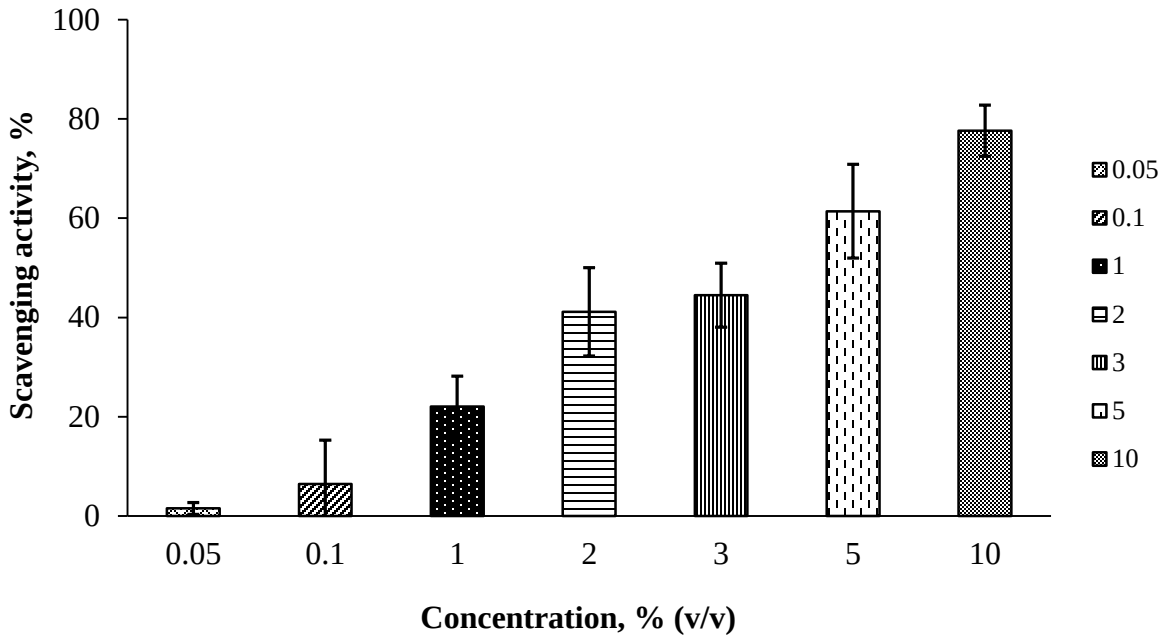


Figure 4.7 DPPH free radical scavenging activity of armoise essential oil.

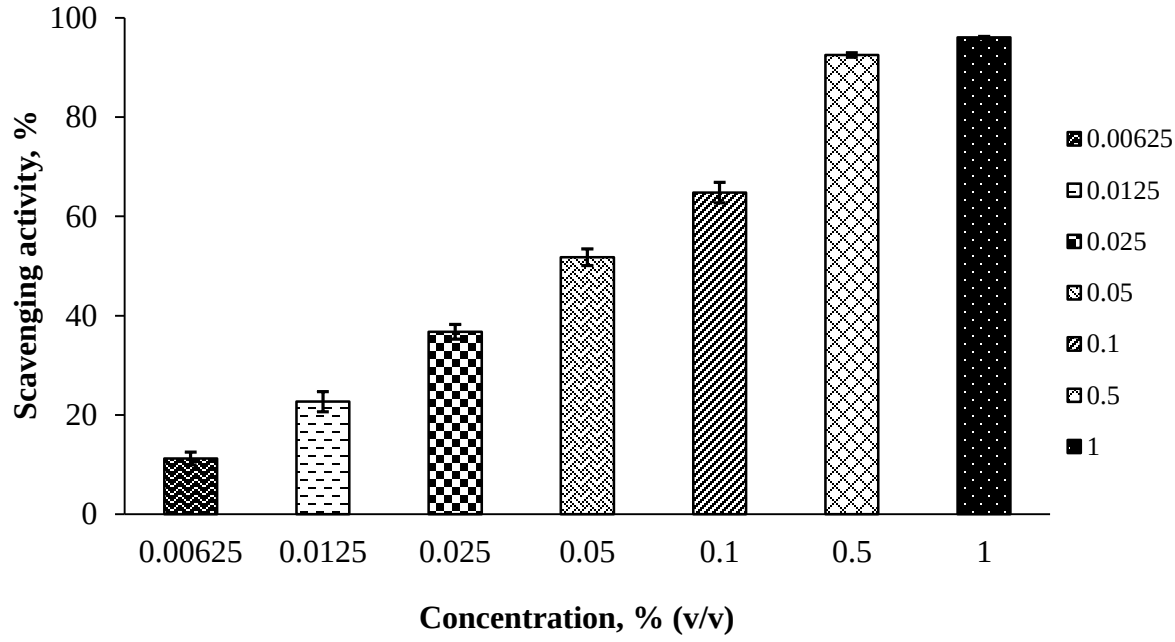


Figure 4.8 DPPH free radical scavenging activity of bay essential oil.

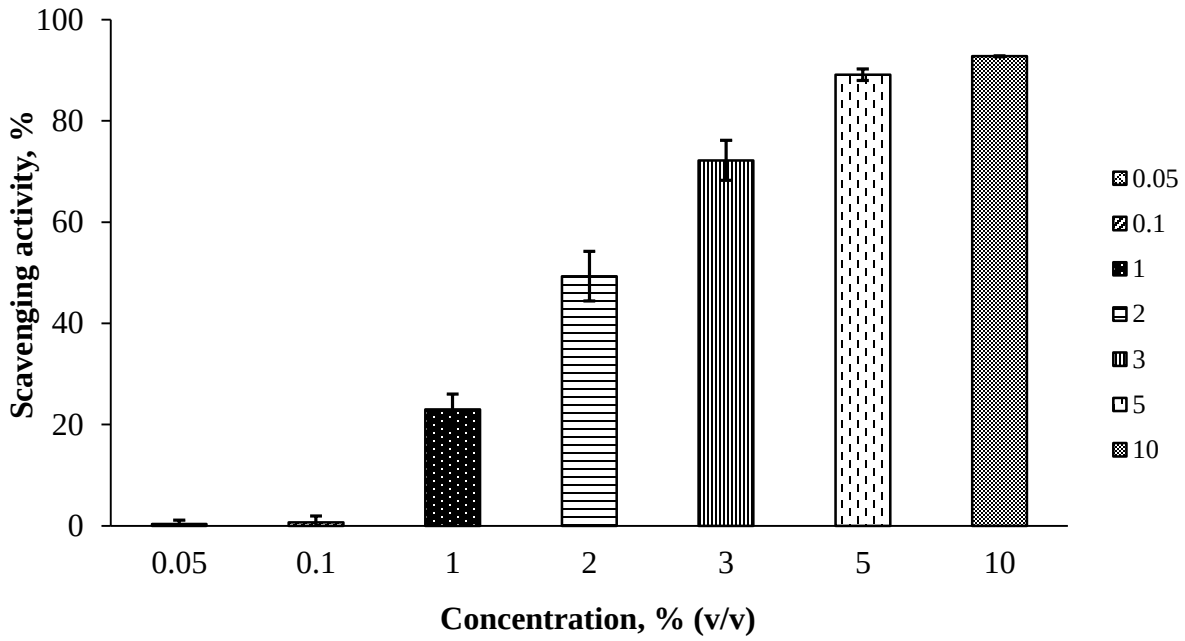


Figure 4.9 DPPH free radical scavenging activity of rose geranium essential oil.

DPPH radical scavenging activity of bay EO is presented in Figure 4.8. Bay EO was found to have very high scavenging activity. At 0.006255% concentration the scavenging activity was 11.21±1.30%. An increase in concentration significantly increased the radical scavenging activity (p<0.05). At 0.0125, 0.025, 0.05 and 0.1% concentrations the scavenging activity was 22.67±2.04, 36.75±1.47, 51.77±1.67 and 64.78±2.06% respectively. Higher scavenging activity was found at 0.5% and 1% concentrations of bay EO which was 92.53±0.44 and 96.04±0.21% respectively.

Figure 4.9 shows the DPPH radical scavenging activity of rose geranium EO. The scavenging activity was concentration dependent. At 0.05% and 0.1%, scavenging activity was found less than 1. The scavenging activity was increased with higher concentrations of rose geranium EO. About 22.99±3.05%, 49.33±4.90% and 72.21±3.97% scavenging activity was found for 1%, 2% and 3% concentration. Higher scavenging activity was found at 5% (89.16±1.14%) and 10% (92.78±0.11%) of rose geranium EO.

The antioxidant activity of each EO was expressed in terms of EC_{50} value (concentration required to inhibit DPPH radical by 50%). Smaller EC_{50} values indicate better DPPH free radical scavenger. Among the three EOs, bay had the lowest EC_{50} value (0.043±0.005, % v/v), which was significantly different (p<0.05) from EC_{50} value of armoise and rose geranium (Table 4.7). Although armoise EO was found to have a slightly higher EC_{50} value than rose geranium EO, the difference was insignificant (p>0.05). EC_{50} value (%, v/v) for armoise EO and rose geranium EO were 2.33±0.47 and 1.85±0.20 respectively. These values suggest that among the three essential oils bay had the strongest capacity in neutralizing free radicals compared to armoise and rose geranium.

Table 4.7

EC_{50} of essential oils against DPPH

Essential oils	EC_{50} (%, v/v)
Armoise	2.33^{A}±0.47
Bay	0.04^{B}±0.005
Rose geranium	1.85^{A}±0.20

Averages with different upper case letters (A and B) in the same column are significantly different (p<0.05)

4.2.2 Reducing Power Activity Assay. The electron donating capacity of essential oils was tested using reducing power assay. Higher absorbance indicates higher reducing power. The results of this method are presented in Figure 4.10. Absorbance of each commercial EO is gradually increased from lower concentrations (10%) to higher concentrations (100%) for the

analysis. At 10% (v/v) concentration, bay EO showed significantly (p<0.05) higher absorbance than armoise EO and rose geranium EO. Absorbance of bay EO was found to be 0.48±0.05 while for armoise EO and rose geranium EO it was 0.08±0.01 and 0.05±0.005 respectively. Although armoise EO showed slightly higher absorbance than rose geranium EO, the difference was not significant (p>0.05). An increase in concentration caused an increase in absorbance reading of all essential oils. At a concentration of 100%, bay EO still showed much higher absorbance as compared to armoise EO and rose geranium EO (p<0.05). The absorbance of bay EO was 2.30±0.35 followed by armoise EO (0.47±0.04) and rose geranium EO (0.25±0.02). Even for 100% concentration, the absorbance value armoise EO and rose geranium EO still had a negligible difference (p>0.05). Higher absorbance reading of bay EO suggests that it has more capacity to donate electrons to free radicals compared to the other two EOs which means that it has more reducing power.

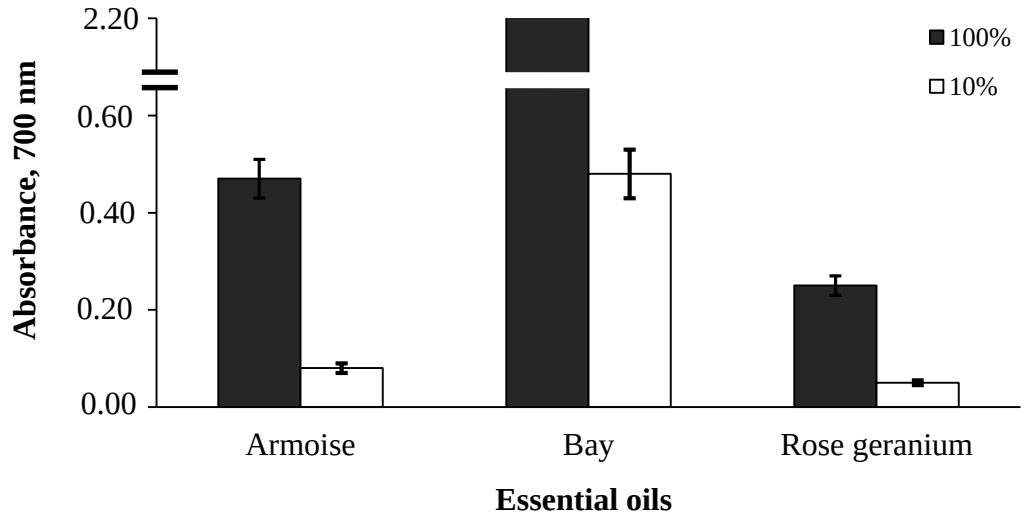


Figure 4.10 Reducing power of essential oils.

4.2.3 Total Antioxidant Activity Test. Results from the total antioxidant activity test are shown in Figure 4.11. Higher absorbance means higher total antioxidant activity. At 0.0001%

(v/v) concentration, bay EO showed higher absorbance than that of armoise EO and rose geranium EO (p<0.05). There was no significant difference between the absorbance of armoise EO and rose geranium EO. The absorbance reading for armoise EO, bay EO and rose geranium EO, which are 0.07±0.03, 0.18±0.05 and 0.10±0.03 respectively, indicate that bay EO had higher total antioxidant activity compared to armoise EO and rose geranium EO.

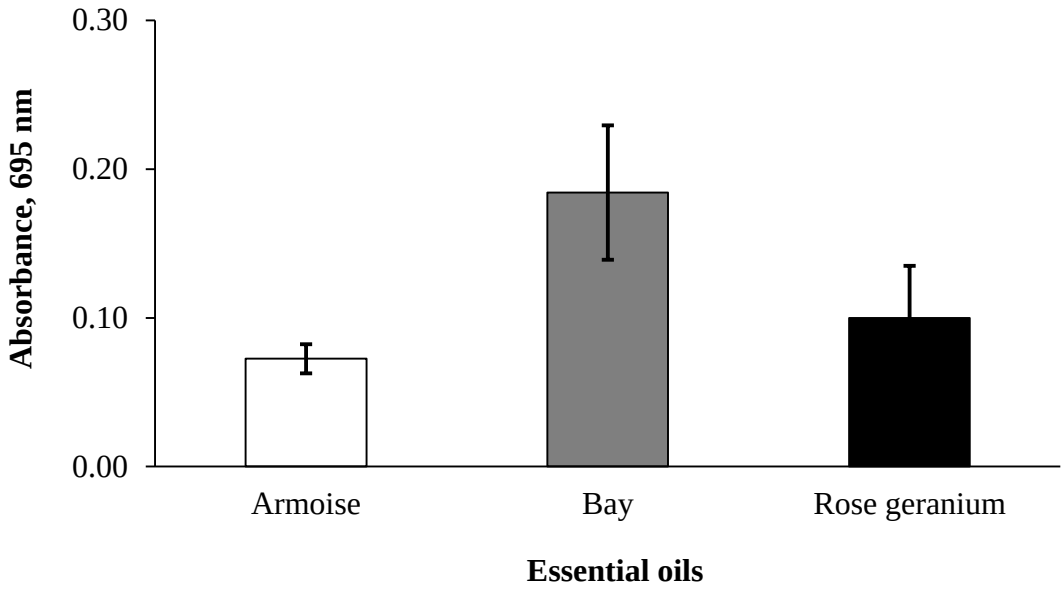


Figure 4.11 Total antioxidant activity of essential oils.

CHAPTER 5

Discussion and Future Research

In this study, antibacterial effects of armoise, bay and rose geranium EOs were tested on *S.* Typhimurium and *E. coli* O157:H7 using growth over time assay and agar diffusion spot technique. The antioxidant activity of the same EOs was additionally assessed using DPPH radical scavenging test, reducing power method and total antioxidant activity assay.

All of the EOs used for this study showed antibacterial activity with the variation depending on both type and concentration of the EO. In both techniques used to determine the antibacterial activity, armoise and rose geranium EOs showed consistent and promising antimicrobial effects, while bay EO showed lower than the others. In growth over time assay, armoise EO was found to be the most effective, and highly sensitive to *S.* Typhimurium. In agar diffusion spot technique rose geranium EO was the most effective and highly sensitive to *E. coli* O157:H7. Sbayou et al. (2014) reported that the MIC and MLC of armoise EO against *E. coli* was around 0.125% (Sbayou et al., 2014). The MIC and MLC obtained from armoise EO treatment in the present study was higher than that of some previous studies. Silveira et al. (2012) reported that the MIC and MLC of bay EO against *E. coli* was about 0.25% and against *S.* Typhimurium it was around 0.5% and 1% respectively (Silveira, Cunha Júnior, Scheuermann, Secchi, & Vieira, 2012). Boukhatem et al. (2013) showed that the MIC and MLC of bay EO against both *E. coli* O157:H7 and *Salmonella sp.* are 0.4% and 2.1% respectively. They reported that rose geranium oil caused inhibition at higher concentration against *E. coli*, while it had no inhibiting effect against *S.* Typhimurium in disk diffusion method. (Boukhatem et al., 2013). Although, the main components of the reported geranium oil are similar to those of the present study the higher inhibition effect of geranium oil might be due to several factors such as the

variation in the percentage of main components, method of extraction, and the different methods used in evaluating the antibacterial activity.

In the present study, among the three EOs, bay was found to be the most effective in DPPH radical scavenging activity. Although, rose geranium and armoise EOs had lower scavenging activity, still rose geranium EO results were comparatively better among the two. The variation in the scavenging activity of the EOs may possibly be due to the dissimilarity in their composition. Gas chromatography analysis of each EO provided by the company revealed the difference in their chemical composition. The primary constituent of bay EO was 1, 8-cineol (40%) and linalool (12.5%) while the same for rose geranium EO was citronellol (22.5%). The chemical composition of armoise EO was dominated by thujone (70%). As bay EO shows strong scavenging activity, the possible reason might be due to the presence of 1, 8- cineole and linalool. However, previous studies on antioxidant activities of 1, 8-cineole and linalool as individual components did not show any promising result (Lee et al, 2005). Another study, on bay EO has shown that it had almost similar activity to standard BHT in terms of DPPH radical scavenging (Paliteo et al, 2007). Cherrat et al. (2014) also found similar results with bay oil (Cherrat et al., 2014). Therefore, the strong activity of bay EO may be due to the presence of other minor components and synergistic effect between the minor and the major components. The antioxidant activities of rose geranium and armoise EOs have been studied previously and compared against BHA. Guerrini et al. (2011) found low scavenging activity of rose geranium EO (Guerrini et al., 2011) whereas, Zouari et al. (2010) found low antioxidant activity for armoise EO compared to BHA, a known synthetic antioxidant (Zouari et al, 2010).

Reducing power method is used to test the electron donating capacity of an antioxidant where higher absorbance indicates higher antioxidant activity. Strong reducing power of bay EO

indicates that the electron donating capacity of bay EO is high. The ease of bay EO to donate electron to free radicals has been described to be the reason for its ability to bring chain termination by neutralizing free radicals (Hsu, Coupar, & Ng, 2006; Yen & Chen, 1995). Armoise EO and rose geranium EO were found to have almost similar reducing power which suggested that both of EOs have similar electron donating capacity. Previous studies have also reported higher reducing power of bay EO compared to the present study. The main components of bay EO in the previous study were 1, 8-cineole, 2-carene, trans-ocimene and sabinene, while the main composition of bay EO in the present study were 1, 8-cineole, linalool, methyl eugenol and eugenol. Their difference in chemical composition might be due to the difference in origin of the plants which reflects their reducing power activity (Cherrat et al., 2014).

In the current study bay EO showed the most promising result in the total antioxidant activity test. Almost similar activity was found for rose geranium and armoise EOs. Total antioxidant activity of bay EO was previously tested by Ekren et al. (2013) who used the same method and also found higher antioxidant activity for bay EO compared to sage, mint and oregano EOs which are known to have good antioxidant activity (Ekren, Yerlikaya, Tokul, Akpınar, & Accedil, 2013).

One of the limitations for the antibacterial study was that the optimum concentration of EOs required to inhibit the growth of bacteria completely in growth over time assay could not be determined. The current research suggests that these EOs can be an alternative for synthetic antimicrobials and antioxidants for the purpose of food preservation. As a result it will act as a healthier option for the consumers. In the current research, the antibacterial activity of the armoise, bay and rose geranium EOs was conducted on foodborne pathogens in laboratory media. Thus, further study should be conducted on the food matrix to find their effectiveness in

food. Studies of these EOs should also be expanded by combining with different types of physical treatments to find the comparative effectiveness in inhibiting the foodborne pathogens.

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