EFFICACY OF ESSENTIAL OIL NANOEMULSION DELIVERY SYSTEM FOR STRONG ANTIMICROBIAL ACTION AGAINST PATHOGEN *LISTERIA MONOCYTOGENES*

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DINA H AL-MANSOORI

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ABSTRACT OF THE DISSERTATION

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By Dina H Almansoori

Dissertation Director:

Paul Takhistov

Listeria monocytogenes has gained increasing attention as a pathogen of public health importance owing to large numbers of foodborne outbreaks of listeriosis, led to this study. A wide range of essential oils (EOs) and natural products have been used to control *Listeria monocytogenes.* The reason for using essential oils is to preserve food by inhibiting the growth and proliferation of microorganisms in food due to its antimicrobial effects against microorganism, but their low water solubility limits their efficacy and application in food. This study included two objectives first, in the present study, 28 different essential oils were evaluated for their antimicrobial activities against *Listeria monocytogenes*.Various concentrations of EOs were introduced into brain heart infusion broth to determine the minimum inhibitory concentration (MIC) for the pathogen. The experimental data were fitted to the modified Gompertz model, and the lag phase duration and maximum growth rate were calculated and compared for each essential oil at various concentrations. Overall, our experimental results indicate that frankincense, eucalyptus, and fire needle oils had the strongest inhibitory effects against *Listeria*

monocytogenes with MICs <2.4 µg/mL. Essential oils with moderate antimicrobial effects included key lime, cedar wood, egyptian geranium, nutmeg, peppermint, valerian, and ylang ylang. Second, the antibacterial effects of the essential oils derived from the extracts of cedar wood, copaiba, fire needle, frankincense, egyptian geranium, nutmeg, peppermint, valerian and ylang ylang against the *Listeria monocytogenes*, was investigated in the presence and absence of different, 17.59, 35.17 and 70.34 µg/ml, concentrations of the casein proteins. The lag phase durations and maximum growth rate of each essential oil were calculated and compared. The effectiveness of individual essential oil varied from one essential oil to the other. The influence of the food matrix such casein proteins, was tested and determined. The effectiveness of the essential oils in absence of casein proteins and in the presence 17.59 µg/ml of casein proteins was similar but further decreased in addition of 35.17 and 70.34 µg/ml of casein proteins.

Keywords: *Listeria monocytogenes*, Essential oils, Nanoemulsion, Growth kinetics, casein protein

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Chapter I: General Background, Significance of the Study, Hypothesis, and Objectives

1.1 Introduction

The food-borne disease listeriosis, caused by the pathogen *Listeria monocytogenes*, has had serious negative human health effects in the United States, so ways are needed to minimize the growth of *L. monocytogenes* on food. Based on rising concerns, today's consumers demand high-quality food free of chemical additives and pathogens that cause food-borne diseases. In this regard, the application of natural preservation such as essential oils (Eos), which have antimicrobial properties, is important to control foodborne pathogen. .EOs are complex mixtures of a broad range of components, and their antimicrobial activity is linked to their composition, configuration, amount, and possibility of interaction. The range of EO activity against bacteria that can restrict the bacterial growth is termed *bacteriostatic*. EOs can be applied at high concentrations and are naturally more aggressive, and their activity results in a reduction of the number of bacterial cells, known as *bactericides* (Faleiro, 2011). According to Kerekes et al. (2015), the phenolic components of EOs are the most active and disrupt bacterial cell membranes, leading to bacterial death; thus, gram-positive organisms such as *L. monocytogenes* are more sensitive to EOs than to gram-negative organisms. The cytotoxic property of EOs is fundamental in the use of EOs on human and animal pathogens and to the preservation of agricultural or marine products, so this attribute makes EOs highly effective against a large variety of organisms, including bacteria (Basile et al., 2006). This study is based on the growing interest in the application of

nanoemulsions of EOs as natural food preservatives in the food industry (Calo, Crandall, O'Bryan, & Ricke, 2015). The literature includes details of EOs' antimicrobial activity. However, the impact of EOs' concentrations as natural preservative agents on the sensory integrity and characteristics of foods has only been marginally addressed. The concentration of EOs required to cause an inhibitory antimicrobial effect in vitro is significantly higher than the concentrations required to cause similar effects in real foods. High concentrations of these natural antimicrobials may alter the organoleptic and sensory characteristics of food products, such as odor and taste (Maté et al., 2017). Through a food model medium, the study will analyze the mechanism of the interaction of the EO nanoemulsions and the food matrix and elucidate the effect of casein protein on the activity of *L. monocytogenes*.

1.2 Significance of the Study

Celikel and Kavas (2008) argued that despite having put in place various measures toward improving food hygiene and food production techniques, food safety is still a concern based on the numerous cases of foodborne diseases resulting from bacterial pathogens. One way to deal with the issues is the use of EOs from medicinal plants and aromatics, which are have antibacterial, antifungal, and antioxidant properties and further beneficial health effects (Zengin & Baysal, 2014). Therefore, this study evaluates the effectiveness of EOs nanoemulsions on restricting the growth and formation of *L. monocytogenes.* Additionally, the study provides a platform for future research on the importance of essential oil nanoemulsion as a food preservative in preventing *L. monocytogenes* in food.

1.3 Hypothesis

We outline a qualitative and quantitative assessment framework to prove two hypotheses. First, we hypothesize that our essential oil emulsion will be effective against planktonic cells of *L. monocytogenes* in vitro. Second, as protein as a food matrix component has been reported to inhibit EOs' hydrophobic properties, we hypothesize that our essential oil emulsion will be less effective against planktonic cells of *L. monocytogenes* in the presence of different concentrations of casein protein as a food model. To determine that the efficacy of EOs as antimicrobial agents depends on the components included in the media (Acevedo-Fani, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2015; Perricone, Arace, Corbo, Sinigaglia, & Bevilacqua, 2015), the media components under investigation for this study are casein protein, regarding the effect of its concentrations on the activity of *L. monocytogenes* on a specified food matrix. The presence of high concentrations of casein protein significantly reduced the efficacy of the antimicrobial activity of EOs.

1.4 Research Objectives:

1. Develop formulation suitable for nanoemulsion preparation for a wide range of EOs
2. Measure and analyze droplet size distribution of EO nanoemulsions
3. Measure the bacterial growth curve
4. Evaluate comparative efficiency of a wide spectrum of natural EOs against *L. monocytogenes*
5. Identify kinetic parameters of the AM efficacy of EO nanoemulsions via the application of a predictive microbiology approach

6. Assess the effect of the food matrix on the efficacy of EO nanoemulsions by determining the efficacy of EO nanoemulsions in the presence of casein protein as a food model and identify the kinetic parameters of the AM efficacy of EO nanoemulsions

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Chapter II. Literature Review

2.1 Foodborne Outbreaks

Food safety requires maintaining the conditions and practices necessary to preserve food quality and prevent contamination by hazards that could lead to public health issues. Currently, there has been an increase of foodborne illnesses associated with food contamination from microbes and poisonous chemicals resulting in major public health problems. The CDC (2016) reported that most of these cases are due to foodborne pathogens. In the United States, foodborne bacteria have been reported to be the most common causes of food-related illnesses leading to hospitalization and death (CDC ,2016). Some bacterial species have been identified as the most common causes of foodborne disease outbreaks: *Listeria monocytogenes, Escherichia coli* O157:H7, *Staphylococcus aureus, Salmonella enterica, Bacillus cereus, Vibrio* spp.*, Campylobacter jejuni,* and *Clostridium perfringens*(CDC ,2016).

The foodborne outbreaks have been linked to increased cases of food contamination due to poor processing and storage of foods (Cartwright et al., 2013). Listeriosis, one of the most common types of foodborne diseases, is caused by *L. monocytogenes*-contaminated foods. Cartwright et al. (2013) stated that the first case of listeriosis outbreak was recorded in Canada in 1981 following the consumption of contaminated coleslaw; the first incidence of listeriosis in the United States was in 1983. Furthermore, Cartwright et al. determined that the intake of contaminated Mexican-style cheese and turkey frankfurters has led to many incidences of listeriosis. Additionally, more than 1,662 new cases of listeriosis have been reported in the United States with more than 1,520 hospitalizations and 266 related deaths (Cartwright et al., 2013).The

increased cases of listeriosis, as a type of foodborne disease, have been linked to improper handling of food, poor food preparation techniques, and unsuitable food storage mechanisms (Zhu, Gooneratne, & Hussain, 2017).

2.2. *L. monocytogenes* Virulence and Pathogenicity

L. monocytogenes is a facultative anaerobic, rod-shaped, Gram-positive bacterium widely distributed in the environment and one of the leading causes of foodborne infections. Additionally, *L. monocytogenes* is neither host specific nor spore forming (Zhu et al., 2017). According to Cahoon and Freitag (2014), the virulence of listeriosis is more severe among individuals with compromised immune systems and pregnant women. Among the four serotypes of *L. monocytogenes*, serotype 4b is the most common among those infected by listeriosis (Gilbreth et al., 2005). *L. monocytogenes* do often induce self-phagocytosis within the host cells, easily replicate within the infected host cells, and migrate to the neighboring host cells, thus making them capable of inducing severe illness (Cahoon & Freitag, 2014). Following the ingestion of *L.monocytogenes* cells, the underlying phagocytic cells will be completely manipulated (Cahoon & Freitag, 2014). Even though there will still be a good number of *L. monocytogenes* involved in the development of listeriosis, some of these cells are always destroyed by neutrophils in collaboration with the Kupffer cells (Lobel et al., 2015).

In the process whereby the host cells fail to induce an adequate T cell-mediated immune response, additional manipulation of the *L. monocytogenes* in the hepatocytes and macrophages will take place (Cahoon & Freitag, 2014).The production of a series of virulent factors by the *L. monocytogenes* cells is one of the most effective mechanisms that these bacterial cells use to ensure that each step of the invasive process takes place

(Lobel et al., 2015). Some of the most common virulent factors for *L. monocytogenes* include ActA proteins, Clp proteases, Protein p60, ATPase, surface protein p104, Listeriolysin O, and phospholipases (Lobel et al., 2015).

2.3. Food Preservation Methods

Nowadays, consumers' demand for high-quality food products, therefore; different types of food preservation such as physical, chemical, and antibacterial methods have been developed to prevent or reduce cases of food contamination. Some of the commonly used methods of food preservation include vacuum packaging, chemical preservation, heat processing, modified atmospheric packaging, and refrigeration. These techniques have been found significantly ineffective on different microbial organisms such as *L. monocytogenes*, which are important causes of food contamination and the outbreak of foodborne diseases (Gutierrez, J., Barry-Ryan, C., & Bourke, P.,2009). To increase the chances of eliminating the *L. monocytogenes*, natural and effective antibacterial agents have been used, which can be grouped into various categories such as synthetically derivative antimicrobials (e.g., organic acids, esters) and naturally occurring antimicrobials, which are developed from animals, plants, and microbial sources such as essential oils (Davidson et al., 2013). EOs has been used in recent years as a food preservative method (Perricone et al., 2015).

2.4. EO Features

EOs are natural, aromatic, and volatile liquid preparations produced from various plant parts and materials (Perricone, Arace, Corbo, Sinigaglia, & Bevilacqua, 2015). EOs are mixtures of spice-derived compounds capable of generating flavor and aroma. The modern use of the term *essential oil* refers to hydrophobic oil or fatty compounds

extracted from plants. Using EOs to preserve food by inhibiting the growth and proliferation of microorganisms in food (Perricone et al., 2015) is also in different industries due to their aromatic features. For example, EOs are used in the production of perfumes, toiletries, and soaps (São Pedro, Santo, Silva, Detoni, & Albuquerque, 2013) and even to repel insects (Isman, 2000). Some well-established modern medical uses rely on the properties of these plant extracts reported to have antifertility, anticancer, antidiabetic, antifungal, antimicrobial, hepatoprotective, cardioprotective, antiemetic, antispasmodic, analgesic, adaptogenic, and diaphoretic properties (Prakash & Gupta, 2005).

EOs can be extracted through application of various methods such as expression, extraction, fermentation, and steam distillation, which is the most preferred method. The extraction of EOs through steam distillation involves charging the plant materials from where the EOs are to be extracted by exposing them to steam (Marzouki, Piras, Marongiu, Rosa, & Dessi, 2008).

2.5. Composition of EOS

Plants do not produce EOs uniformly between all components. The flowers, leaves, roots, seeds, stems, and other plant parts can store different quantities and compositions. Identifying and quantifying the components requires highly sensitive tools (Ester et al., 2012). Plants propagated in different locations or conditions can also have different constituent compounds (Oezcan & Chalchat, 2002). For example, the EOs of the mace or nutmeg plant, *Myristica fragrans*, differ between the seeds and fruit of the same plant (Singh, Kiran, Marimuthu, Isidorov, & Vinogorova, 2008; Spricigo, Pinto, Bolzan, & Novais, 1999). Another example of different EO properties involves the dill plant, with

dry leaves, flowers, and fruit with different compounds and different ratios of the same major compounds (Rădulescu, Popescu, & Ilieş, 2010). In one study over 40 constituent compounds were discovered in the EO of a single basil plant (Raut & Karuppayil, 2014). Determining the constituents of EO extracts can be critical for research and human health, yet it is extremely time consuming and difficult. There are several pharmacologically active compounds in EOs, and most are relatively low molecular weight terpenoids and phenylpropanoids (Raut & Karuppayil, 2014). According to Ghirardo (2011), terpenoids (i.e., isoprenoids) are defined as the ever-present group of compounds found in all living organisms and the biggest collection of natural compounds present in nature with over 40,000 structures (Keeling & Bohlmann, 2012). Terpenoids can be explained as modified terpenes through oxidation. The name *terpenoid* originates from turpentine based on the isolation of the first members of the class (Croteau, Kutchan, & Lewis, 2000; Howlett, 2014). Terpenoids are the consequence of repetitive synthesis of branched five-carbon units dependent on isopentane skeletons. Terpenoids can be categorized into esters, alcohol, ketones, ethers, and aldehydes. Examples of terpenoids are thymol, carvacrol, linalool, and piperitone (Keeling & Bohlmann, 2012).

In contrast, *terpenes* are a large cluster of natural products occurring in advanced plants as secondary metabolites (Rahman-ur-A., 1998). Terpenes can also be termed hydrocarbons formed from the blending of numerous isoprene units. Terpenes contain a hydrocarbon backbone that can be reorganized into cyclic formations by cyclases to create monocyclic or bicyclic structures (Hyldgaard, Mygind, & Meyer, 2012). The major terpenes are monoterpenes and sesquiterpene.

While EOs contain acids, alcohols, aldehydes, aliphatic hydrocarbons, acyclic esters, nitrogen, and sulfur compounds lactones, coumarones, and phenylpropanoids, EOs' primary components are terpenoids and terpenes (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). The major probable antibiotic compounds in many EOs are these terpenes and terpenoids. The modes of action in many bactericidal terpenes are unknown. In nature, many terpenoids in plants used to prevent bacterial infections in living tissues (Arendt, Pollier, Callewaert, & Goossens, 2016). Those that have been characterized change the lipid fraction of the plasma membrane (Trombetta et al., 2005). Because terpenes can cross the lipid membranes of cells, they enter and easily disrupt those cells (Trombetta et al., 2005).

Other small monoterpenes can enter the bacterial cell and interact with internal cellular structures to cause cell death (Trombetta et al., 2005). In addition some terpenoids interact directly with the cell wall or cell membrane of bacteria and change permeability to adenosine triphosphate (ATPs) or similar essential molecules (Nazzaro et al., 2013), which leads to cell death or an inability to move. They can also change the thermal resistance to change in the cell membrane by acting like aberrant cholesterols (Nazzaro et al., 2013). Some phenylpropanoids are also antibiotics and have medicinal properties (Shalaby et al., 2014). Their activity is linked to the side chain molecules (Nazzaro et al., 2013). These are possibly cytotoxic and kill cells including bacterial cells (Wangchuk, Pyne, Keller, Taweechotipatr, & Kamchonwongpaisan, 2014). One known mode of action is disrupting cell membranes' pH gradients (Nazzaro et al., 2013).

2.6. Antimicrobial Activity of EOs

EOs are complex solutions containing a wide range of hydrophobic proteins, aromatic compounds, and lipids (Mahmud, 2008). Many EOs have antibacterial properties (Hammer, Carson, & Riley, 1999). Some are even useful in controlling fungal pathogens (Kalemba & Kunicka, 2003). Some of these relate to the oils' toxic properties and have little medicinal use; some even present a threat to human health (Papachristos & Stamopoulos, 2002; Tisserand & Young, 2013). For example, the EO of *Origanium vulgare* ssp, *hirtum* is extremely toxic to both bacterial and human cells (Sivropoulou et al., 1996). Other EOs such as in tea can be toxic to human tissues at higher concentrations than those in traditional foods (Söderberg, Johansson, & Gref, 1996). The many examples of food additives of EOs include cinnamon, anise, sage, and even flower oils such as rose. Some have bacteriostatic or bactericidal properties (Baydar, Sağdiç, Özkan, & Karadoğan, 2004). Baydar et al. demonstrated that EOs of oregano and wild savory would suppress the growth of over a dozen microbial pathogens. The use of EOs of plants already used in food as flavoring such as fennel and caraway show promise in protecting human health by killing pathogenic bacteria and slowing food spoilage (Gutierrez, Rodriguez, Barry-Ryan, & Bourke, 2008). As a result, mass screening projects aimed at testing wide ranges of EOs on specific high-threat microbes have been repeatedly conducted (Sacchetti et al., 2005).

2.7. Mechanism of Antimicrobial Activity of EOs

EOs exhibits a broad array of potential antibacterial effects against different types of both gram-negative and gram-positive bacteria. Gram-positive bacteria are more susceptible to antimicrobial effects of EOs than are gram-negative. This observation is

attributed to gram-negative bacteria's having hydrophilic lipopolysaccharides (LPS), which restrict macromolecules and hydrophobic compounds (Hyldgaard, Mygind, & Meyer, 2012). A gram-positive bacterium has a thicker peptidoglycan layer than the gram-negative bacterium linked to other molecules, so the cell wall allows hydrophobic molecules to easily penetrate the cells (Nazzaro et al., 2013). Gram-positive bacteria also display susceptibility to antimicrobial activity by phenolic compounds present in EOs. At low concentrations, these phenolic compounds interfere with the enzymes involved with energy production, and at high concentration, they cause the cell proteins to denature (Nazzaro et al., 2013).

The mechanism of EO antimicrobial activity is attributed to a tandem of reactions within the whole cell, which generally inhibits the growth and production of toxic bacterial metabolites. EOs perturb cell membrane potential and permeability by causing rapid concentration-dependent depolarization of the cell plasma membrane and increasing the leakage of cellular ATP and K^+. The hydrophobic nature of EOs leads to disruption of the bacterial structure by damaging the cytoplasmic membrane, cytoplasmic coagulation, and membrane proteins, thereby increasing permeability and causing alteration to functionality (Nazzaro et al., 2013).

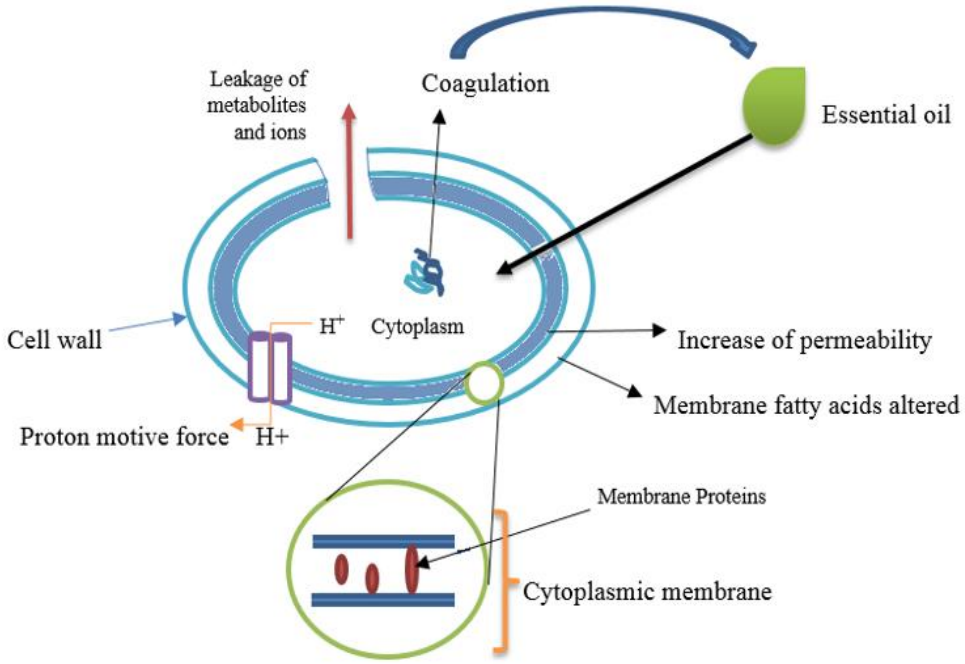

Figure 1. Mechanism of essential oil in bacterial cell (source: Shoughy & Tabbara, 2014,p301).

The antimicrobial activity of EOs relies on their composition because their inhibitory effects are linked to their major components. Nonetheless, other minor components may modulate the antimicrobial activity of the main elements because numerous elements of EOs participate in fixation to cell walls and cellular distribution (Basile et al., 2006). Some of the main elements that have been linked to the strong effects of EOs include thymol, eugenol, p-cymene, and 1.8-cineole (Campos, Castro, Gliemmo, & Schelegueda, 2011). The composition of EOs is influenced by various factors such as plant species and subspecies, geographical location, harvesting season, drying method, and extraction method (Burt, 2004; Mith et al., 2014).

2.8. Challenges Associated with the Application of Essential Oils in Food

The application of the essential oil nanoemulsions in the elimination of *L. monocytogenes* cells has been found effective because the nanoemulsions can easily penetrate the inner structures of the *L. monocytogenes* cells (Sugumar, Mukherjee, & Chandrasekaran, 2015). According to Sugumar et al., the challenges associated with the application of EOs are a result of the fact that the EOs have low water stability and interactive binding with the other forms of food components such as proteins. This will therefore jeopardize their antibacterial activities in the presence of complex food matrices. Additionally, the continuous interaction between the EOs and food components can affect the overall quality of the food components to be preserved (Sugumar et al., 2015). Reducing the sizes of the nanoemulsions is an important strategy to increase the penetrating ability of the nanoemulsions through the *L. monocytogenes* cells (Zhu et al., 2017). The effectiveness of EOs in food components is determined by the OEs' structural and chemical components. Sugumar et al. (2015) described food matrices as complex, multi-component systems composed of different compounds such as fibers, fats, carbohydrates, and proteins. All are situated in an interconnecting microenvironment. Food proteins are the major component of the food matrix (Sugumar et al., 2015).

Different natural extracts such as EOs have low water stability and poor interactive levels with different components of the food matrix such as proteins. Therefore, food made up of high protein contents can reduce the effectiveness of the antibacterial activities of EOs (Sugumar et al., 2015). Protein properties of a hydrophobic nature and the presence of a three-dimensional matrix layer act as a barrier to penetration of the EOs into the inner structures of the bacterial cells (García-Díez et al., 2017).

Different forms of proteins, such as whey and casein proteins, have been derived from animals and used in the formulation and improvement of the stability of various food emulsions (Gandomi, Abbaszadeh, JebelliJavan, & Sharifzadeh, 2014).

The casein proteins can be described as the phosphor-proteins sporadically contained in mammalian milk. In milk from a cow, the casein proteins compose 80% of the total milk components and from 25–40% of the human milk components (Triprisila, Suharjono Suharjono, & Fatchiyah, 2016). The ability of EOs to effectively interact with the protein content within the food matrix is facilitated by the existence of high hydrophobicity within the components of the EOs as well as the presence of a short extension in their carbon chains (Hyldgaard et al., 2012).

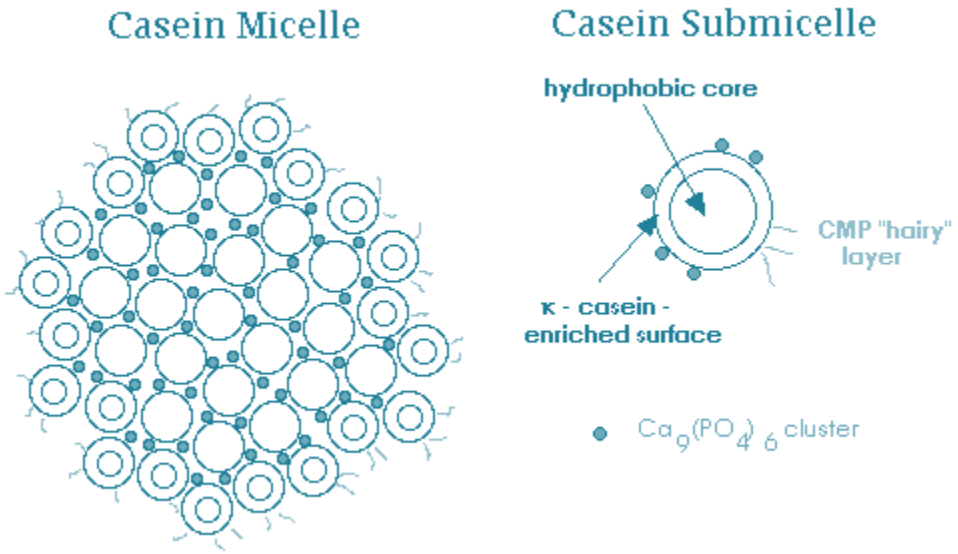

Figure 2. Casein protein structure (source: Głąb and Boratyński.,2017).

Additionally, EOs' metabolites can easily penetrate bacterial cell membranes and thus bind to the cellular proteins to inhibit the cellular activities that take place within the cells of the bacteria (Hyldgaard et al., 2012). The application of EOs in the elimination of the *L. monocytogenes* cells is possible because they have the ability to easily penetrate

into the cellular structure of the bacterial cells from where they exert their antibacterial activities (Xue, 2015), in part because gram-negative bacteria have lipopolysaccharides that lead to a decreased peptide affinity during the binding process between the antibacterial agent and the bacterial cells.

2.9. EO Delivery Systems

The delivery mechanism to transfer EOs as bactericidal is a critical component of any antibiotic product. Many products have been proposed for EOs as antibiotics, including nanoparticles, emulsifications, vapors, sprays, washes, and direct paint-on products (Saraf, 2010). One established system for formulating and delivering EOs is by using liposomes.

2.9.1. Liposomes

Liposomes are phospholipid-enclosed bubbles that can be artificially made to encapsulate drugs and other chemicals. The fact that they can deliver the essential oil prior to cooking and still see activity indicates that this method has promise in protecting the activity of the oils in harsh environmental conditions. One of the major benefits to liposome encapsulation is that it helps prevent evaporation of EOs (Martín, Varona, Navarrete, & Cocero, 2010). Liposome encapsulation can also pack EOs and possibly reduce the size of liposome (Sherry, Charcosset, Fessi, & Greige-Gerges, 2013). Liposomes are also dryable and can be reduced to a powder. These encapsulated EOs exhibited higher antimicrobial activity and minimal effect on the organoleptic quality of food than Eos-loaded liposomes (Donsì, Annunziata, Sessa, & Ferrari, 2011). Because a stable dry essential oil technology has been demonstrated with gum Arabic to produce an encapsulated cardamom (Al-Ismail, Mehyar, Al-Khatib, & Al-Dabbas, 2014), it might be

a beneficial combined product. Encapsulated EOs can also be further stabilized with a polymer monolayer, as demonstrated by Van Vuuren, du Toit, Parry, Pillay, and Choonara (2010).

EOs' mode of action continues while in liposomes. Liposome delivery systems are large nanoparticles. Because the liposome can bind to or interact with living cell membranes, it can facilitate transport of the encapsulated protected EO, and because different molecules can be combined in a single liposome, synergistic effects might be amplified (Nazzaro et al., 2013). While this could be technically difficult at first, once the process is worked out it would allow different compounds to be combined in a single treatment. If specific components of the EO are of biological interest, they are often purified for laboratory testing, as when the terpenoids of an essential oil are purified by high-performance liquid chromatography (Gaysinski, Ortalo-Magné, Thomas, & Culioli, 2015).

2.9.2. Biopolymeric Nanoparticles

An additional option for controlling any microorganism is using a biopolymeric nanoparticle with the essential oil. These particles can be loaded with EOs or their constituent chemicals (Kao et al., 2012; Sundar, 2010). The etymology of *biopolymeric* implies that the material is a polymer of biologically produced material. Both micro- and nanobiopolymeric particles have been fabricated in the laboratory, often from the same backbone molecules (Joye & McClements, 2014). The particles can be constructed using a range of possible technologies including electrostatic spraying, solvents, and coacervation (Sundar, 2010). These particles are typically constructed of proteins or polysaccharides, especially if they are intended as a food additive or to be used on food

preparation surfaces because these backbone molecules may be edible (Joye & McClements, 2014). In addition, because these are biological materials, they can be readily degraded by microbial or chemical modes of degradation and are not environmentally persistent and do not pose an ecological threat (Sundar, 2010). Indeed, one option with biopolymeric nanoparticles is to use them in oral delivery of drugs, including EOs (Elgindy, Elkhodairy, Molokhia, & ElZoghby, 2011).

Biopolymeric nanoparticles have been successfully used to deliver drugs to humans. These particles act as carriers for a drug and can enhance delivery to a targeted site or organ, as demonstrated with several antidepressant drugs (Margret & Aishwarya, 2012).Biopolymeric nanoparticles also have promise for protecting and delivering EOs. Knowing the target organs and methods of delivery is critical for nanoparticle development with essential oil delivery systems (Bilia et al., 2014). This is especially true because not all volatile molecules in an EO will bind or be encapsulated by the biopolymeric nanoparticles (Bilia et al., 2014). Chen (2014), demonstrated that zein nanoparticles could deliver EOs. Other biopolymeric nanoparticles include an alginate/cashew gum used to encapsulate EOs, but this method had low efficiency overall (de Oliveira, Paula, & de Paula, 2014). A very real concern about encapsulated EOs is the effect of the oil on the structure and solubility of the nanoparticles because these oils can be good solvents (Torrieri, Cavella, & Masi, 2015).

2.9.3. Emulsions

A final option for delivery of an essential oil is as an emulsion. This could be as a mixture of two colloidal fluids. These emulsions of EOs might include a nanoparticle component where the emulsifier and essential oil are both in microquantities (Xue, 2015).

Emulsion can be classified according to the spatial distribution of two phases (oil-in-water and water-in-oil) or by the size of the droplets in the emulsion. Emulsions of EOs are colloidal solutions with two or more liquids that are immiscible (Slomkowski et al., 2011). Many essential oil products are emulsions of oil in water known as colloidal suspensions and are used commercially because these are more stable than pure oils (Bylaite, Nylander, Venskutonis, & Jönsson, 2001).

2.10. Nanotechnology and EO Nanoemulsion

Current developments in nanotechnology have led to the establishment of different methods for inhibition of microbial growth through the application of environmental control (Amaral & Bhargava, 2015). The presence of high levels of complexities of the EOs during interaction with the food matrix that can lead to ineffective inhibition against the targeted microbes together with the negative influences they have on the foods' organoleptic quality are some of the common problems faced during the application of EOs as food preservatives. However, the development of EO nanoemulsions has been able to present a possibly beneficial alternative for application in the food matrix (Amaral & Bhargava, 2015).

Nanoemulsion is an emulsion system containing very small droplets (i.e., mean diameters of ca. 100nm; Tadros, Izquierdo, Esquena, & Solans, 2004). Even though they have higher stability to gravitational separation and aggregation than do macroemulsions, they are still thermodynamically unstable because the free energy of the colloidal dispersion is higher than that of the separate phases (oil and water). Increasing the amount of *L. monocytogenes* cells being eliminated by the nanoemulsion EOs, requires reducing the size and increasing the concentration of the nanoemulsion oils (Chouhan,

Sharma, & Guleria, 2017). The antibacterial activity of the nanoemulsion oils is influenced by their ability to bind to the lipid bilayer found in the bacterial cell membrane, leading to the destabilization of the membrane integrity as a result of the lysis of the bacterial cellular contents (Chouhan et al., 2017). Table 1 shows the MIC values from various studies on different EOs against different microorganisms.

Table 1 *Effects of Essential Oil Nanoemulsions Against Various Bacteria Reported in the Literature*

Formulation	**Oil**	**Targeted Microorganism**	**Purpose**	**Minimum Inhibitory Concentration**	**Reference**
Nanoemulsion	Cinnamon bark	*Listeria monocytogenes* *Escherichia coli*	Flavoring agent for food products, cosmetics, and medicine	6 (ppm) 6 (ppm)	(Hilbig Cox, Rajauria, Jaiswal, & Abu-Ghannam, 2016)
Nanoemulsion	Eucalyptus	*Proteus mirabilis*	Pharmaceuticals		(Saranya, Chandrasekaran, & Mukherjee, 2012)
Nanoemulsion	Peppermint	*L. monocytogenes*, *Staphylococcus aureus*	Food	0.5 (%v/v)	(Liang et al., 2012)
Nanoemulsion	Oregano	*L. monocytogenes*,	Flavoring agent	0.625 μL/mL	(Bhargava et al., 2015)
Nanoemulsion	Egyptian geranium	*L. monocytogenes*	Fragrances, cosmetics, medicine, and aromatherapy	149.7 μg/mL	(Giongo et al., 2015)

Use of surfactants can result in metastable nanoemulasions by providing a sufficiently large energy barrier between the two phases (McClements, 2012). However, microemulsions are thermodynamically stable liquid solutions where the free energy of

the colloidal dispersion is lower than the free energy of the separate phases. They typically contain droplets with diameters less than 50 nm (McClements, 2010). They are optically isotropic and transparent because the droplet size is much smaller than that of the wavelength of light (Lawrence & Rees, 2000).

Surface-active components (i.e., surfactants) play a crucial role in the fabrication and stabilization of nanoemulsions. In an emulsion, surfactant molecules are absorbed by a freshly formed oil-and-water interface and retard the rate of coalescence and phase separation (Jafari, Assadpoor, He, & Bhandari, 2008). In the present study, cetyltrimethylammonium bromide (CTAB) was used as a surface-active component. At the oil-and-water interface, the hydrophobic tail of CTAB interacts with oil droplets through the van der Waals force, whereas the cationic head resides in the continuous water phase. Due to this surfactant layer, which surrounds each oil droplet in the emulsion, the electrostatic repulsion by cationic head interaction prevents oil droplets from coming into contact and preventing coalescence (Jafari, Assadpoor, He, & Bhandari, 2008)..

Moreover, the use of a cationic surfactant in the present study provides an advantage in the cell-essential oil interaction. Because *L. monocytogenes* detains a highly negatively charged lipid bilayer (Briandet, Meylheuc, Maher, & Bellon-Fontaine, 1999), the positive charge on the oil droplet surface, attained by CTAB, enables a strong electrostatic attraction between *L. monocytogenes* and oil droplets. The previous study conducted by our group demonstrated that the surface charge of curcumin nanoparticles contributed by surfactants has a major effect on the antimicrobial efficacy of the curcumin nanoparticles against *L. monocytogenes* growth (No et al., 2017). The

nanoemulsions can be prepared through the application of both high-energy and low-energy emulsification methods (Sokolov, 2014).

2.10.1. High-energy emulsification.

This method relies on the use of the mechanical devices that lead to the production of strong disruptive forces, such as high shear stirrers, high-pressure homogenizers, and ultrasound generators. The production of the nanoemulsions using high-energy methods, such as high-pressure homogenization, sonication, and microfluidization, was described by Sokolov (2014) as a straightforward mechanism because it involves the application of high-energy inputs that lead to the formation of smaller-sized droplets. The application of the sonication method for the preparation of the nanoemulsions involves the application of energy to disrupt the required forces and reduce the sizes of the particles (Amaral & Bhargava, 2015).

2.10.2. Low-energy emulsification.

The low-energy approaches depend on the spontaneous formation of tiny essential oil droplets within the oil-water-emulsifier mixture following the alteration of the components of the EOs or the environmental conditions (Sokolov, 2014). Some of the most common types of low-energy methods used in the production of nanoemulsion EOs include spontaneous emulsification (SE), phase inversion temperature (PIT), emulsion inversion point (EIP), and phase inversion composition (PIC) methods.

2.11. Predictive Bacterial Growth and the Gompertz Model

Bacterial behavior in foods can be determined by mathematical models that enable determination of microorganism behavior in a particular time frame contributed by several factors. Kinetic studies use mathematical models (Belda-Galbis et al., 2014),

and their application has facilitated the establishment of different methods that can be used in the analysis of different types of substances. The kinetic studies will provide a platform that will be used to examine different conditions that can affect the antibacterial activity of the nanoemulsion EOs and the establishment of relevant information about the mechanism of the reaction (Buchanan, Whiting, & Damert, 1997). The Gompertz model is one of the commonly applied mechanisms for determining primary growth because it enables our understanding of the bacterial growth from the initial stages up to maturity (Buchanan et al., 1997). Therefore, this technique can be applied in the quantitative modeling of the nanoemulsion EOs that can enhance the elimination of *L. monocytogenes* in the presence of casein proteins in the food matrix.

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Chapter III Quantitative modeling and design of essential oil nanoemulsion delivery systems for strong antimicrobial action against Listeria monocytogenes

Abstract

Food safety remains a concern owing to numerous cases of foodborne diseases resulting from bacterial pathogens. *Listeria monocytogenes* is one of the three most serious foodborne pathogens. Essential oils (EOs) are volatile compounds found in the secondary metabolites of aromatic plants. Owing to their high terpenoid and phenolic compound content, these oils are potential natural antimicrobial agents for food preservation, but their low water solubility limits their efficacy and application in food. In the present study, 28 different EOs were evaluated for their antimicrobial activities against *L. monocytogenes*. Various concentrations of EOs were introduced into brain heart infusion broth to determine the minimum inhibitory concentration (MIC) for the pathogen. To quantitatively evaluate the effect of each oil on *L. monocytogenes* from a kinetic viewpoint, the experimental data were fitted to the modified Gompertz model, and the lag phase duration and maximum growth rate were calculated and compared for each EO at various concentrations. Overall, our experimental results indicate that frankincense, eucalyptus, and fire needle oils had the strongest inhibitory effects against *L. monocytogenes* with MICs <2.4 μg/mL. EOs with moderate antimicrobial effects included key lime, cedar wood, Egyptian geranium, nutmeg, peppermint, valerian, and ylang ylang.

Keywords: Food safety, Essential oils, Nanoemulsion, *Listeria monocytogenes*, Growth kinetics

1. Introduction

Food safety, which is essential for promoting health and wellness, can be achieved through preventing or reducing the growth and formation of microorganisms in foods (Belda-Galbis et al., 2014). Microorganisms play a role in the spoilage of food, and after ingestion, they cause health issues such as foodborne illnesses. Contamination of food in most cases is natural; hence, it is vital to treat the food before packaging and consumption. Treatment makes food microbiologically stable and safe; however, some treatments may neutralize the sensorial and nutritional qualities of food. Consequently, an alternative approach is the application of natural preservatives combined with freezing and refrigeration (Belda-Galbis et al., 2014).

Consumers demand fresh produce, despite its short shelf life, or products with no chemical additives. The traditional method of using high temperatures to control foodborne pathogen results in the loss of flavor, odor, texture, color, and nutritional value by food; thus, the use of natural substances with bacteriostatic and bactericidal properties such as essential oils (EOs) has been promoted. Such treatments can prevent the growth of pathogens considered harmful to humans from both health and socioeconomic viewpoints, such as *Listeria monocytogenes* (Belda-Galbis et al., 2014).

L. monocytogenes is a gram-positive, non-spore forming; rod-shaped bacterium that causes listeriosis (Farber and Peterkin, 1991; Fouladynezhad et al., 2013), a foodborne disease that has critical negative effects on certain groups in populations. Specifically, listeriosis can lead to miscarriages in pregnant women and cause fatal outcomes in immune-compromised individuals and the elderly. Conversely, the disease is mild in healthy people. *L. monocytogenes* can be found throughout the environment and

it has been isolated from wild and domestic animals, soil, birds, fodder, vegetation, drains and wet areas of food-processing factories (McLandsborough, 2015).

Currently, there is growing interest in the use of EOs as natural preservatives owing to their characteristic inhibitory effects against fungi, viruses, and bacteria. Campos et al. (2011) described EOs as oily liquid mixtures of volatile and complex compounds extracted from distinct parts of aromatic plants such as flowers, roots, and buds (Padalia et al., 2015). EOs are synthesized by plants as secondary metabolites, and they can be retrieved through steam distillation or supercritical fluid extraction (Muñoz et al., 2009). EOs contain 20–60 components based on the source and method of extraction (Li et al., 2014). Amaral and Bhargava (2015) demonstrated that EOs contain photo-chemicals that exhibit antimicrobial properties and the ability to prevent lipid oxidation, therefore extending the shelf life of food products. The majority of EOs are classified as terpenoids and terpenes, whereas others are categorized as aromatic and aliphatic compounds of low molecular weight (Faleiro, 2011).

Recent advances in nanotechnology have significantly benefited the techniques for microbial inhibition through environmental control. However, there have been considerable challenges regarding the use of antimicrobials such as EOs for food preservation due to their complex interaction with the food matrix, leading to inefficient inhibition against the targeted microbes and negative effects on the organoleptic quality of food. For these reasons, more efficient methods for delivering EOs at concentrations that inhibit microbial activities while having minimal effects on food integrity are needed.

Amaral and Bhargava (2015) suggested that EO nanoemulsions represent a potentially good alternative for application in food matrices. Nanoemulsions are two-phased colloidal systems composed of water, oil, and surfactant in nano-sized droplets of less than 100 microns. Nanoemulsion systems are more advantageous than conventional emulsions conserving the delivery of hydrophobic bioactive compounds because they have large surface areas and kinetic stability against coagulation. Molecular dispersion allows for solubilization of EOs in the water phase, making the EO nanoemulsion systems applicable in food (Amaral and Bhargava, 2015). High-energy emulsification is one of the techniques used to create nanoemulsions through high-pressure homogenization, microfluidization, and sonication, for which energy is essential to provide the required disruptive forces and reduce particle size (Amaral and Bhargava, 2015). Ultrasonic emulsification, a high-energy emulsification method, has been reported to have the most potential for application in food because it generates EO nanoemulsions with smaller particles, high physical stability, optical transparency, and high bioavailability. The other method is low-energy emulsification in which the nanoemulsion is formed through spontaneous mixing of the components. The composition of the mixtures and environmental factors are altered to attain the desired nanoparticle size (Amaral and Bhargava, 2015).

The antimicrobial activity of plant oils and extracts has been extensively studied in recent years. However, few studies have compared the effects of large numbers of oils and extracts against *L. monocytogenes* in the form of unified formulations and under similar conditions. In previous studies, EO nanoemulsions exhibited strong antibacterial effects; however, study of their antimicrobial activity through kinetic analysis has been

limited. Therefore, it is important to determine the most effective EO nanoemulsions for use in food preservation.

The use of kinetic studies has assisted in the development of strategies such as the use of EOs as preservatives and treatments for bacteria as alternatives to high-temperature techniques. The methods satisfy the needs of customers for fresh produce with long shelf lives. The Gompertz model is one of the commonly used primary growth methods. The model enables understanding of the growth and development of bacteria from the initial stages to maturity (Buchanan et al., 1997).

Increased attention has been devoted to *L. monocytogenes* in public health because of the large numbers of foodborne outbreaks of listeriosis. For these reasons, the objectives of the present study were to (i) develop formulations suitable for nanoemulsion preparation for a wide range of EOs, (ii) measure and analyze droplet size distribution of EO nanoemulsions, (iii) determine the comparative efficiency of wide spectrum of natural EOs against *L. monocytogenes*, and (iv) clarify the kinetic parameters of the antimicrobial efficacy of EO nanoemulsions by applying a predictive microbiology approach.

2. Materials and Methods

2.1. Preparation of bacterial cultures

To prepare bacterial cultures, we used a technique reported by Jiang (2011) with a few modifications. The method involved broth microdilution to assess antimicrobial activity against *L. monocytogenes*. The Scott A strain of *L. monocytogenes* was used in the study and purchased from D. Portnov, University of California, Berkeley. The Scott

A strain was stored in brain heart infusion (BHI) broth supplemented with 30% glycerol at −20°C until use. Preparation of the BHI broth entailed dissolving 39 g/L in deionized water, and the broth was heated in an autoclave for approximately 45 min. The cells used in different experiments were subcultured from the same original culture (Balouiri et al., 2016).

2.2. Fabrication of essential oil nanoemulsions

To prepare EO emulsions, cetrimonium bromide (1%, w/w) was dissolved in 6 mL of deionized water in a test tube (i.e., 13 mm). To prepare a stock solution of EO, each EO (15×10^3 µg/mL) was added to surfactant solution. The resulting emulsion was mixed well and subjected to size reduction performed using a 20-kHz ultrasonicator (Fisher Scientific, Hampton, NJ, Model 505 Sonic Dismembrator; nominal power 500 W). This system consisted of a generator, converter, and horn tip (diameter, 3 mm). The horn tip was immersed in the coarse emulsion, and sonication was performed at 30% amplitude in pulse mode with a 30-s pulse-on period followed by a 15-s interval for a total of 2.75 min. Each experiment was performed in triplicate.

2.3. Particle size distribution of emulsion droplets

A particle analyzer (Model Delsa™ nano C; Beckman Coulter) was used to analyze the droplet size distribution of the EO nanoemulsions. This device performs size measurements via dynamic light scattering, which measures the Brownian motion of particles and relates this variable to particle size on the premise that larger particles have slower motion. Calculations are based on the Stokes–Einstein equation (1) as follows:

$$d_H = \frac{\kappa T}{3\pi\eta D}, \quad \textbf{(1)}$$

where d_H is the hydrodynamic diameter, D is the translational diffusion coefficient, κ is Boltzmann's constant, T is the absolute temperature, and η is the viscosity of the medium, in this case pure water (η = 0.89 cP at 25°C). Size distribution results represent an average of three measurements of three freshly prepared emulsions.

2.4. Microplate assay procedure

Flat-bottomed 96-well sterile microliter plates with lids to prevent cross-contamination were used in this research (Sultanbawa et al., 2009). The procedure followed was that described by Sultanbawa et al. (2009) with a few modifications. First, a culture of *L. monocytogenes* 1×10^8 colony forming unit (CFU)/mL had been allowed to grow overnight in BHI medium (Difco, Sparks, MD, USA), and 10-µL aliquots were transferred from storage overnight to a 15-mL test tube containing 10 mL of BHI broth to achieve a concentration 1×10^6 CFU/mL. The broth microdilution procedure was conducted by dispensing a 50-µL volume of BHI broth into each well of a 96-microtiter plate. The stock solution of each essential oil at 15×10^3 µg/mL was diluted to 23.7 µg/mL; then, aliquots of EOs (150 µL) from 23.7 µg/mL were added to the first row of a 96-well plate. Then, 0.75-fold dilutions of EOs were made along each column by transferring 150 µL of the EOs in the first-row wells into the second row and repeating the process to produce eight concentrations ranging from 17.8 to 2.4 µg/mL (Thapa et al., 2012). Frankincense, fire needle, and eucalyptus were tested at concentrations ranging from 17.8 to 1.002 µg/mL. Finally, 50-µL aliquots of bacterial dilutions containing 1×10^6 CFU/mL were added to all wells of the microtiter plates to achieve a total volume of 100 µL in each well. The last two wells served as negative and positive controls. The negative control contained EO solutions and sterile growth medium only, whereas the positive

control contained growth medium inoculated only with a bacterial dilution. After thorough mixing, a perforated plate seal (TREK Diagnostic System Inc., Cleveland, OH) was used to seal the 96-well microplates to avoid evaporation. The plates were then incubated under standard conditions at 37°C.The absorbance was measured at 590 nm using a microplate reader spectrophotometer (Labsystems Mutliskan Ascent Model 354 Photometric Plate Reader, USA) every 30 min over a 19-h incubation period.

2.5. Modeling microbial growth and determination of kinetic parameters

The modeling method was carried out as described by Belda-Galbis et al. (2014) with some modifications. Observations regarding the growth of *L. monocytogenes* were recorded to determine the kinetic parameters. During the recording of microbial growth, data pertaining to optical density were transformed into counts given as $\log_{10}$ CFU/mL. After the transformation process, the resulting data were fitted to a modified Gompertz model developed by Gibson et al. (1988):

$$\log_{10} N_t = A + CXe^{-BX(t=M)} \quad (2)$$

where N_t represents the number of microorganisms at a particular time t, A is the natural logarithm of the initial count, C represents the difference between the final and initial counts, B is the growth rate when t = M, and M is the time taken to reach the maximum growth rate (μ_{max}).

The data were also fitted to a nonlinear regression model using a Levenberg–Marquardt algorithm. The regression model helped to determine the parametric values by

reducing the residual sum of squares as previously described by Zwietering et al. (1990). Based on previous methods described by McMeekin et al. (1993), the λ and μ_{max} attained by the microorganism under each scenario are given by Equations 2 and 3, respectively, as follows.

$$\lambda = M - \left(\frac{1}{B}\right) + \frac{\log_{10} N_0 - A}{\mu_{\max}} \quad \textbf{(3)}$$

$$\mu_{\max} = \frac{B \times C}{e} \quad \textbf{(4)}$$

The data were fitted using the statistical software OriginLab.

3. Results

3.1. Fabrication of EO nanoemulsion

Table 2 shows the diameter value (D90) and average droplet size of each essential oil nanoemulsion. Depending on the chemical composition and physical properties (e.g., viscosity, solubility), the EOs exhibited unique droplet size distributions which allowed them to be divided into three groups. The majority of oils had extremely similar and uniform size distributions, and they were included in group 1. This group, which consisted of 16 oils, featured an average size of approximately 98.5 nm with a D90 value of 128.0 nm. Some oils including allspice, citronella, and lemon eucalyptus (group 2) displayed smaller droplets with an average size of approximately 44.7 nm and a D90 of approximately 55.6 nm after ultrasound-assisted size reduction. EOs in group 3 featured larger droplets with an average size and D90 of approximately 135.4 nm and 176.8 nm,

respectively. It was noteworthy that group 3 EOs had higher viscosity than those in the other two groups.

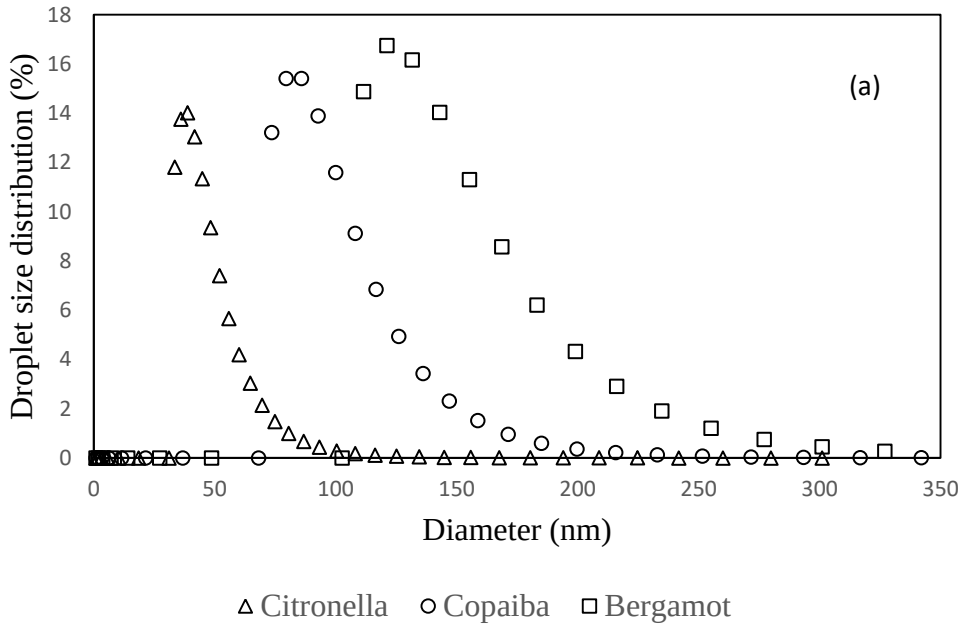
Table 2. D90 and average droplet size of the nanoemulsions of 28 essential oils

		Average Size (nm)	D90 (nm 0)
Group 1	Carrot	92.3 ± 24.0	121.8 ± 10.6
	Cedar wood	116.9 ± 28.9	145.9 ± 1.6
	Cinnamon bark	90.2 ± 24.9	117.1 ± 0.2
	Copaiba	98.8 ± 24.9	120.4 ± 8.1
	Fire needle	107.4 ± 30.4	146.0 ± 11.8
	Frankincense	99.2 ± 29.0	130.8 ± 11.2
	Geranium	96.7 ± 26.4	115.0 ± 15.4
	Ginger	94.2 ± 23.7	122.1 ± 0.6
	Juniper berry	98.8 ± 25.0	131.8 ± 5.6
	Key lime	107.0 ± 27.4	156.6 ± 16.0
	Lavender	107.9 ± 30.7	150.7 ± 26.7
	Nutmeg	98.5 ± 25.2	121.9 ± 9.9
	Oregano	95.8 ± 26.7	121.2 ± 23.8
	Turmeric	95.1 ± 25.0	120.4 ± 8.1
	Valerian	94.1 ± 23.9	116.6 ± 6.7
	Ylang ylang	82.7 ± 22.2	109.7 ± 3.1
Group 2	Allspice	21.6 ± 2.8	23.6 ± 0.1
	Citronella	52.1 ± 13.9	64.6 ± 3.8

	Lemon eucalyptus	60.4 ± 15.3	78.8 ± 3.3
Group 3	Basil	148.4 ± 31.6	178.7 ± 11.3
	Bergamot	142.3 ± 40.2	182.0 ± 4.6
	Coriander	130.3 ± 34.8	170.0 ± 0.01
	Eucalyptus	145.2 ± 35.1	184.5 ± 25.6
	Fennel	141.3 ± 29.9	181.3 ± 28.2
	Peppermint	129.0 ± 34.4	183.5 ± 28.7
	Rosemary	120.2 ± 31.1	165.1 ± 15.9
	Sweet orange	124.4 ± 34.4	175.3 ± 18.6
	Wintergreen	137.4 ± 40.3	171.1 ± 27.0

[1]D90 value indicates the diameter below which 90% of the droplets in the emulsion are distributed. Data are presented as the mean ± SD.

The droplet size distributions of EOs (citronella, copaiba, and bergamot) for each group are depicted in **Figure 3.** The graph shows that each group has a narrow size distribution range, indicating that ultrasound-assisted size reduction resulted in the uniform droplet size in each emulsion. Despite differences in droplet size distribution in each group, the size distribution of all essential oil emulsions fell below the criteria for a nanoemulsion, which allowed the comparative study of the antimicrobial effect of each essential oil.


Droplet size distribution (%)
18
16
14
12
10
8
6
4
2
0
0
50
100
150
200
250
300
350
(a)
Diameter (nm)
Citronella
Copaiba
Bergamot

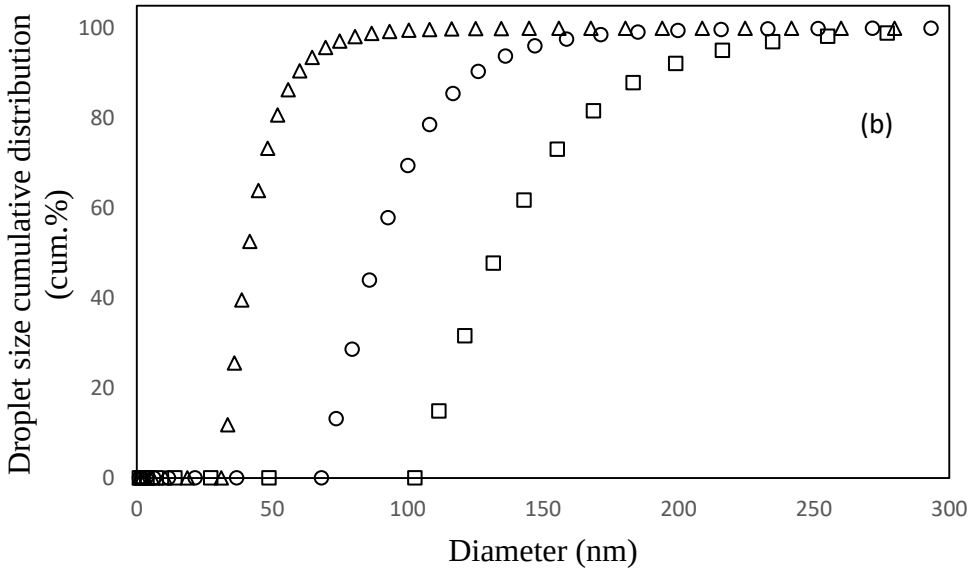

Droplet size cumulative distribution (cum.%)
100
80
60
40
20
0
0
50
100
150
200
250
300
(b)
Diameter (nm)
Citronella
Copaiba
Bergamot

Figure 3. Droplet size distribution (top) and cumulative size distribution (bottom) of citronella oil (Δ), copaiba (○), and bergamot oil (□) nanoemulsions prepared using cetrimonium bromide.

Essential oils were sonicated by an ultrasonicator and then the droplet size of essential oil nanoemulsions was measured by a particle analyzer (Beckman Coulter).

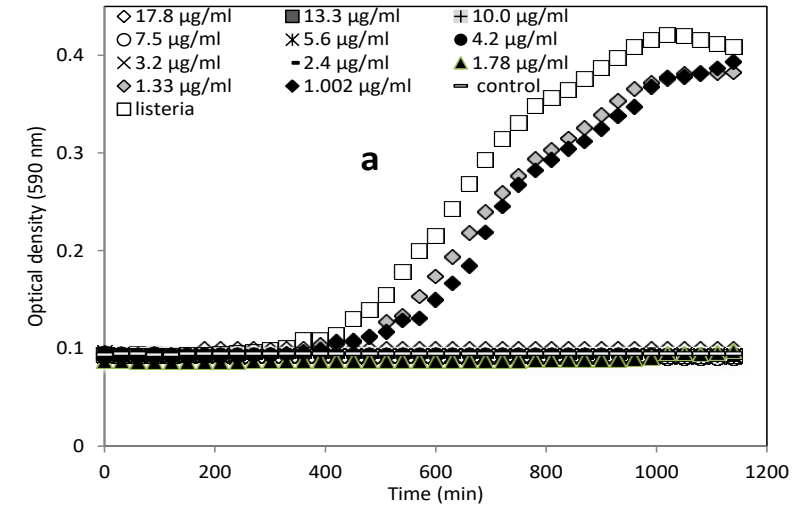
3.3.2. Bacterial Growth Curve & MIC

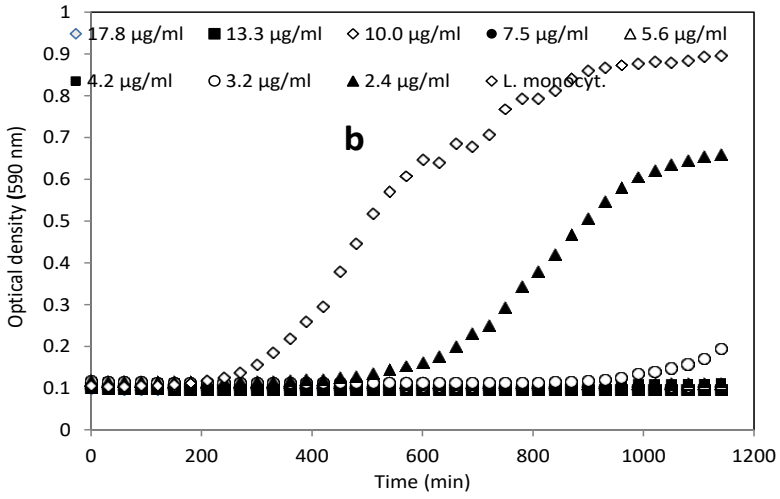
This section presents the minimum inhibitory concentration (MIC) of all tested EOs. Different concentrations of 28 EOs were tested against *L. monocytogenes* planktonic cells. Frankincense EO was observed to have a very high inhibitory effect against *L. monocytogenes* with an MIC value of approximately 1.78 µg/mL. The MIC of eucalyptus and fire needle that inhibited visible growth of *L. monocytogenes* was 2.09 *µg/mL,* as shown in Figure 2(a) and Table 2.

The results in Figure 2(b) shows the growth curve of *L. monocytogenes* in nutmeg; the inhibitory effects were only observed at two concentrations of 2.4 and 3.2 µg/mL, at which the bacterial growth rate was effectively retarded. No visible growth was observed at concentrations of 4.2, 5.6, 7.5, 10.0, 13.3, and 17.8 µg/mL. The MIC value of nutmeg, Egyptian geranium, cedar wood, ylang ylang, and peppermint was 4.2 µg/mL which was the lowest concentration at which complete inhibition was observed, whereas the MIC values of key lime, bergamot, sweet orange, and valerian were 2.4, 4.9, 4.4, and 3.7 µg/mL, respectively, as shown in Table 2.

Figure 2 (c,d) shows that there was only a slight antibacterial effect against *L. monocytogenes* at concentrations of 2.4 and 3.2 µg/mL for fennel and copaiba oils. No visible growth was observed at 10.0, 13.3, and 17.8 µg/mL. The MIC of fennel, basil, carrot, cinnamon bark, citronella, coriander, rosemary, and turmeric was 10.0 µg/mL,

whereas the MIC value of copaiba, juniper berry, ginger, lemon eucalyptus, and oregano was 13.3 µg/mL. The MIC values of allspice, lavender, wintergreen, and ginger were 7.9, 7.5, 8.75, and 11.65 µg/mL, respectively, as shown in Table 3.





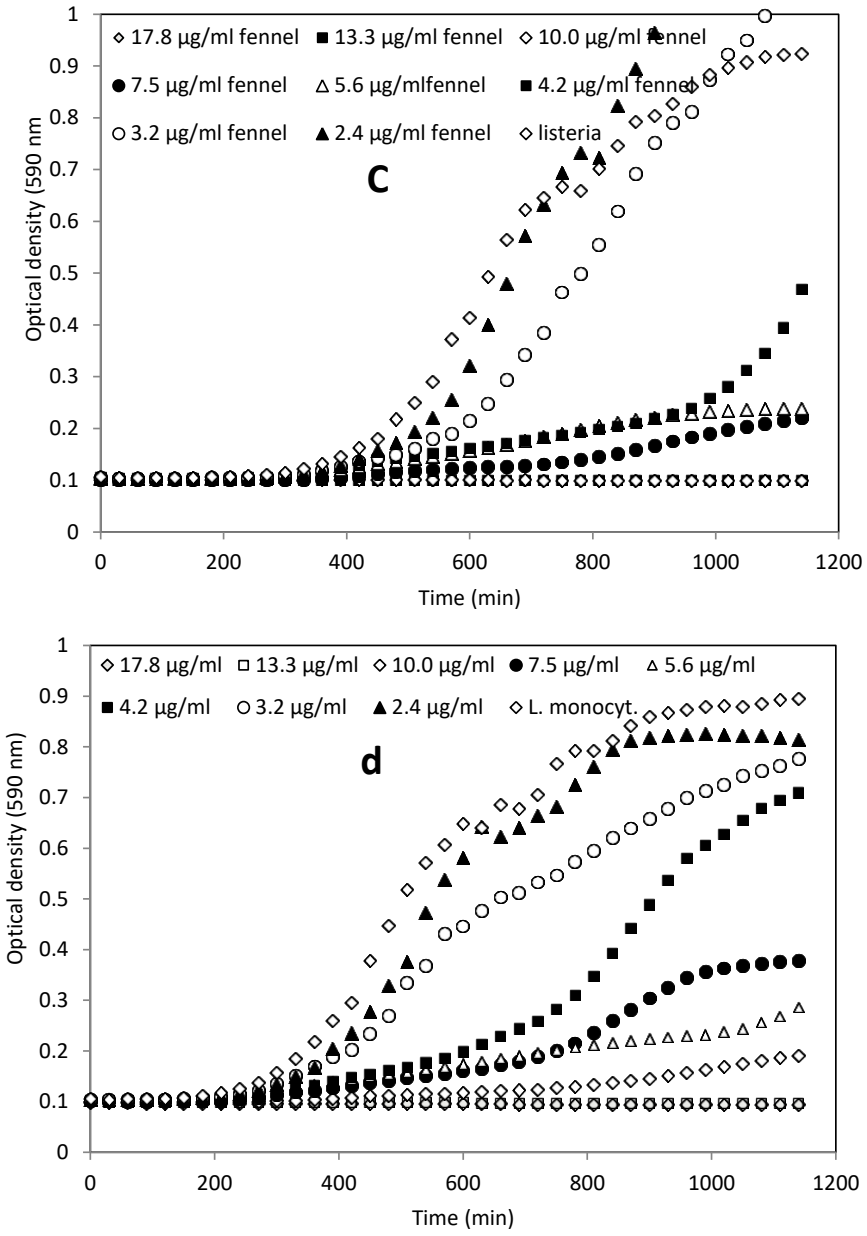


Figure 4 Growth curves of *L. monocytogenes* in the presence of a) eucalyptus, b) nutmeg, c) fennel, and d) copaiba nanoemulsions.

3.3.3. Quantitative modeling of RO nanoemulsions

This study provides the results of a predictive food microbiological model of *L. monocytogenes* bacteria by using 28 EO nanoemulsions. Growth data obtained from optical density measurements were transformed to counts given as $\log_{10}$cfu/mL. Then,

data were fitted using a Gompertz model to find the values for lambda and maximum growth rate in the presence of EOs. The lag phase (λ) is the early stage of the bacterial growth cycle, which refers to the time before exponential growth. Based on the results, Table 3 showed the lag phase and maximum reached by *L.monocytogenes* at 3.2 µg/mL of 28 essential oil nanoemulsions.

Table 3: Lag time and maximum growth rate of *Listeria monocytogenes* in the presence of various essential oil nanoemulsions at 3.2 µg/mL

EO nanoemulsions	**Lag time (h)**	**Max. growth rate** log10 CFU	**MIC** (µg/mL)
Frankincense	0.00	0.00	1.78 ± 0.00
Fire needle	0.00	0.00	2.09 ± 0.438
Eucalyptus	0.00	0.00	2.09 ±0.438
Nutmeg	16.734 ± 0.00	0.111 ± 0.00	4.2 ± 0.00
Key lime	13.781 ± 0.00	0.135 ± 0.00	2.4 ± 0.00
Egyptian geranium	13.775 ± 1.097	0.202 ± 0.00	4.2 ± 0.00
Cedar wood	11.125 ± 4.221	0.246 ± 0.026	4.2 ± 0.00
Sweet orange	10.315 ± 0.00	0.124 ± 0.155	4.4 ± 1.697
Valerian	10.215 ± 7.447	0.101 ± 0.135	3.7 ± 0.707
Bergamot	10.200 ± 0.303	0.240 ± 0.007	4.9 ± 0.989
Ylang ylang	9.832 ± 1.146	0.229 ± 0.007	4.2 ± 0.00
Peppermint	8.491 ± 0.842	0.208 ± 0.00	4.2 ± 0.00
Allspice	8.176 ± 0.262	0.131 ± 0.004	7.8 ± 3.111

Wintergreen	7.581 ± 0.602	0.132 ± 0.002	7.5 ± 0.00
Cinnamon bark	7.486 ± 0.017	0.162 ± 0.021	10 ± 0.00
Basil	7.309 ± 0.094	0.302 ± 0.391	10 ± 0.00
Oregano	7.368 ± 0.262	0.219 ± 009	13.3 ± 0.00
Fennel	7.244 ± 0.138	0.191 ± 0.056	10 ± 0.00
Turmeric	7.131 ± 0.142	0.251 ± 0.086	10 ± 0.00
Citronella	7.098 ± 0.129	0.281 ± 0.047	10 ± 0.00
Coriander	7.035 ± 0.108	0.217 ± 0.0006	10 ± 0.00
Lemon eucalyptus	6.920 ± 0.160	0.193 ± 0.009	13.3 ± 0.00
Carrot	6.860 ± 0.038	0.219 ± 0.132	10 ± 0.00
Juniper berry	6.556 ± 0.261	0.224 ± 0.010	13.3 ± 0.00
Rosemary	5.471 ± 0.00	0.254 ± 0.00	10 ± 0.00
Lavender	6.429 ± 0.867	0.117 ± 0.038	8.75 ± 1.767
Ginger	6.263 ± 0.00	0.134 ± 0.015	11.65 ± 2.333
Copaiba	6.087 ± 0.00	0.228 ± 0.103	13.3 ± 0.00
Control	6.718 ± 0.141	0.231 ± 0.215	

CI indicates the complete inhibition of microbial growth.

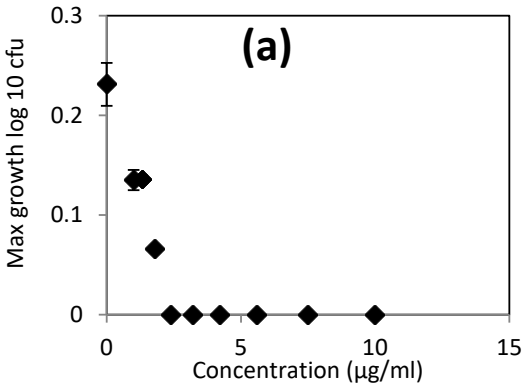
Based on the results of this study, the EOs of fire needle, eucalyptus, and frankincense are very effective against *L. monocytogenes*. The λ values for fire needle, eucalyptus, and frankincense were 13.325, 12.373, and 13.450, respectively, at 1.78 µg/mL, while the control value was 6.718. The μ_{max} of *Listeria* for the EOs of fire needle, eucalyptus, and frankincense decreased with a rise in the EO concentration from 0.063, 0.065, and 0.00, respectively, at 1.78 µg/mL to 0.00, at 2.4 µg/mL, compared with the

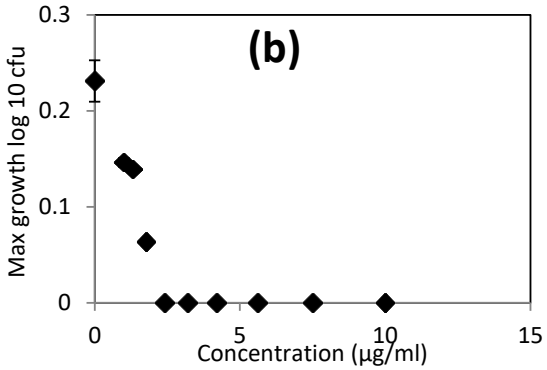
control value of 0.231. The findings indicate that these EOs are very effective against *L. monocytogenes*.

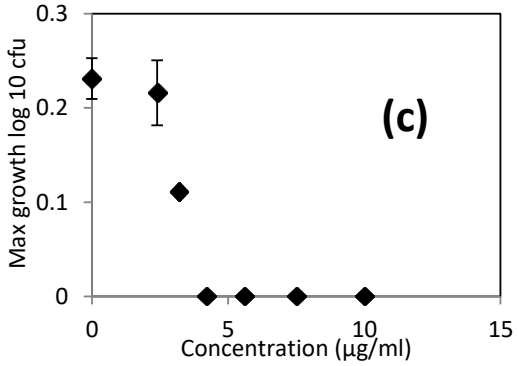
Figure 5 and **Figure 6** (c–f) show that for EOs of bergamot, ylang ylang, Egyptian geranium, and nutmeg, the λ values of *L. monocytogenes* at 2.4 µg/mL were 7.8, 8.52, 9.92, and 10.939, respectively, which increased with increasing EO concentration; at 10, 7.5, 5.6, and 4.2 µg/mL, the growth of *L. monocytogenes* was completely inhibited compared with that of the control, which was 6.718. The μ_{max} value decreased with an increase in the concentration of bergamot, yiang yiang, egyptian geranium, and nutmeg from 0.245, 0.226, 0.200, and 0.215,respectively at 2.4 µg/mL to 0.00 at (5.6, 7.5, and 10)µg/mL compared with control which was 0.231; this indicates that these EOs completely inhibited the growth of *Listeria* at concentrations 5.6, 7.5, and 10 µg/mL. The results indicate that these EOs were effective against the growth of *L. monocytogenes*. Moderate effectiveness was also observed for cedar wood, valerian, sweet orange, and peppermint; the λ of *L. monocytogenes* with these EOs increased with an increase in the EO concentrations. The μ_{max} values also decreased with a rise in the EO concentration.

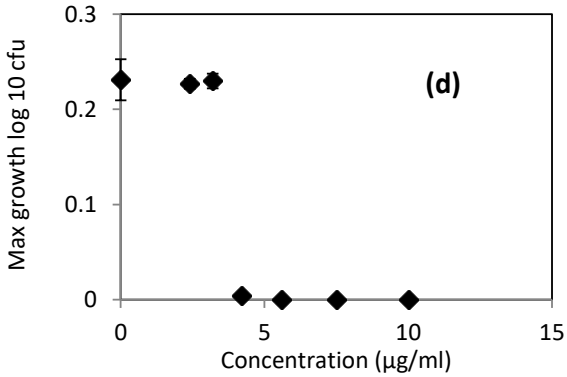
Figure 5 and Figure 6 (g–i) show that the μ_{max} of *L. monocytogenes* decreased with an increase in EO concentration for lemon eucalyptus, fennel, and copaiba, but there was a slight increase in the duration of the lag phase of *Listeria* with a rise in the concentrations of these oils. At 2.4 µg/mL, the λ values of *Listeria* for lemon eucalyptus and copaiba were 6.910 and 6.651 respectively, whereas at 10 µg/mL the λ values, were 7.416 and 10.528,respectively which contrasted with the value in the control of 6.718. The λ in fennel oil was 7.423 at 2.4 µg/mL and increased with an increase in EO

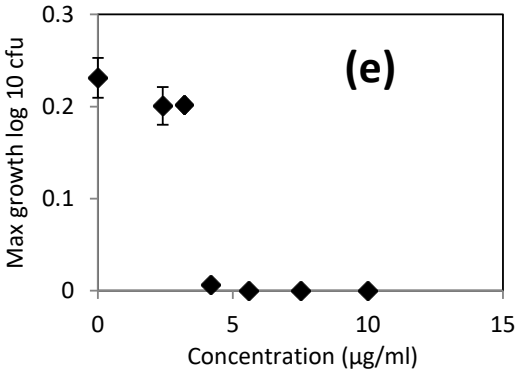
concentration; at 10 µg/mL, there was no observable growth. This indicates that this concentration results in complete inhibition of Listeria. The μ_{max} values decreased with a rise in the concentrations of EO of lemon eucalyptus, fennel, and copaiba from 0.205, 0.246, and 0.283 at 2.4 µg/mL to 0.122, 0.00, and 0.114 at 10 µg/mL, respectively, which differed from the value in the control of 0.231. The results indicate that these EOs are less inhibitory against *L. monocytogenes*. The majority of the EOs including basil, cinnamon, ginger, copaiba, lemon eucalyptus, wintergreen, turmeric, coriander, citronella, oregano, carrot, lavender, fennel, and Jupiter berry were less effective at reducing the growth rate and lag time of *L. monocytogenes*. The λ values of these EOs also increased with an increase in the EO concentrations, but the increase was slight, whereas the μ_{max} values decreased with a rise in the EO concentrations.

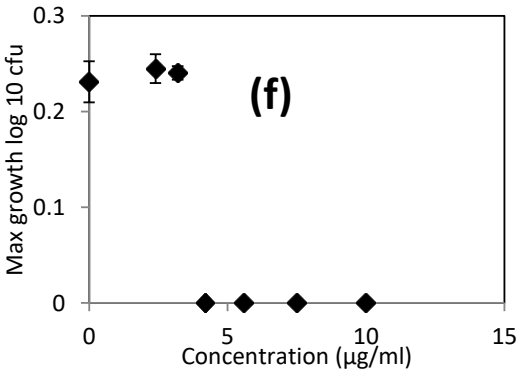

(a)
Max growth log 10 cfu
0.3
0.2
0.1
0
0
5
10
15
Concentration (µg/ml)


(b)
Max growth log 10 cfu
0.3
0.2
0.1
0
0
5
10
15
Concentration (µg/ml)


(c)
Max growth log 10 cfu
0.3
0.2
0.1
0
0
5
10
15
Concentration (µg/ml)


(d)
Max growth log 10 cfu
0.3
0.2
0.1
0
0
5
10
15
Concentration (µg/ml)


(e)
Max growth log 10 cfu
0.3
0.2
0.1
0
0
5
10
15
Concentration (µg/ml)


(f)
Max growth log 10 cfu
0.3
0.2
0.1
0
0
5
10
15
Concentration (µg/ml)

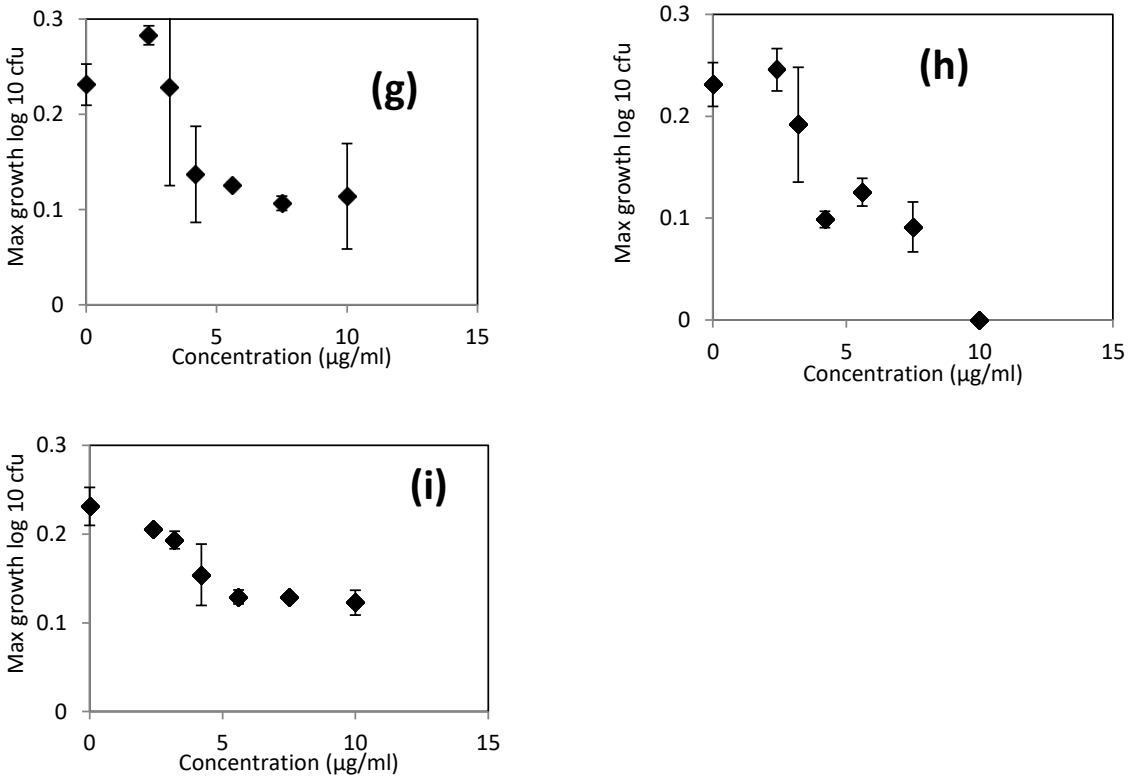
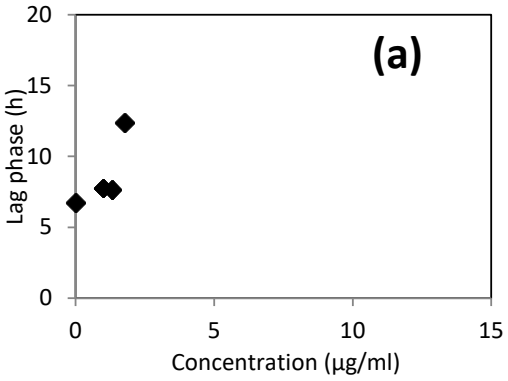
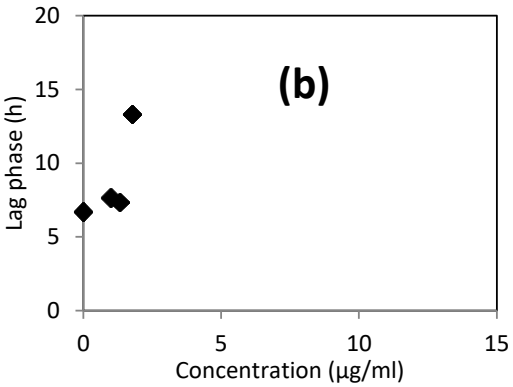
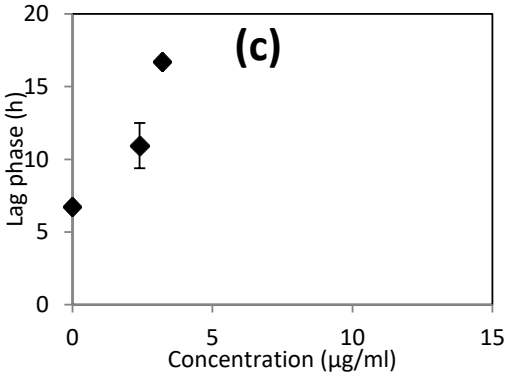
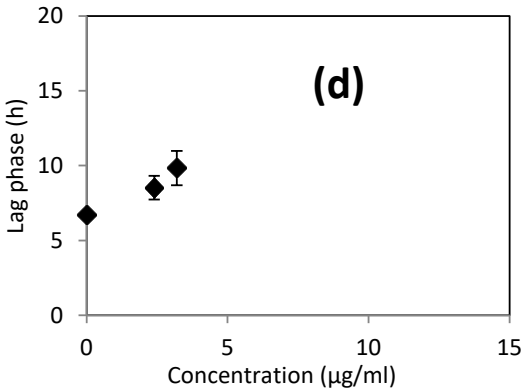


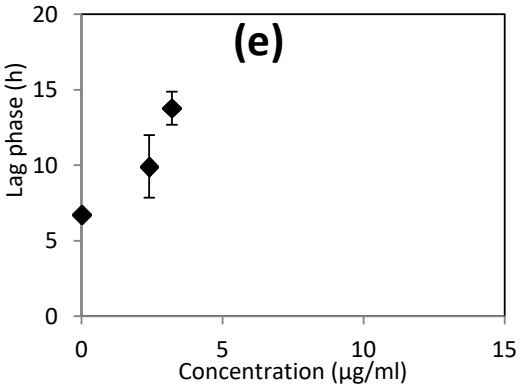
Figure 5. Maximum specific growth of *L. monocytogenes* of a) eucalyptus, b) fire needle, c) nutmeg, d) ylang ylang, e) Egyptian geranium, f) bergamot, g) copaiba, h) fennel, and i) lemon eucalyptus.

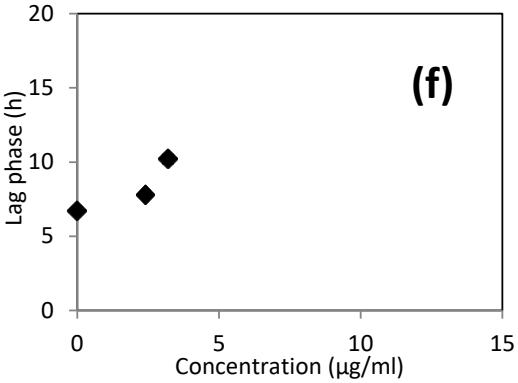

(a)
Lag phase (h)
Concentration (µg/ml)
0
5
10
15
20


(b)
Lag phase (h)
Concentration (µg/ml)
0
5
10
15
20


(c)
Lag phase (h)
Concentration (µg/ml)
0
5
10
15
20


(d)
Lag phase (h)
Concentration (µg/ml)
0
5
10
15
20


(e)
Lag phase (h)
Concentration (µg/ml)
0
5
10
15
20


(f)
Lag phase (h)
Concentration (µg/ml)
0
5
10
15
20

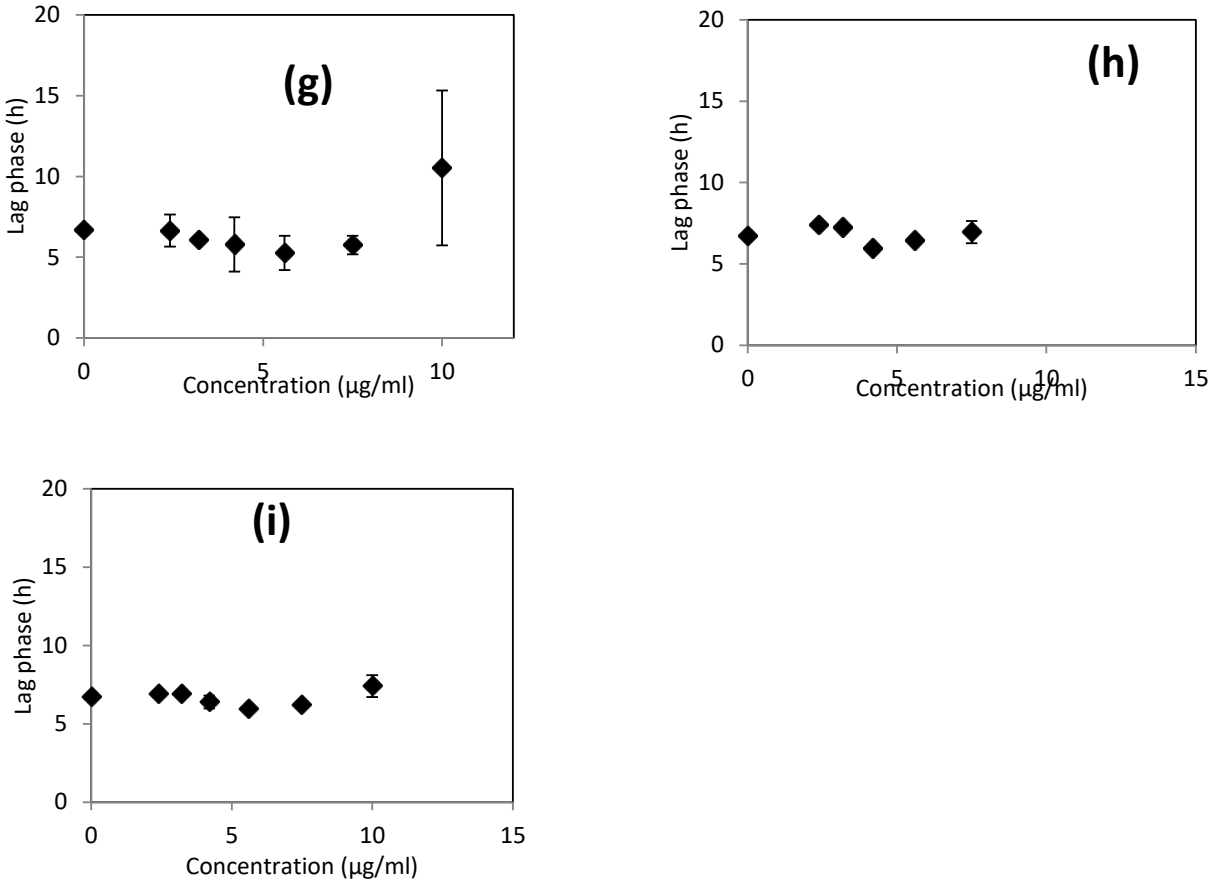


Figure 6. Lag phase of *L. monocytogenes* for a) eucalyptus, b) fire needle, c) nutmeg, d) ylang ylang, e) Egyptian geranium, f) bergamot, g) copaiba, h) fennel, i) lemon eucalyptus.

As shown in Figure 7, cinnamon bark, bergamot, ylang ylang, Egyptian geranium, cedar wood, peppermint, nutmeg, valerian, allspice, frankincense, sweet orange, wintergreen, eucalyptus, key lime, and fire needle had lag phase that increased exponentially with increase in concentration, indicating that these EOs are highly effective against *Listeria*. Meanwhile, the EOs copaiba, lavender, carrot, citronella, rosemary, oregano, lemon eucalyptus, coriander, fennel, Jupiter berry, basil, ginger, and turmeric had lag phase that increased slowly with increase in concentration.

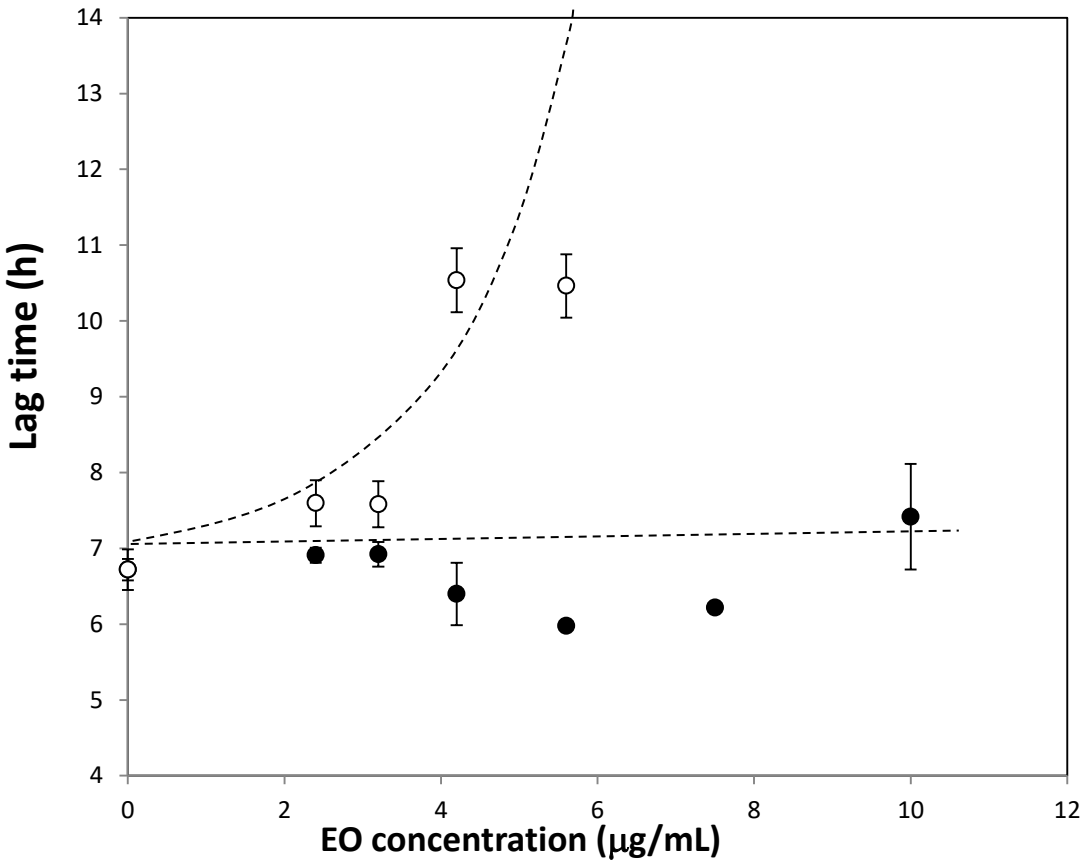


Figure 7 The shape of the lag phase of *L. monocytogenes* for all EO nanoemulsions.

Based on the results**,** Figure 8 shows that the growth rate associated with bergamot, ylang ylang, egyptian geranium, cedar wood, peppermint, nutmeg, valerian, allspice, frankincense, sweet orange, wintergreen, eucalyptus, key lime, and fire needle decreased steeply between concentrations 1.78 and 2.40 µg/mL, indicating that these EOs are highly effective against *Listeria*. Meanwhile, the growth rate associated with cinnamon bark, lavender, carrot, citronella, rosemary, oregano, lemon eucalyptus, coriander, fennel, Jupiter berry, basil, ginger, and turmeric decreased in a steady manner as concentration increased throughout the entire concentrations tested in this study, indicating that these EOs are less effective against *Listeria*.

Based on the results of this study, almost all EOs exhibited antibacterial activity against *L. monocytogenes,* and the observed differences were based on the concentration of EO nanoemulsions.

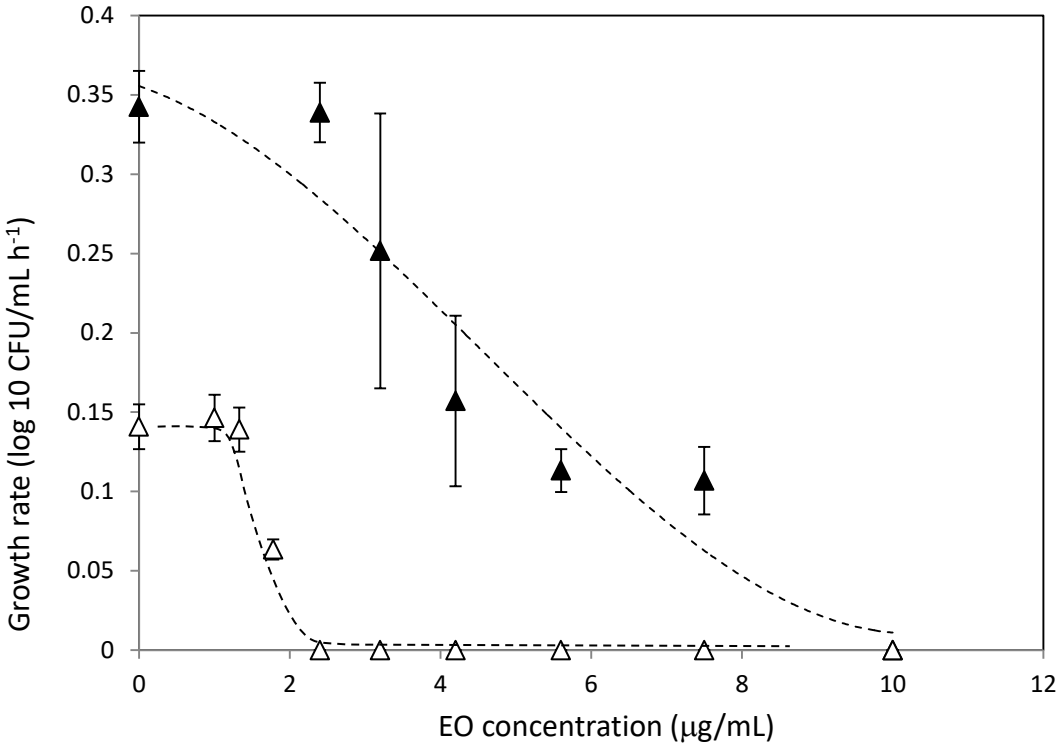


Figure 8 the maximum specific growth of *L. monocytogenes* for all EO nanoemulsions.

3.4. Discussion

Two parameters of bacterial growth were examined in relation to the effects of EOs, namely, λ and μ_{max}. The lag phase is a process of equilibration regulated by unknown mechanisms that allows bacterial cells to begin utilizing new environmental conditions for growth (Madigan et al., 2000; Rolfe et al., 2012).

The inhibitory effects of EO nanoemulsion against *L. monocytogenes* can be identified using λ and the growth rate (μ_{max}) (Donsì et al., 2011). As the lag phase is considered the initial period of a bacterium's life, during which it adjusts to the new environmental conditions, interference with the normal cell functionality would strongly affect such adaptation (Donsì et al., 2011). The nanoemulsion droplets have the ability to bind to the lipid bilayer of bacterial cell membranes (Bhargava et al., 2015). This fusion results in the destabilization of membrane integrity. This clearly indicates that the mode of bactericidal action of the nanoemulsion against *L. monocytogenes* is membrane disruption, which leads to further cell death. Apart from the reduced concentrations of the EOs, the sizes of the nanoemulsions played a significant role in determining their antibacterial activities. Small EO droplets exhibit stronger antibacterial activity against *L. monocytogenes* than oils with large droplets (Schultz and Kishony 2013). This is because a reduced droplet size provides a large surface area to volume ratio, hence improving the fusion between EOs and the bacterial cell membranes, and enabling the nanoemulsion oil contents to quickly penetrate into the cells, thus causing cellular rapture (Sugumar et al., 2015).

In all tests, increasing the concentration of EOs consequently extended the lag phase of *L. monocytogenes*, as shown in Figure 7. This observation indicated a bacteriostatic effect followed by a decline of the specific μ_{max}, which may be partial. A similar observation was made in a study by Faleiro (2011). Extension of the lag phase may have been caused by an increase in antibiotic stress against *L. monocytogenes* cells due to a bacteriostatic effect (Faleiro, 2011).

Figure 6 showed that the Eos demonstrated an increased lag phase of listeria, indicating that these oils effectively affected on *L. monocytogenes* during the lag phase. In the presence of these EOs, *L. monocytogenes* extended its lag phase to overcome the elevated antibiotic stress (Li et al., 2016). In line with this finding, there was no observable growth at higher concentrations, indicating complete growth inhibition by the EO being tested.

The μ_{max} curves in Figure 5 reveal that the growth rate declined as the EO concentration increased. ATPase inhibition may have been one of the factors behind the observed decline in *L. monocytogenes* growth at sublethal concentrations (Nazzaro et al., 2013). Additionally, small hydrophobic molecules that formed as a result of changes in the protein conformation could have caused nonspecific inhibition of membrane-bound and embedded enzymes to inhibit bacterial growth (Oosterhaven et al., 1995; Nazzaro et al., 2013).

As shown in Table **3**, our study findings was in agreement with those of Panahi et al. (2011) and Pattnaik et al. (1994), who observed that eucalyptus EO had a significant antimicrobial effect, the MIC concentration was revealed to be 1.95 µg/mL against *Staphylococcus aureus*.This observation was in agreement with other previous studies;

for example, Zengin and Baysal (2014) reported that eucalyptus oil extended the λ of *E. coli* O157, *Salmonella typhimurium*, H7, and *S. aureus* at concentrations of 0.6%, 0.7%, and 1%, respectively.

The findings of this study are consistent with those of Carrizo et al. (2014), who found that lime EO inhibited the growth and increased the lag phase of *L. monocytogenes*. These authors also found that lime EO extended λ for *L. monocytogenes* by more than 292.7%, compared with that for control juice. The chemical components of EOs affect the cell membranes of bacteria, thus inhibiting their growth (Nazzaro et al., 2013). Some studies found that bacterial membranes contain enzymes involved in the regulation of cellular pH and ATP generation (Magi et al., 2015). Treating of some pathogens with carvacrol, eugenol, and cinnamaldehyde, disrupted the bacterial cell membrane and inhibited the ATPase activity of bacterial cell ,thus leading to ATP loss (Nazzaro et al. ,2013).The effects of EOs on the growth and lag phase of *L. monocytogenes* were additionally studied by Silva-Angulo et al. (2015), who identified significant differences in λ and μ_{max} between low and high concentrations of citral oil in both bacteria.

Based on the findings of this experiment, it is noted that each EO has a specific MIC at which it has noticeable effects on the lag time of *L. monocytogenes* (as shown in Table 3). Our study findings are consistent with those of Firouzi et al. (2007) who reported that nutmeg EO had stronger inhibitory activity (MIC = 25 µL/mL) than oregano EO (MIC = 26 µL/mL) on the growth of *L. monocytogenes* in a broth culture system. However, the MICs were inconsistent with those reported by Firouzi et al. (2007), which may have been attributable to factors such as inoculum volume, incubation time, and

temperature (Mith et al., 2014), as shown in Table 3.The results in Figure 5 and Figure 6, showed that the changes in (μ_{max}) and (λ) were dependent on both the concentrations and the components of the EOs, as well as the overall nature of the bacteria.

EOs can be categorized into four groups based on chemical structure: terpenoids, phenylpropenes, terpenes, and others (Hyldgaard et al., 2012). Identifying the most effective EOs is difficult because they are complex mixtures of up to 45 components (Hyldgaard et al., 2012). In addition, the components of EOs are derived from low-molecular-weight compounds that have different levels of antimicrobial activity (Hyldgaard et al., 2012). The time needed by EOs to interact and interfere with bacteria depends on their composition and the nature of the bacterial cell wall (Donsì et al., 2011). Therefore, the varying levels of antimicrobial activity exhibited by EOs in this study might have resulted from differences in their chemical composition. Overall, the results indicated that the λ of *L. monocytogenes* increased with increasing concentration of EOs, whereas the μ_{max} decreased.

3.5. Conclusion

Overall, the findings of this study indicate that EOs play a significant role in inhibiting growth and prolonging the lag phase in *L. monocytogenes.* Thus, it is advisable to use effective EOs to control the growth of *L. monocytogenes* and other foodborne pathogens. Effective control of *L. monocytogenes* significantly reduces the risk of listerosis, a major health issue, as well as losses resulting from food damage .Although all EOs were effective, there were major differences in λ and μ_{max} among the tested EOs. This study illustrates that effective inhibition of *L. monocytogenes* requires the use of high concentrations of EO

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Chapter IV Efficacy of Essential Oil Nanoemulsion against *Listeria monocytogenes* in the Presence of Casein Protein as a Food Matrix model

Abstract

Listeria monocytogenes is a food borne pathogen causes listeriosis, which can be acquired from processed food that have been contaminated. Ensuring supply of safe food is important for the protection of public health. Therefore, this study aimed to evaluate quantitatively the antimicrobial activity of essential oils nanoemulsion as food preservative against foodborne-pathogens in the presence of food ingredients such protein as a major component of food components. The study used casein protein as a food model. The antibacterial effects of the essential oils derived from the extracts of cedar wood, copaiba, fire needle, frankincense, Egyptian geranium, nutmeg, peppermint, valerian and ylang ylang against *Listeria monocytogenes*, was investigated in the presence and absence of different, 17.59, 35.17 and 70.34 µg/ml, concentrations of the casein proteins. Quantitative evaluation of the antibacterial activity of each essential oil on the *L. monocytogenes* was achieved through fitting the collected data onto to the modified Gompertz model to calculate the lag phase durations and maximum growth rate of each essential oil.The effectiveness of the essential oils in absence of casein proteins and in the presence 17.59 µg/ml of casein proteins was similar but further decreased in addition of 35.17 and 70.34 µg/ml of casein proteins, indicating that presence of high concentration of protein play a role in reducing the antimicrobial activity of Eos**.**

Keywords: Listeria monocytogenes, essential oils, Nanoemulsion, casein proteins

1.Introduction

Food safety involves the process of making food products safe for consumption by the general public, through encouraging practices and procedures which are aimed at making the foods free from foodborne pathogens and hence, preserving food quality and preventing contamination of food by pathogens which can lead to development of foodborne diseases (De Silva, Kanugala et al. 2016). Ensuring supply of safe food is important for the protection of public health. Food contamination and spoilage can be as a result of different factors such as microbial, chemical and physical contaminants, it is therefore important for food safety management to understand the specific nature of contamination, its sources, and risks to the consumers, and methods which can be used to eliminate or reduce the contamination levels (Hussain 2016). As much as treatment of these foods is seen as an option for making the foods safe, the process might at times lead to neutralization of the essential nutrient contents present in the foods (Baer, Miller et al. 2013). To achieve a complete food safety scenario, it is important for all of the stakeholders such as government, food processing industries, and consumers to share the responsibility (Baer, Miller et al. 2013).

There are different groups of pathogenic microorganisms which cause foodborne diseases that affect the general public (Cassini, Colzani et al. 2016). The pathogenic microorganisms are the key factors that lead to food-borne infections, and they largely include viruses, parasites, molds, and bacteria (Arisseto-Bragotto, Feltes et al. 2017). Foodborne diseases are usually toxic or infectious and are instigated by agents who enter the human body through ingestion of contaminated foods (Farmer, Keenan et al. 2012). The increasing levels of foodborne diseases in the public domain have made the

consumers to raise a lot of concerns about the status of the processed foods which they eat (Kuehn 2012).

The pathogenic microorganisms which cause foodborne diseases can be categorized into the various groups such as; (i) Infectious pathogens such as *Salmonella spp.* which causes salmonellosis, *Listeria monocytogenes* which causes listeriosis, *Campylobacter* which causes campylobacteriosis and *Escherichia coli* which cause Escherichia coli O157: H7 Infection. (ii) Toxigenic pathogens; *Bacillus cereus*, Staphylococcus aureus, and *Clostridium botulinum*. (iii) Toxico-infectious pathogens such as *Clostridium perfringens* and E. coli (Farmer, Keenan et al. 2012). The infectious pathogens are capable of invading and colonizing the body of the host then eventually manifesting their side effects (Belda-Galbis, Leufvén et al. 2014). Regardless of the developed novel strategies and technologies, the occurrence of foodborne diseases is not decreasing in the recent years (Cassini, Colzani et al. 2016).

Foodborne pathogens lead to food contamination which leads to health threats on the general public (Farmer, Keenan et al. 2012).*Listeria monocytogenes* is one of the most common pathogenic microorganisms which are found in the food processing industries (Kowalik, Lobacz et al. 2013). It is a Gram-positive bacteria which is rod-shaped, and that can survive in the presence or absence of oxygen. It is broadly disseminated in the environment, and it also does not form spores (Kowalik, Adamczewski et al. 2014). This bacteria has no defined particular host hence it is identified as a non-host specific pathogen. *L. monocytogenes* causes listeriosis which can occur epidemically or sporadically. This bacterium has in the past isolated from different forms of milk such as pasteurized milk, raw milk, and mastitis milk (Shoughy and

Tabbara 2014). Apart from milk, there are also some foodstuffs which are prone to listeriosis outbreak; soft cheese, coleslaw, ice cream, sliced cold cuts among others (Kowalik, Lobacz et al. 2013) .There are thirteen *L. monocytogenes* serotypes, and from them, only three are serotypes have been mostly involved in spreading human diseases. They include; 1/2a, 1/2b and 4b serotypes (Kowalik, Adamczewski et al. 2014).

Consumers are always demanding for high-quality food products hence it is essential to take into practice the most relevant food preservation methods which would not compromise their overall qualities (De Silva, Kanugala et al. 2016). This is the reason behind the development of various food preservation methods. These methods include physical, chemical and biochemical preservation methods and they have been used to prevent microbial activities in food. In the recent years, there has been a lot of developments in food preservation methods which include heat processing, vacuum packaging (VP), chemical preservation, modified atmospheric packaging (MAP) and refrigeration (Gandomi, Abbaszadeh et al. 2014).

Nevertheless, these techniques have not been able to completely eradicate food spoilage associated with microbial contaminations from bacteria such as *L. monocytogenes* (Gutierrez, J., Barry-Ryan, C., & Bourke, P.,2009). To improve on this, there has been development of more advanced techniques such as new non-thermal procedures and naturally derived antimicrobial ingredients applications in food safety and preservation mechanisms (Kasi, A Hatamleh et al. 2017). In relation to the many types of food preservation methods, the food antibacterial plays important role in ensuring growth inhibition of pathogenic microorganisms which promote food spoilage (Larkin 2004) .They can be categorized into two groups such as synthetically derived antimicrobial

which include organic acids and esters (for example, acetic acids and acetates, benzoic acid and benzoates, and lactic acid and lactates) and naturally occurring antimicrobial which is derived from the animals, plants and microbial sources (Davidson et al., 2013). Natural antimicrobials are considered to be more appropriate for the purpose of satisfying the demand for natural foods from the customers (Davidson, Taylor et al. 2013) .Essential oils include large groups of natural plant origin antimicrobial which have been in the resent years used to eliminate or inhibit the growth of pathogenic microorganisms (Kasi, A Hatamleh et al. 2017).

In the recent years, there have been increasing concerns about application of herbal products which have additional functional properties as alternatives to synthetic chemical food preservatives (Ebrahimi and Darani 2013). Among the natural antimicrobials, essential oils have been widely used as a result of their antimicrobial (antifungal, antiviral and antibacterial), anti-mutagenic, antioxidants and anti-carcinogenic properties (Ebrahimi and Darani 2013). Essential oils (also referred to as volatile oils) are aromatic oily liquids which are obtained from plant materials such as flowers, buds, bark, wood, fruits, seeds, herbs, roots and leaves that are made of complex mixture of secondary metabolites (Ebrahimi and Darani 2013). The EOs can be produced through various methods such as fermentation, expression or extraction and of all; steam distillation becomes the most commonly used method. The components of EOs have the ability to offer antimicrobial effects on both the Gram-positive and Gram- negative bacteria (broad spectrum) hence can be used to eliminate *L. monocytogenes* and general food spoilages caused by different microbes (Arisseto-Bragotto, Feltes et al. 2017).

As natural and effective antimicrobial agents, different types of essential oils have been used to control the growth of *L. monocytogenes* and therefore enhance food safety(Said N El 2013).The Gram-positive bacteria such as *L. monocytogenes* are considered to be very vulnerable during the inhibition process by the EOs. The antibacterial activity of the EOs against *L. monocytogenes* depends on their respective MIC value, which is the most important factor in determining the inhibition levels of growth of bacteria (Cheng, Yang et al. 2012).

The antibacterial activity of every essential oil against *L. monocytogenes* depends on two or more major components of the essential oils. The ability of the essential oils to be effective on the *L. monocytogenes* depends on their capacity to reach the maximum inhibitory concentration faster. In a situation whereby the cells of *L. monocytogenes* come into contact with the essential oils, there will be the release of the cytoplasmic contents which can be determined by measuring their respective absorbance (Sugumar, Mukherjee et al. 2015).

Food matrices are complex, multicomponent systems which are made up of different components, such as proteins, fibers, fats and carbohydrates, all with interconnecting microenvironments. Direct fusion of essential oils in the food system are perceived to face a lot of challenges; this is based on the fact that they have low water stability and interactive binding with other food components such as proteins, hence reducing their antibacterial activity in complex food matrices (Sugumar, Mukherjee et al. 2015). This factor is caused by the level of interaction that occurs between the food matrix and EOs. To ensure high efficiency of the essential oils against bacteria, there is need to use them in high concentration (Gandomi, Abbaszadeh et al. 2014).

Proteins are considered as major food components. Hence food with high protein content has the ability to protect bacteria against the antibacterial activity of the selected EOs (Larkin 2004). This is due to the presence of the hydrophobic properties of the proteins and the presence of the three-dimensional matrix layer which functions as a barrier that prevents entry essential oils into the inner structures of the bacterial cells (Sugumar, Mukherjee et al. 2015). Essential oil metabolites also have the ability to cross the cell membrane and bind to the proteins to inhibit their respective activities (Beeby 1970). The existence of a high hydrophobicity within the EOs constituents together with the short extension of their carbon chains ensure strong interaction with the proteins (Tongnuanchan and Benjakul 2014).

A variety of the proteins are derived from plants and animals, for example, milk proteins which include whey and casein protein, and they have frequently been used to facilitate the formation and improvement of the stability of food emulsions (Gandomi, Abbaszadeh et al. 2014). Casein proteins include phosphoproteins which are occasionally found in mammalian milk, composing of up to 80% of the proteins found in the cow's milk and between 25% and 40% of the proteins which make up the human milk (Tongnuanchan and Benjakul 2014).

The resistance properties of several bacteria to various types of antibacterial compounds have in the recent years created the need of researching and finding various developments which can be made of the existing agents (Chardigny and Walrand 2016). This has been possible through the application of nanotechnology and more specifically, nanoemulsion of essential oils which have improved their solubility, stability, and efficacy. Nanoemulsions are dispersions of nano-scale droplets which are formed by

shear-induced rupturing. These properties when formulated into the essential oils, they are made to be least sensitive to physical and chemical changes (Zorzi, Carvalho et al. 2015). Due to the challenges associated with food contaminations, there have been advanced developments in the application of EOs present photo-chemicals such as vanillin, carvone, Citral, cinnamaldehyde, thymol, eugenol among others in the elimination or prevention of microbial contamination (Gupta, Eral et al. 2016).

The formulation of nanoemulsion can be done by through use of low-energy methods which include Spontaneous Emulsification (SE), Phase Inversion Composition (PIC), Emulsion Inversion Point (EIP) and Phase Inversion Temperature (TIP) methods and high-energy methods such as high-pressure homogenization, micro fluidization, and sonication (Nanjwade, Kadam et al. 2013). The most recent application of essential oil emulsification has been observed in plums whereby the lemongrass oil nanoemulsion was used to evaluate the different antibacterial properties. These nanoemulsions were able to prevent the increase of *L. monocytogenes* and *E. coli* without altering the flavor, factorability, and glossiness of the product (São Pedro, Santo et al. 2013).

The smaller the size and a higher concentration of the nanoemulsion oils, the higher the volume of cellular content which is released from the cells (Tongnuanchan and Benjakul 2014). The nanoemulsion droplets have the ability to bind with the lipid bilayer which is present in the bacterial cell membrane (Ebani, Nardoni et al. 2016).The fusion leads to compromising of the cell membranes hence causing destabilization in the membrane integrity. This whole process is as a result of the lysis of the bacterial cells (Zorzi, Carvalho et al. 2015). The changes which take place in the structure of *L. monocytogenes* as a result of treatment with nanoemulsion oils alter the cellular permeability and hence

promoting the eventual release of the intercellular contents (Maté, Periago et al. 2016). The antimicrobial activity of essential oils has been previously studied. However, few studies have compared their antimicrobial activity quantitively through kinetic analysis against *L. monocytogenes* through their application as nanoemulsion in the food matrix and in the presence of food proteins.

The kinetic studies have made it possible for the development of different methods which can be applied during the analysis of different substances (Kessick 1974). Kinetic studies will, therefore, enhance the investigation involving how different conditions will influence the rate of essential oil nanoemulsion reactions and produce information regarding the reaction mechanism and transmission states (Kessick 1974). Gompertz model is one of the most commonly applied original growth models. In this model, it is possible to analyze how different cellular growth take place in different conditions (Tjørve and Tjørve 2017). .This study can, therefore, be applied in the process of quantitative modeling of essential oil nanoemulsion against *L. monocytogenes* in the presence of casein protein as a food matrix to show the rate of reaction of different types of essential oil nanoemulsions on *L. monocytogenes* in the presence of casein protein as a food matrix. In a situation whereby there is the need for application of EOs in food preservation process, it is important to employ primary studies in the individual food models to determine the potential interactions between EOs and the food products (Falade and Oyeyinka 2015).

To effectively achieve this, it will be important for this study to fulfill the following objectives adequately. (i) To develop suitable formulations for the essential oil nanoemulsion preparation (ii) To measure and determine the droplet size distribution of

nanoemulsion of essential oils. (iii) To determine the comparative efficiency of the wide spectrum of natural nanoemulsion oils against *L. monocytogenes* in the presence of different concentrations of casein protein.(iv) To identify kinetic parameters that influences the effectiveness of EO nanoemulsions through the application of predictive microbiology approach in the presence of different concentrations of casein protein.

2. Materials and Methods.

2.1. Preparation of Bacteria Culture

A technique reported used by Jiang (2011) ,with little modifications, was used in the preparation of the bacterial cultures. Scot A strains of the L. monocytogenes (obtained from D. Portnoy, University of California, Berkey) were used in this study. The storage of Scot A was done in the Brain Heart Infusion (BHI) broth supplemented with 30% glycerol at -20^{0}C until it was used. BHI broth was prepared by dissolving 39g/L in the deionized water and autoclaved for 45 minutes while the bacterial cells used were sub-cultured from the original culture (Balouiri, Sadiki et al. 2016).

2.2. Fabrication of essential oil nanoemulsions

Nanoemulsions of 8 different essential oils – i.e., cedar wood, copaiba, fir needle, frankincense, Egyptian geranium, nutmeg, valerian and ylang ylang oils –were prepared by first preparing (15×10³ μg/mL) of each essential oil and added to 6 mL of cetrimonium bromide aqueous solution (1.0%, w/w). The resultants were subsequently followed by ultrasonication to allow size reduction of droplets in the emulsions using a 20 kHz ultrasonicator (Fisher Scientific, Hampton, NJ, Model 505 Sonic Dismembrator, nominal power 500W), equipped with a generator, a converter, and a horn tip (diameter, 3mm). All experiments were performed in a small test tube (i.d., 13mm). The

ultrasonication was carried out with an amplitude of 30% in pulse mode with 30- s of pulse-on and 15- s of off interval for a total of 2.75 min. Each experiment was carried out in triplicate.

2.3. Particle size distribution of emulsion droplets

A particle analyzer (Model Delsa™ nano C; Beckman Coulter) equipped with a size cell was used to analyze the droplet size distribution of essential oil nanoemulsions.This device performs size measurements by Dynamic Light Scattering (DLS), which measures the Brownian motion of particles and relates this to particle size on the premise that larger particles have slower motion. Calculations are based on the Stokes–Einstein Equation (1) as follow:

$$d_H = \frac{\kappa T}{3\pi\eta D} \qquad (1)$$

Where d_H is the hydrodynamic diameter, D is the translational diffusion coefficient, κ is the Boltzmann's constant, T is the absolute temperature, and η is the viscosity of the medium, in this case pure water (η = 0.89 cP at 25 °C).

2.4. Casein protein stock solution preparation

The study was used 2% casein protein powder to prepare a stock solution using buffer solution.PH solution was adjusted to 7.2 using1 M HCl .Afterward, the solution was left for five minute under magnetic sterile .The stock solution then was centrifuged for 30 min at 3000 r.p.m and -4 c. The supernatant was collected using filtration with filter paper. Centrifuged process was conducted twice to remove any residual traces of insoluble protein in order to get clarity of casein protein solution and prevent turbidity

through absorbance measurement. Model media containing casein protein 2% were autoclaved prior to use. A stock solution containing 2%casein protein was then diluted by 2 fold dilution to two concentrations (LaClair and Etzel 2009).

2.5. Determination of Protein content

A modified Bradford Assay which uses bovine serum albumin as the relevant standard was used in the determination of the quantity of the protein contents which was present in the casein protein after centrifuging. In this assay, there is the application of the micro-plate absorbance reader.Based on the description of the Bradford Proteins Assay by Ernst and Zor (2010) ,this study was conducted on the same principle but with little modifications. 0.1 mg/ml of bovine serum albumin was used as the standard stock solution, diluted to three concentrations of 0.05, 0.025 and 0.0125 mg/ml respectively. Dilution of the Bradford reagent by 2.5 fold using the deionized water. Aliquot of 50 µL of every concentration of BSA was added to 96-well microplate in triplicate followed by addition of 50 µL of deionized water to every well contain BSA. In the next step, there was the addition of 100 µL of the unknown proteins (casein protein 2%) in triplicates to a different well of 96 micro-plate. This was then followed by the addition of 100 µL of diluted Bradford reagent into all of the wells to make a total volume of 200 µL/well. Control well was made of 200 µL of deionized water. Based on the findings by Ernst and Zor (2010), the absorbance was measured at 450 nm and 595 nm after 10 to 30 minutes. The calibration graph was then prepared by dividing the final absorbance values. The last step involved the calculation of the values of the unknown proteins through the application of the linear equation calibration curve.

2.6. Effect of food ingredients on the efficacy of EO nanoemulsions

The effect of food ingredients on the antimicrobial efficacy of Eos was conducted as described by Gutierrez, Barry-Ryan et al. (2008) with some modification. Model media was prepared of the following: casein from bovine milk, technical grade (0, 2, 1, or 0.5%, Sigma-Aldrich Ireland Ltd) in DI water.

The application of flat bottom 96-well, as described by Sultanbawa, Cusack et al. (2009),The proceduare involved a culture of *L. monocytogenes*, that had been allowed to grow, overnight in BHI medium to 15 mL test tube containing 10 mL of BHI broth to obtain a concentration 1×10^{8} CFU/mL,then the bacterial suspension was diluted by transferring 10µL of overnight to a 15 mL test tube containing 10 mL of BHI broth to obtain a 1×10^{6} CFU/mL.A50-µL aliquots of BHI broth was dispensed into each well of a 96-microtiter plate The stock solution of each essential oil at 15×10^{3} µg/mL was diluted to produce 23.7 µg/mL, followed with the addition of the aliquots of Eos (150µL) from 23.7 µg/mL in the first row of the 96-well plate. Thereafter, the production of 0.75 fold dilutions of EOs was done through transferring 150 µL of the EOS in the first row wells into the second row. The process was repeated to produce eight concentrations which ranged from 17.8 µg/mL to 2.4 µg/mL (Thapa, Losa et al. 2012). To achieve a total volume of 100 µL in each well, 50 µL aliquots of bacterial dilutions comprising 1×10^{6} CFU/mL were introduced to all wells of the micro-titer plates. The last two wells were made to serve as a negative (containing EO solutions and sterile growth media only) and positive (containing growth media inoculated only with a bacterial dilution) control respectively. To avoid evaporation, a perforated plate seal (TREK Diagnostic System Inc. Cleveland, OH) was used to cover the 96-well micro-plates and incubated at 37^{o}C. The

absorbency state of the essential oil was determined at 590 nm using a microplate reader spectrophotometer (Biotek Instrument Inc., USA) every 30 min over a 24-hr incubation period.

2.7. Modeling Microbial Growth and Determination of Kinetic Parameters

The modelling process was done as described by Belda-Galbis, Leufvén et al. (2014) and incorporation of some modifications. To determine the kinetic parameters, the growth rate of the L. monocytogenes were recorded. The data collected after measuring the optical density were then transferred into counts given as $\log_{10}$ CFU/mL and the resulting data were then fitted onto a Gompertz model.

$$\log_{10} N_t = A + CXe^{-BX(t=M)}$$

(1)

In the above equation, N_t is the number of microorganisms at a given time (t), A represent the natural logarithm of the initial count, C is the difference between the final and the initial counts, B represent the growth rate when t=M while M is the time taken to reach the maximum growth rate (μmax).

The application of the Levenberg-Marquardt algorithm enhanced fitting the data to a nonlinear regression model. This regression model facilitated the determination of the parametric values by reducing the residual sum of squares as described by Zwietering, Jongenburger et al. (1990). The λ and μmax produced by the Listeria monocytogenes under each experiment was determined using the equation 2 and 3 respectively as shown below.

$$\lambda = M - \left(\frac{1}{B}\right) + \frac{\log_{10} N_0 - A}{\mu_{\max}}$$

(2)

$$\mu_{\max} = \frac{B \times C}{e}$$

(3)

The fitting of the data were made possible through the application of OriginLab statistical software.

3. Results

3.1. Fabrication of essential oil nanoemulsions

Table 1 shows D10, D50 and D90 values for 8 essential oil nanoemulsions after ultrasound-assisted size reduction. D10, D50 and D90 values indicate diameters below which 10, 50 and 90 percent of the droplets in an emulsion lie. These values represent the size distributions of each emulsions measured by a dynamic light scattering particle analyzer. All the essential oil resulted in very similar size range between ca. 59 nm and 146 nm. The average droplet size of all the essential oil emulsions was ca. 86.7 nm, which was within the desired nano emulsion range. Cedar wood essential oil resulted in the largest droplet sizes (D50=107.1 nm) in the emulsion after ultrasonication compared to the others, whereas ylang ylang essential oil showed the smallest size (D50=73.8 nm). In addition, all the essential oil showed narrow polydispersity, indicating droplets within an emulsion had been broken down evenly through our size reduction process. The small droplet size as well as narrow polydispersity of an emulsion are critical in terms of the stability of an emulsion, since flocculation, coalescence, and creaming process can be

significantly delayed depending on these two factors. Also, compared to conventional emulsions, nanoscale emulsions have been reported to exert improved delivery of bioactive compounds, and thus functionalities such as antimicrobial activities (Donsì, Annunziata et al. 2012). Overall, even though there was a slight difference in the droplet size distribution between emulsions, the size distribution of all essential oil emulsions fell below the criteria for nanoemulsion, which allowed comparative study of the antimicrobial effect by each essential oil.

Table 4. [1]D10, D50 and D90 values of essential oil nanoemulsions after ultrasonication

EOs	D10 (nm)	D50 (nm)	D90 (nm)
Cedar Wood	85.0 ± 1.3	107.1 ± 1.4	145.9 ± 1.6
Copaiba	70.1 ± 5.0	85.0 ± 1.3	120.4 ± 8.1
Fir Needle	81.2 ± 0.2	96.0 ± 0.1	139.9 ± 7.7
Frankincense	73.9 ± 1.2	87.9 ± 1.7	124.5 ± 3.2
Egyptian geranium	65.7 ± 8.5	79.8 ± 7.0	115.1 ± 19.5
Nutmeg	65.6.7 ± 8.4	85.8 ± 5.9	116.9 ± 7.2
Valerian	62.3± 2.9	78.1 ± 3.8	113.6 ± 6.0
Ylang ylang	58.5 ± 2.2	73.8 ± 2.7	108.8±3.8

[1]D10, D50, and D90 values indicate diameters below which 10, 50 and 90 percent of the droplets in an emulsion lie.

3.2. Determining the Minimum Inhibitory Concentration (MIC) Of Eos

The MIC values for each EO are represented in Table 5 and Figure 9. Eight concentrations of each EO 2.4µg/mL, 3.2µg/mL, 4.2µg/mL, 5.6µg/mL,7.5µg/mL,10.0µg/mL, 13.3µg/mL, and 17.8µg/mL were tested against *L.monocytogenes* in the absence and the presence and of three different concentrations of the casein proteins, 17.59, 35.17 and 70.34 µg/ml.

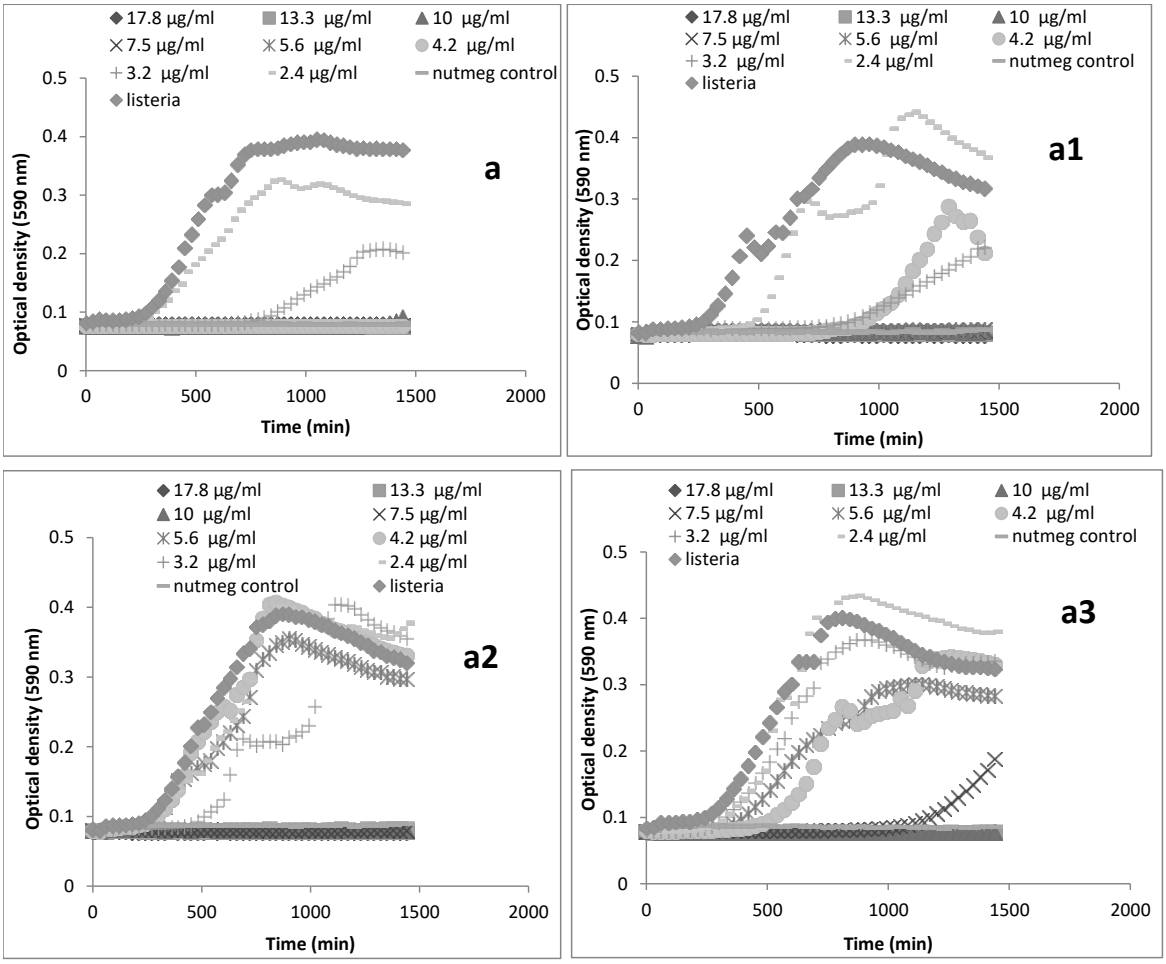
Frankincense, peppermint, ylang ylang ,nutmeg showed the most effective EO nanoemulsions against listeria, In the presence of 0 casein protein (control),the MIC of nutmeg was 4.9 µg/mL, whereas the MIC of frankincense, peppermint, ylang ylang was observed at 5.6µg/mL. Refer to Figure 9, at concentrations 5.6µg/ml, 7.5µg/ml, 10µg/ml, 13.3µg/ml, and 17.8µg/ml there was no observable growth indicating complete inhibition of *L. monocytogenes* grown in frankincense, peppermint, ylang ylang, nutmeg nanoemulsion containing 17.59 µg/mL, the MIC was 5.6µg/mL. The MIC values of EOs was increased with an increase in casein protein,when listeria was exposed to peppermint, ylang ylang, frankincense,and nutmeg nanoemulsions containing, 70.34 µg/ml of casein protein, the MIC values were determined as 10.0,10.0,10.0,and 8.75 µg/mL, respectively, as shown in Table 5**.**,the results indicate that nutmeg nanoemulsion showed the most effective EO against *L. monocytogenes* in the absence and in the presence of high concentration of protein.

The MIC of cedarwood and egyptian geranium, in the presence of 0 casein (control) , was determined at 7.5µg/mL,whereas the MIC of valerian and fire needle was 5.6 and 6.55 µg/ml, respectively .In EO nanoemulsions of cedar wood, egyptian geranium, fire needle, valerian containing 17.59µg/ml of casein, complete inhibition was evident in 7.5µg/ml, 10.0µg/ml, 13.3µg/ml, and 17.8µg/ml. Therefore, the MIC value was determined as 7.5µg/ml Figure 9 **.** The MIC of EOs was increased with an increase in casein protein concentrations, when *L. monocytogenes* were exposed to EO nanoemulsions containing 70.34µg/ml of casein protein ,the MIC of cedar wood, egyptian geranium ,fire needle, valerian was 10.0, 10.0,13.3,and 13.3µg/mL, respectively, indicate that presence of high concentration of protein contributed in

reducing the antimicrobial activity of EO nanoemulsions.The MIC values of copaiba in the present of (0, 17.59, 35.17, 70.34) µg/mL casein are 10.0, 10.0, 10.0, 13.3 µg/mL, respectively.

Table 5 MIC values of Eo nanoemulsions

oils	**Control**	**17.59 (µg/ml)casein**	**35.17 (µg/ml)casein**	**70.34 (µg/ml)casein**
Nutmeg	4.9 ±0.98	5.6 ± 0.00	7.5 ± 0.00	8.75 ± 1.76
Frankincense	5.6 ± 0.00	5.6 ± 0.00	8.75 ± 1.76	10.0 ± 0.00
YIang YIang	5.6 ± 0.00	5.6 ± 0.00	7.5 ± 0.00	10.0 ± 0.00
Peppermint	5.6 ± 0.00	5.6 ± 0.00	7.5 ± 0.00	10.0 ± 0.00
Egyptian geranium	7.5 ± 0.00	7.5 ± 0.00	7.5 ± 0.00	10.0 ± 0.00
Cedar wood	7.5 ± 0.00	7.5 ± 0.00	10.0 ± 0.00	10.0 ± 0.00
Valerian	5.6 ± 0.00	7.5 ± 0.00	10.0 ± 0.00	13.3 ± 0.00
Fire needle	6.55 ± 1.34	7.5 ± 0.00	10.0 ± 0.00	13.3 ± 0.00
Copaiba	10.0 ± 0.00	10.0 ± 0.00	10.0 ± 0.00	13.3 ± 0.00


17.8 µg/ml
13.3 µg/ml
10 µg/ml
7.5 µg/ml
5.6 µg/ml
4.2 µg/ml
3.2 µg/ml
2.4 µg/ml
nutmeg control
listeria
Optical density (590 nm)
Time (min)
a
a1
a2
a3

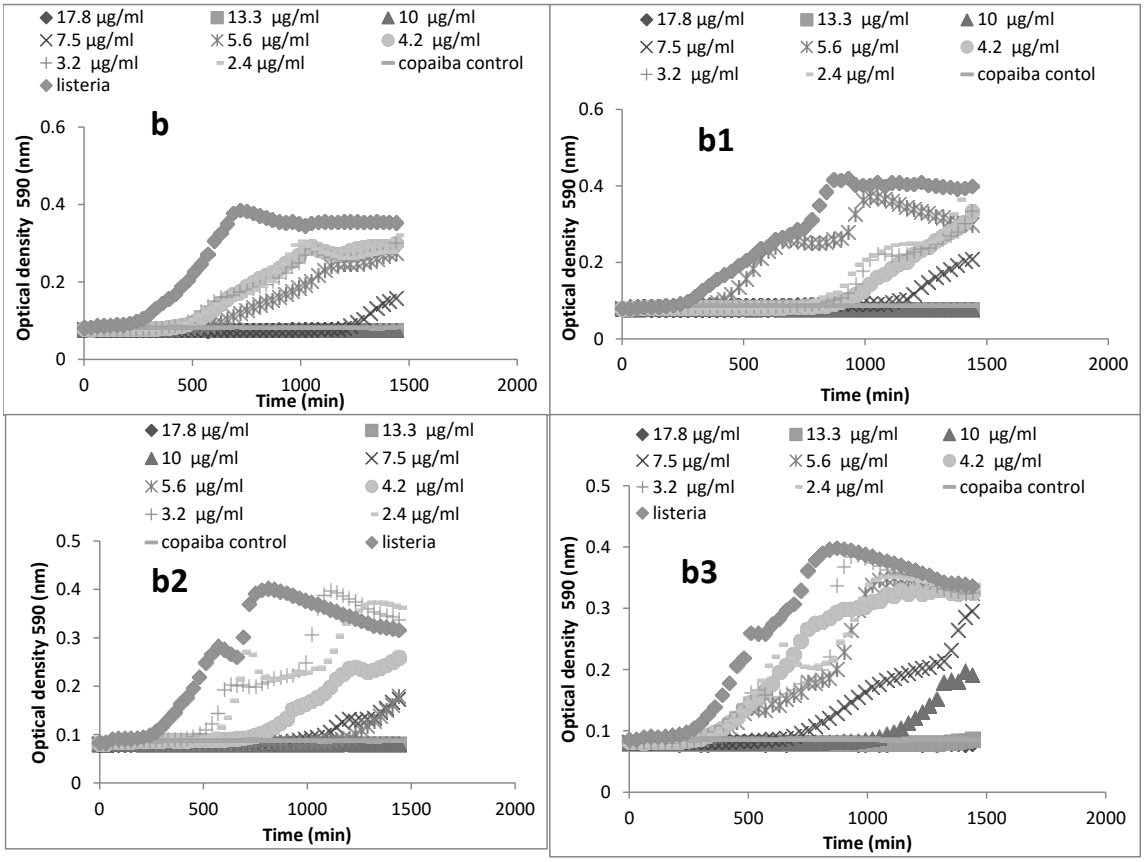


Figure 9 . Growth curves of *L.monocytogenes* and MIC values observed in EO nanoemulsions in the presence of 0,17.59,35.17,70.34 µg/mL casein protein, respectively of (a,a1,a2,a3) nutmeg and (b,b1,b2,b3) copaiba .

Microdilution assay was prepared to test different concentration of essential oils nanoemulsions against L.monocytogenes ,the absorbance was measured at 570 nm every 30 min over a 24 h incubation period, MIC was calculated . The experiment was repeated three times.

3.3. Quantitative modeling of EO nanoemulsions

Nine essential oils (EOs) were tested on the *L. monocytogenes* and analyzed using a Gompertz model to find the values of lambda (λ) and maximum growth rate (μmax).The ability of the essential oils used in the experiment to demonstrate inhibitory effects on the *L. monocytogenes* was obtained after assessing the various concentrations of the essential oils on the *L. monocytogenes*. Different essential oils can inhibit bacterial growth at different concentrations (Sadeghi, Mohammadi et al. 2016). The inhibitory state of the essential oils on the *L.monocytogenes* was therefore determined through evaluation of the values of the maximum growth rate (μmax) and lambda (λ) as utilized in the experiment.

Refer to Figure 10 and Figure 11, the lag phase (λ) values increased with an increase in the essential oils (EO) concentrations while the maximum growth rate (μmax) decreased with the increase in the essential oils (EO) concentrations. In that case, it is important to note the essential oils that have high maximum growth rate values (μmax) and low lag phase values (λ) are less effective against the *L. monocytogenes* while those that have low maximum growth rate values (μmax) and high lag values (λ) are more effective against the *L. monocytogenes*.

Based on the results, ylang ylang, nutmeg, frankincense showed high effectiveness against *L. monocytogenes* in both the absence and presence of different concentrations of casein protein. Refer to Table 7 and Figure 10, In the absence of casein protein at 2.4 μg/mL of ylang ylang, nutmeg, frankincense, the μmax values were 0.09, 0.14 and 0.17,respectively compared to control which was 0.16 ,whereas at 7.5 μg/ml of these

EOs, the µmax value was 0.00 compared to control which was 0.16 as shown in Table 9,indicating that µmax value decreased with an increase in EOs concentrations. λ values of *L.monocytogenes* in the present of ylang ylang, nutmeg, frankincense increased with the increase in EOs concentrations which were 6.38, 5.13, and 6.05 respectively at 2.4µg/mL. A complete inhibition being achieved at 5.6, 7.5, and 10 µg/ml compared to the control value being at 3.98 as shown in Table 6 , Table 8 , and Figure 11.

Even though the effectiveness of ylang ylang, nutmeg, frankincense decreased with the addition of the different concentrations of casein proteins, it still demonstrated high effectiveness against *L. monocytogenes* than most of the other essential oils. In the presence of 17.59 µg/ml of casein protein and at 2.4 µg/ml of ylang ylang, nutmeg, frankincense ,the µmax values of *L. monocytogenes* were 0.20, 0.25 ,and 0.14 at 2.4 µg/mL respectively higher than *L. monocytogenes* grown without adding casein protein as shown in Table **7**, while they inhibited completely the growth of *L. monocytogenes* at 7.5 µg/ml of these Eos compared to the control value was at 0.16 as shown in Table 9**,** indicating that the µmax value decreased with an increase in EOs concentrations .The λ values of *L. monocytogenes* in the present of yiang yiang, nutmeg, frankincense at 2.4 µg/ml were 9.23, 11.58,and 5.74, respectively compared to the control value being at 4.71 as shown in Table 6. YIang yIang, nutmeg, frankincense inhibited completely the growth of *L. monocytogenes* at 5.6, 7.5, and 10 µg/ml Table **8**.

Moreover, when *L. monocytogenes* grown in a medium containing ylang ylang, nutmeg, frankincense at 2.4 µg/ml and supplemented with 35.17 or 70.34 µg/ml of casein protein , the µmax values of *L. monocytogenes* increased by comparison with of those grown in a medium of 0 casein protein, which were 0.15, 0.15, and 0.16

respectively, whereas at a medium containing 70.34 µg/ml of protein, the µmax values were 0.25, 0.16, and 0.20, respectively compared to control which was 0.17 respectively as shown in Table 7,indicating that the higher concentrations of casein protein results in decreasing the antimicrobial activity of EOs thus increasing the µmax values by comparison with low concentrations of casein protein. The high concentrations of ylang ylang, nutmeg, frankincense (7.5 µg/ml), inhibited completely the growth of *L. monocytogenes* in the present of 35.17 µg/ml compared to control which was 0.15.At a medium containing 70.34 µg/ml of casein, the nutmeg oil nanoemulsion inhibited completely the growth of *L. monocytogenes* ,whereas the µmax values *L. monocytogenes* under ylang ylang, frankincense were 0.20, and 0.10, respectively compared to control values which were 0.17 as shown in Table 9,the results indicate that nutmeg was the most effective EOs against *L. monocytogenes* and inhibited the growth of *L. monocytogenes* at all its concentrations and even in the presence of higher concentration of casein protein..

Based on the results that shown in Table 6, *L. monocytogenes* grown in a medium contaning 2.4 µg/ml of ylang ylang, nutmeg, frankincense nanoemulsions supplemented with 35.17µg/ml of protein, the λ values of *L. monocytogenes* increased with an increase in EOs concentrations, which were 5.01 , 7.19, 4.47,respectively compared to control which was 4.16 , whereas in the presence of 70.34 µg/ml of protein, the λ values were 6.49, 5.13, and 5.57, respectively compared to, control which was 4.6, indicating that the presence of high concentration of casein protein play a role in reducing antimicrobial activity of EOs. As shown in Table 8, At 7.5 µg/ml of these EOs, a complete growth inhibition being achieved in the present of 35.17 µg/ml of protein .The nutmeg oil nanoemulsion at 7.5 µg/ml in the presence of 70.34 µg/ml casein proteins inhibited

completely the growth of *L. monocytogenes* ,whereas the λ values of ylang ylang, frankincense were 17.17 and 10.62 compared to control value which were 4.16 and 4.64 in the present of 35.17 and 70.34 µg/ml of casein respectively by comparison of a medium containing 0 casein protein as shown in Table 8, hence the presence of higher concentrations of casein proteins making the ylang ylang and frankincense less effective than nutmeg oil against *L.monocytogenes* as shown in Table 8 and Figure 11.

Cedar wood, valerian, fire needle, peppermint, Egyptian geranium demonstrated less inhibitory effect on the *L. monocytogenes* compared to the other essential oils, the µmax values of *L.monocytogenes* decreased, whereas the λ values increased with an increase in the Eos concentrations Figure 10 and Figure 11. The antimicrobial efficacy in the present of higher concentration of casein protein was reduced making these EO nanoemulsions less effective. Based on our results that shown in Figure 10 and Figure 11, copaiba showed the least antibacterial activity against the *L. monocytogenes* in the absence and in the present of the different concentrations of the casein protein. Overall, the results indicate that copaiba was the least effective EO nanoemulsion and nutmeg was the most effective Eos nanoemulsion against listeria .Also, the presence of high concentrations of casein protein resulted in reducing the antimicrobial activity of EO nanoemulsions, therefore the antimicrobial activity of EOs reduced in the presence of food components.

Table 6.Lag phase values of *L. monocytogenes* observed in EO nanoemulsions at 2.4 and 4.2 µg/mL in the presence of 0, 17.59, 35.17, 70.34 µg/mL casein protein, *lag phase is expressed in hours.

Eos con	**2.4 (µg/ml)**				**4.2 (µg/ml)**			
protein con / EOS	**0**	**17.59 (µg/ml)**	**35.17(µg/ml)**	**70.34(µg/ml)**	**0**	**17.59 (µg/ml)**	**35.17(µg/ml)**	**70.34(µg/ml)**
Yiang Yiang	6.38±1.51	9.23±1.84	5.01±1.13	6.49±1.44	15.50±0.83	17.76±4.18	5.43±1.50	7.12±0.03
Frankincense	6.05±0.95	5.74±0.63	4.47±0.46	5.57±1.45	14.17±3.65	4.66±0.00	5.69±1.64	5.06±1.50
Nutmeg	5.13±1.77	11.58±3.59	7.19±3.47	5.13±1.34	13.79±0.60	14.89±2.29	11.55±2.63	7.83±1.55
Cedar wood	6.19±0.23	7.33±0.32	8.73±0.08	5.03±0.21	13.43±3.24	10.29±4.21	7.90±0.15	5.98±1.05
Valerian	4.57±0.06	9.69±0.90	6.22±0.69	5.68±0.39	12.54±3.09	15.13±3.88	9.89±1.97	8.97±2.20
Fire needle	6.27±1.00	9.14±2.23	6.83±0.53	6.17±1.48	12.62±3.51	11.75±7.24	9.47±1.51	5.67±1.53
Peppermint	8.85±0.65	8.82±0.95	4.82±0.57	5.48±0.43	11.00±2.90	13.92±0.00	4.33±0.14	5.42±0.96
Egyptian geranium	5.34±0.35	8.44±1.76	7.92±0.92	5.11±1.16	7.69±1.97	15.86±0.00	11.54±1.65	6.12±0.73
Copaiba	5.95±1.11	9.14±6.82	9.06±1.82	5.22±0.45	8.74±1.56	13.60±3.88	10.31±1.55	5.14±1.84
CONTROL	3.98±0.01	4.71±1.21	4.16±0.35	4.64±0.7	3.98±0.01	4.71±1.21	4.16±0.35	4.64±0.7

Table 7 Maximum growth rate values of *L. monocytogenes* observed in EO nanoemulsions at 2.4 and 4.2 µg/mL in the presence of 0, 17.59, 35.17, and 70.34 µg/mL casein protein, *Maximum specific growth rate is expressed in hours.

Eos con	**2.4 (µg/ml)**				**4.2(µg/ml)**			
protein con EOS	**0**	**17.59 (µg/ml)**	**35.17(µg/ml)**	**70.34(µg/ml)**	**0**	**17.59 (µg/ml)**	**35.17(µg/ml)**	**70.34(µg/ml)**
Yiang Yiang	0.09±0.01	0.20±0.04	0.15±0.03	0.25±0.10	0.00±0.00	0.19±0.02	0.12±0.03	0.21±0.07
Frankincense	0.17±0.06	0.14±0.03	0.16±0.00	0.20±0.10	0.00±0.00	0.00±0.00	0.16±0.00	0.15±0.06
Nutmeg	0.14±0.02	0.25±0.08	0.15±0.03	0.16±0.06	0.03±0.04	0.00±0.00	0.04±0.06	0.16±0.00
Cedar wood	0.10±0.03	0.18±0.05	0.16±0.04	0.14±0.03	0.00±0.00	0.10±0.01	0.08±0.02	0.15±0.01
Valerian	0.14±0.02	0.11±0.00	0.13±0.00	0.18±0.09	0.05±0.08	0.14±0.05	0.17±0.01	0.12±0.09
Fire needle	0.13±0.02	0.23±0.02	0.19±0.07	0.22±0.06	0.12±0.00	0.15±0.01	0.16±0.00	0.17±0.06
Peppermint	0.10±0.00	0.22±0.07	0.17±0.06	0.15±0.00	0.04±0.06	0.00±0.00	0.11±0.01	0.15±0.02
Egyptian geranium	0.11±0.01	0.19±0.07	0.12±0.00	0.14±0.04	0.09±0.02	0.04±0.05	0.10±0.05	0.17±0.01
Copaiba	0.11±0.01	0.14±0.01	0.11±0.03	0.10±0.00	0.11±0.02	0.12±0.02	0.10±0.01	0.10±0.03
CONTROL	0.16±0.02	0.16±0.03	0.15±0.01	0.17±0.01	0.16±0.02	0.16±0.03	0.15±0.01	0.17±0.01

Data represents the means of experiment performed in duplicate and replicated at least twice.

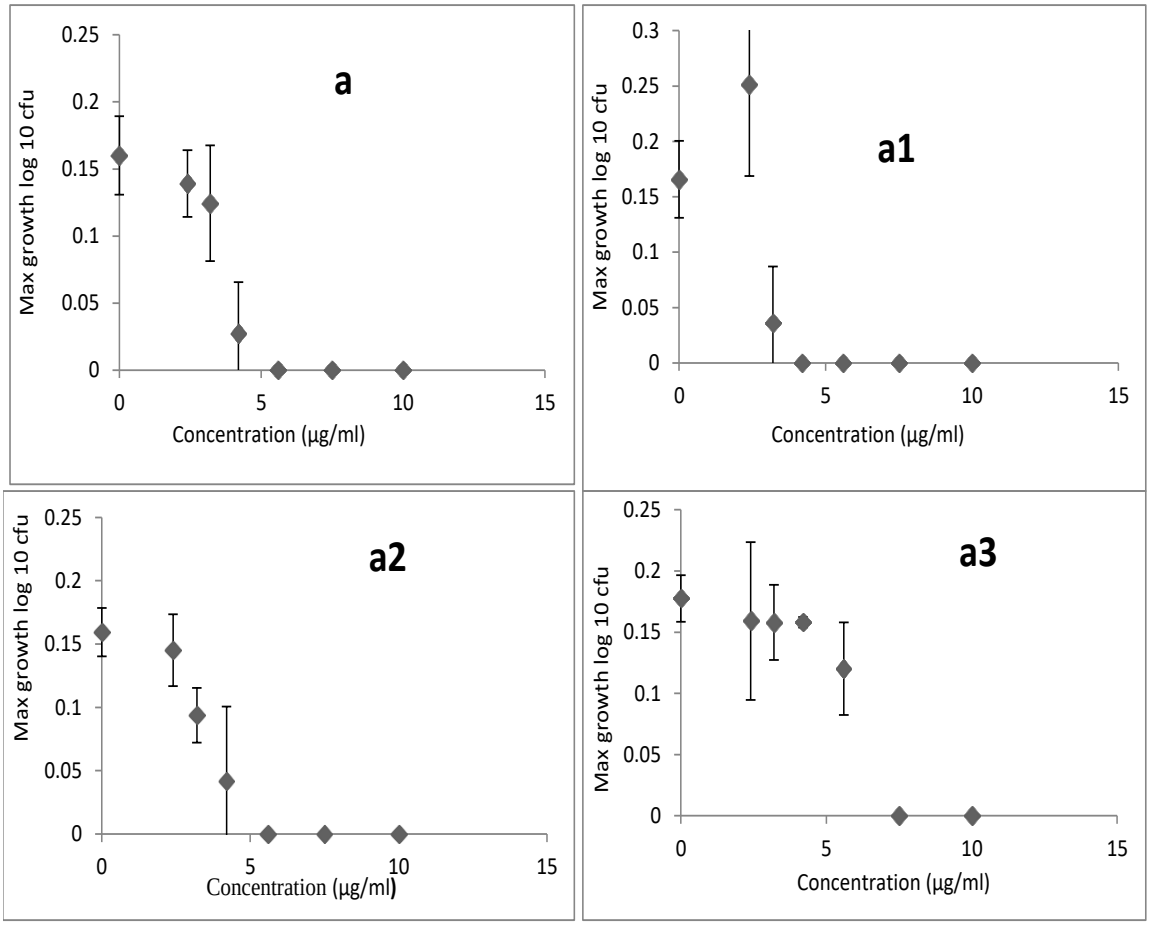
Table 8. Lag phase values of *L. monocytogenes* observed in EO nanoemulsions at 5.6 and 7.5 µg/ml *in the presence of* 0, 17.59, 35.17, 70.34 µg/mL casein protein, *lag phase is expressed in hours

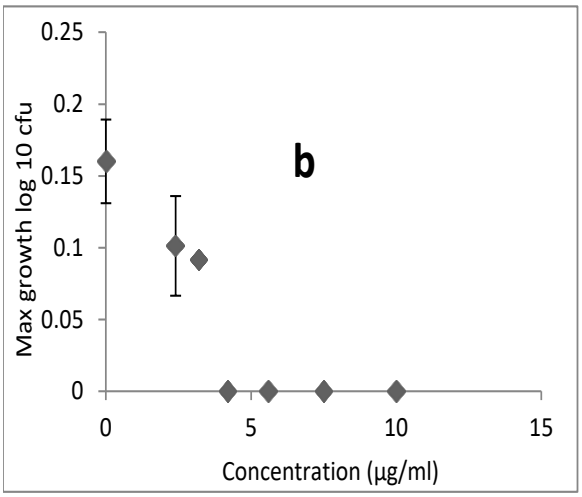
Eos con	**5.6 (µg/ml)**				**7.5 (µg/ml)**			
EOS protein con	**0**	**17.59 (µg/ml)**	**35.17(µg/ml)**	**70.34(µg/ml)**	**0**	**17.59 (µg/ml)**	**35.17(µg/ml)**	**70.34(µg/ml)**
Yiang Yiang	CI	CI	8.04±5.42	6.60±0.21	CI	CI	CI	17.17±8.74
Frankincense	CI	CI	5.83±3.10	7.19±0.23	CI	CI	CI	10.62±4.11
Nutmeg	CI	CI	9.38±6.55	11.148±6.22	CI	CI	CI	CI
Cedar wood	CI	CI	14.37±1.27	5.58±0.19	CI	CI	CI	8.37±0.83
Valerian	CI	CI	15.04±3.32	9.69±1.11	CI	CI	CI	13.17±4.19
Fire needle	CI	CI	15.33±3.05	7.04±0.70	CI	CI	18.75±0.19	17.43±4.28
Peppermint	CI	CI	5.50±0.10	6.63±0.35	CI	CI	CI	12.66±0.78
Egyptian geranium	CI	CI	CI	7.91±0.77	CI	CI	CI	10.56±1.49
Copaiba	11.48±3.09	17.23±0.00	16.15±1.86	7.21±1.55	CI	CI	17.69±2.98	10.73±1.13
CONTROL	3.98±0.01	4.71±1.21	4.16±0.35	4.64±0.7	3.98±0.01	4.71±1.21	4.16±0.35	4.64±0.7

*Data represents the means of experiment performed in duplicata and replicated at least twice, *CI, Completely inhibited.

Table 9. Maxiumum specific growth rate values of *L. monocytogenes* observed in EO nanoemulsions at 5.6 and 7.5 µg/ml *in the presence of* 0, 17.59,35.17,70.34 µg/mL casein protein, *Maximum specific growth rate is expressed in hours.

Eos con	**5.6 (µg/ml)**				**7.5(µg/ml)**			
protein con / EOS	**0**	**17.59 (µg/ml)**	**35.17(µg/ml)**	**70.34(µg/ml)**	**0**	**17.59 (µg/ml)**	**35.17(µg/ml)**	**70.34(µg/ml)**
Yiang Yiang	0.00±0.00	0.00±0.00	0.11±0.04	0.19±0.09	0.00±0.00	0.00±0.00	0.00±0.00	0.20±0.09
Frankincense	0.00±0.00	0.00±0.00	0.10±0.04	0.16±0.09	0.00±0.00	0.00±0.00	0.00±0.00	0.10±0.02
Nutmeg	0.00±0.00	0.00±0.00	0.00±0.00	0.12±0.04	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Cedar wood	0.00±0.00	0.00±0.00	0.09±0.01	0.14±0.02	0.00±0.00	0.00±0.00	0.00±0.00	0.11±0.02
Valerian	0.00±0.00	0.00±0.00	0.07±0.10	0.14±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.08±0.01
Fire needle	0.00±0.00	0.00±0.06	0.14±0.00	0.15±0.09	0.00±0.00	0.00±0.00	0.08±0.11	0.05±0.07
Peppermint	0.00±0.00	0.00±0.00	0.06±0.08	0.16±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.14±0.04
Egyptian geranium	0.00±0.00	0.00±0.00	ND*	0.15±0.02	0.00±0.00	0.00±0.00	0.01±0.00	0.00±0.05
Copaiba	0.09±0.00	0.00±0.02	0.12±0.00	0.10±0.02	0.00±0.00	0.00±0.00	0.04±0.05	0.09±0.01
CONTROL	0.16±0.02	0.16±0.03	0.15±0.01	0.17±0.01	0.16±0.02	0.16±0.03	0.15±0.01	0.17±0.01


a
Max growth log 10 cfu
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
Concentration (µg/ml)
a1
Max growth log 10 cfu
0.3
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
Concentration (µg/ml)
a2
Max growth log 10 cfu
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
Concentration (µg/ml)
a3
Max growth log 10 cfu
0.25
0.2
0.15
0.1
0.05
0
0
5
10
15
Concentration (µg/ml)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0.2
0.15
0.1
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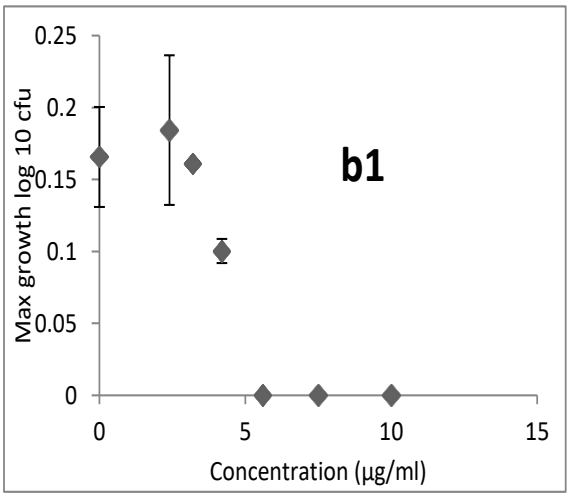
0
0
5
10
15
Concentration (µg/ml)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0.1
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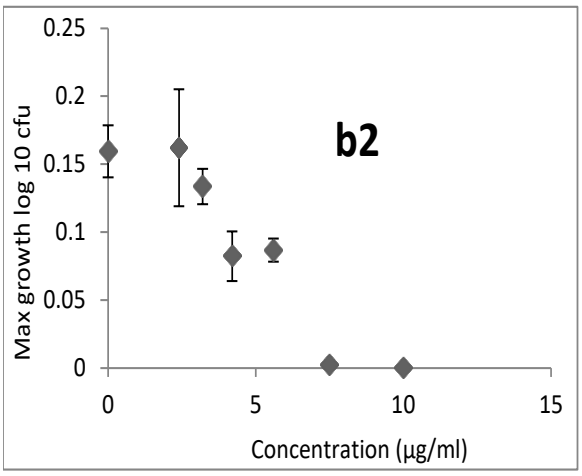
0
0
5
10
15
Concentration (µg/ml)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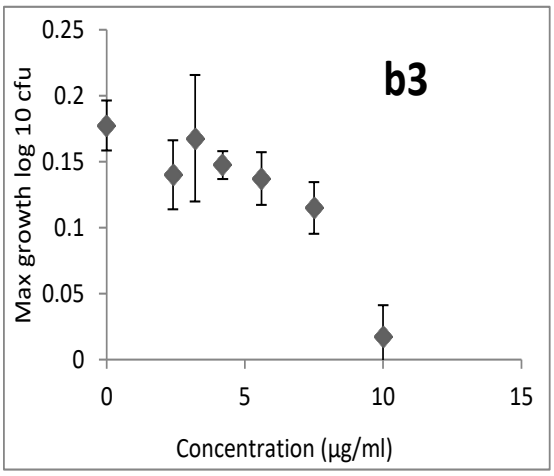
0.15
0.1
0.05
0
0
5
10
15
Concentration (µg/ml)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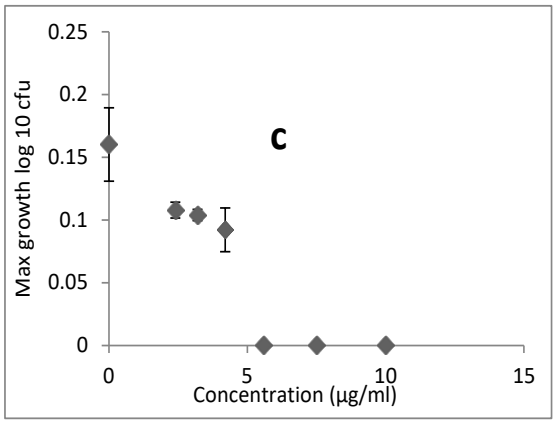
0.1
0.05
0
0
5
10
15
Concentration (µg/ml)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Max growth log 10 cfu
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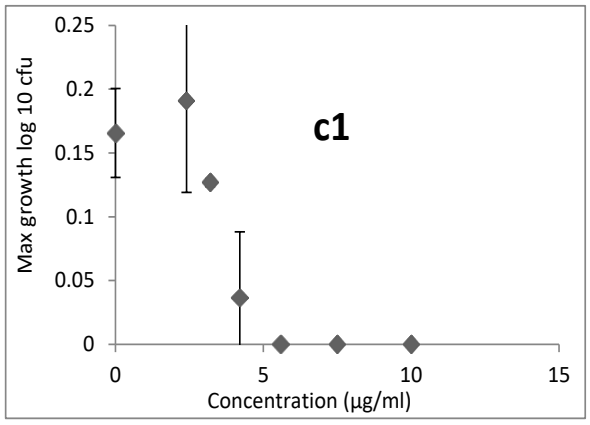
0.15
0.1
0.05
0
0
5
10
15
Concentration (µg/ml)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Max growth log 10 cfu
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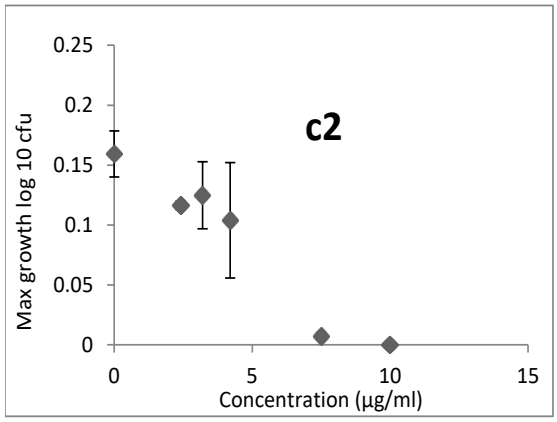
0.15
0.1
0.05
0
0
5
10
15
Concentration (µg/ml)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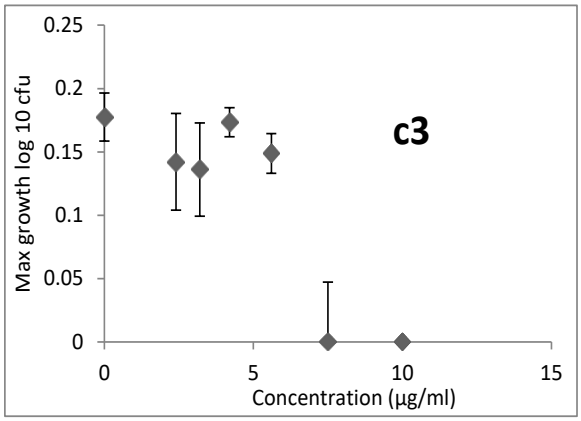
0.15
0.1
0.05
0
0
5
10
15
Concentration (µg/ml)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0
0
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10
15
Concentration (µg/ml)

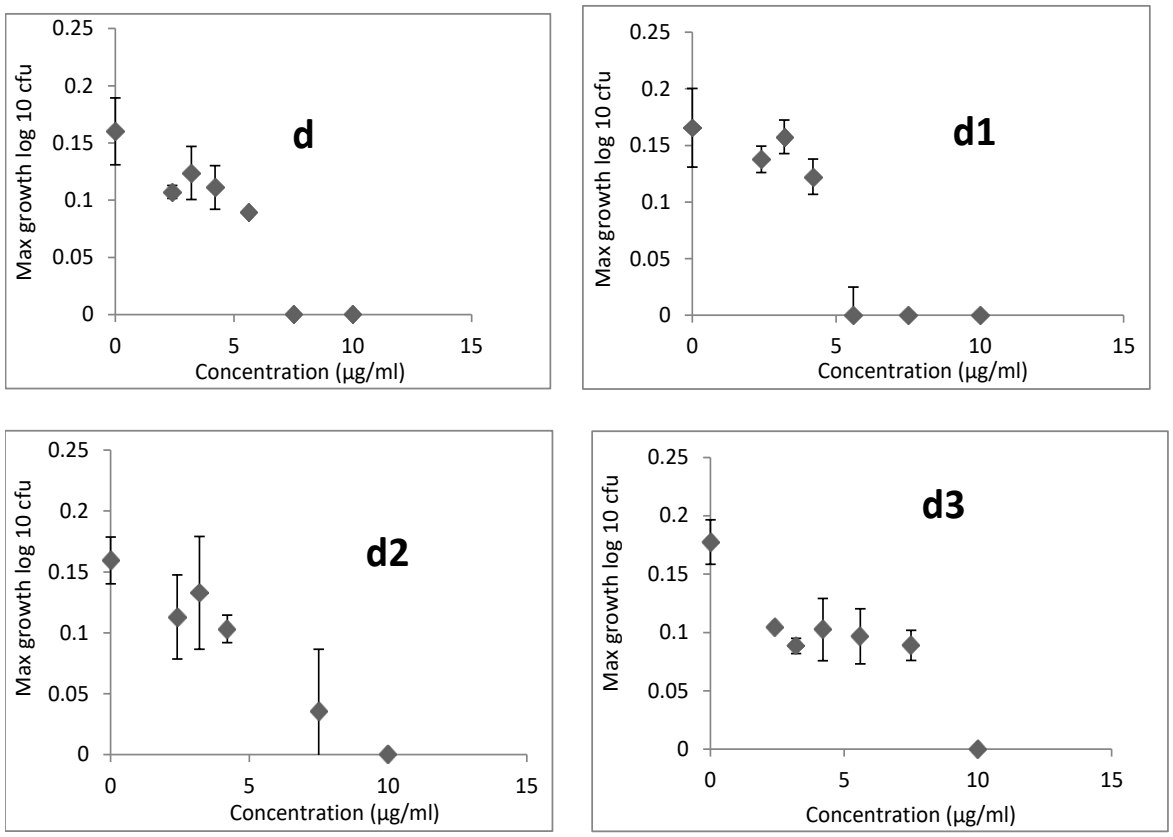
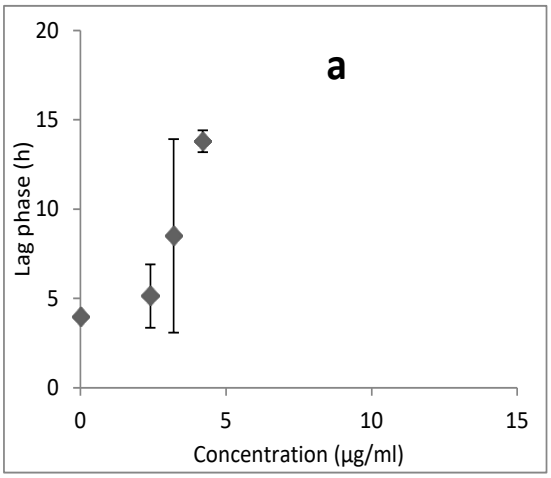


Figure 10. Maximum Specific growth of *L.monocytogenes* in the presence of 0, 17.59, 35.17, 70.34 of casein protein for nutmeg (a,a1,a2,a3), (b,b1,b2,b3) cedar wood, (c,c1,c2,c3) Egyptian geranium,(d,d1,d2,d3)copaiba


a
Lag phase (h)
Concentration (µg/ml)
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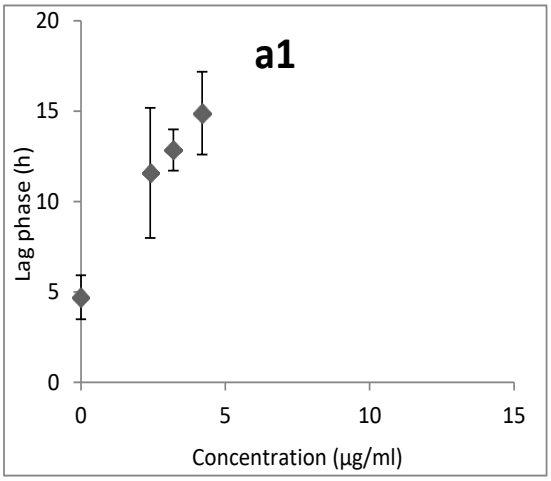
a1
Lag phase (h)
Concentration (µg/ml)
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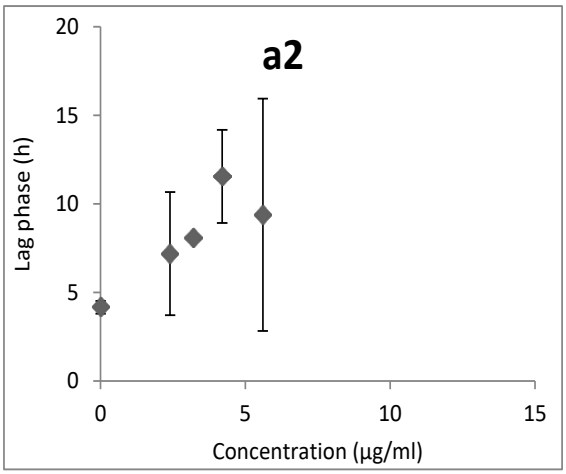
a2
Lag phase (h)
Concentration (µg/ml)
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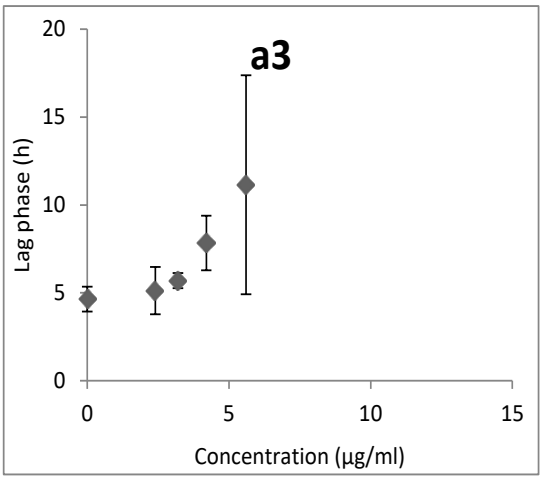
a3
Lag phase (h)
Concentration (µg/ml)
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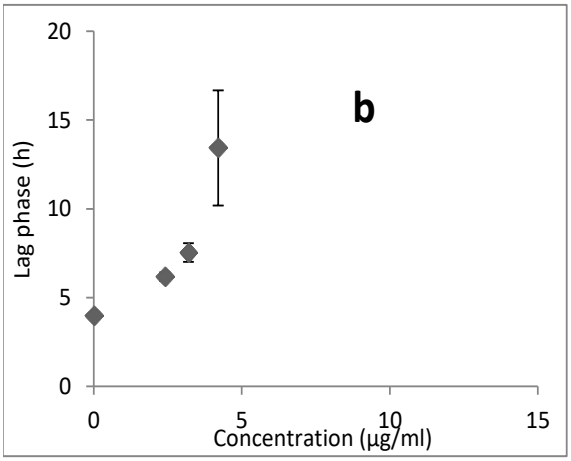
b
Lag phase (h)
Concentration (µg/ml)
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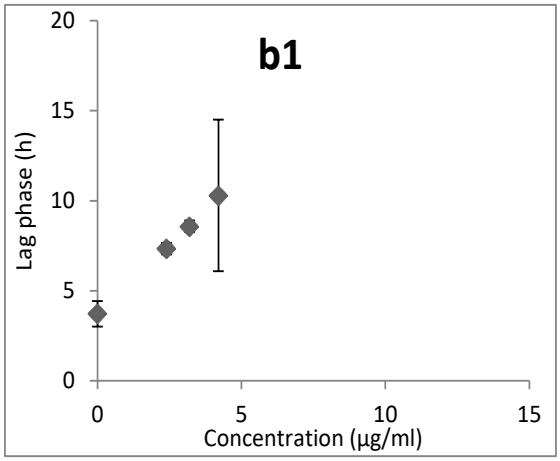
b1
Lag phase (h)
Concentration (µg/ml)
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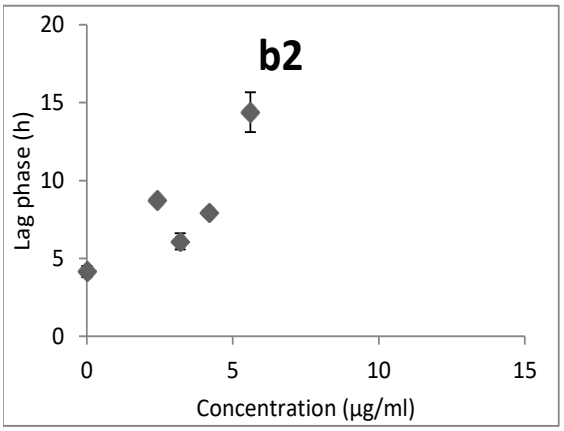
b2
Lag phase (h)
Concentration (µg/ml)
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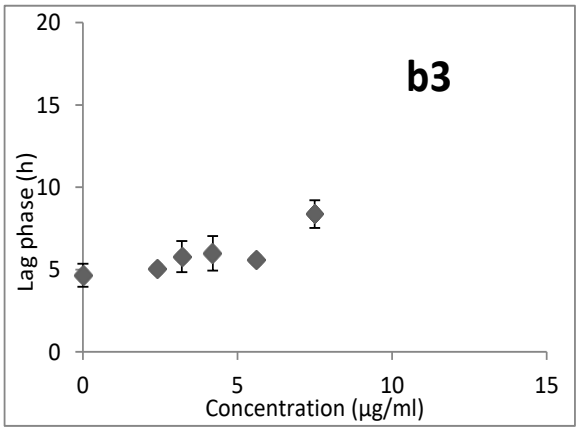
b3
Lag phase (h)
Concentration (µg/ml)
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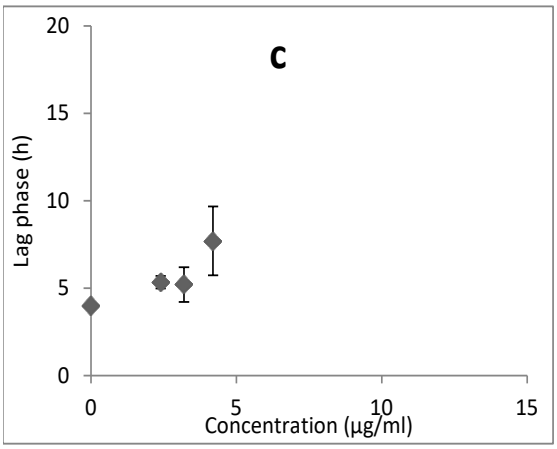
c
Lag phase (h)
Concentration (µg/ml)
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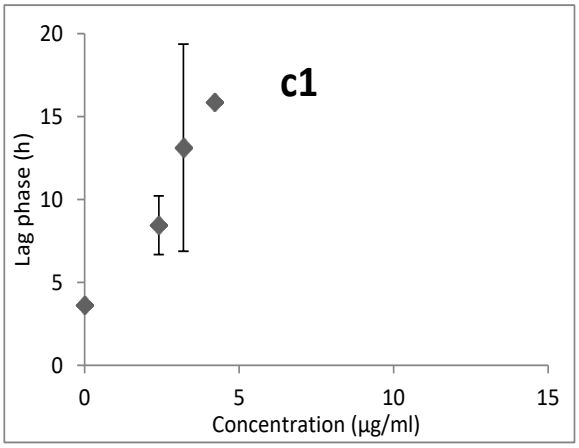
c1
Lag phase (h)
Concentration (µg/ml)
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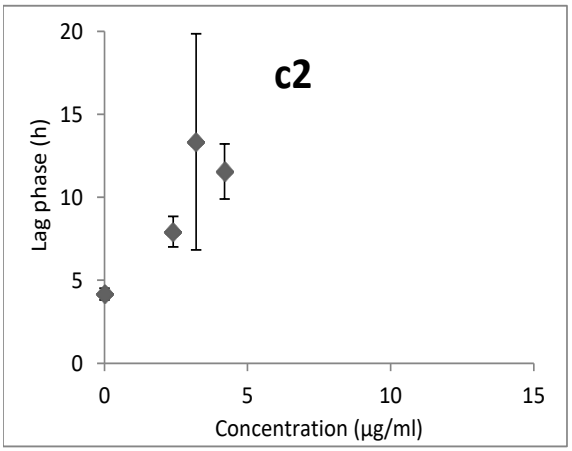
c2
Lag phase (h)
Concentration (µg/ml)
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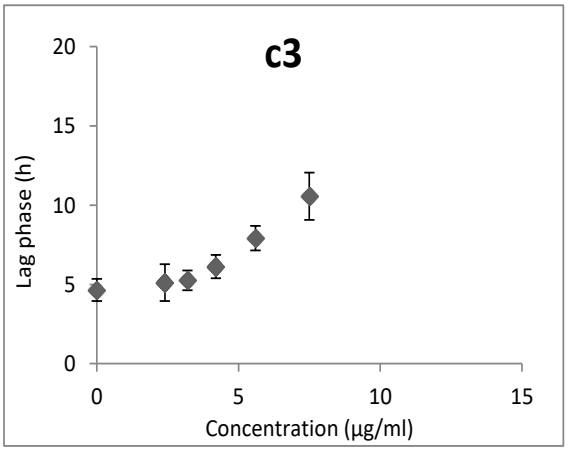
c3
Lag phase (h)
Concentration (µg/ml)
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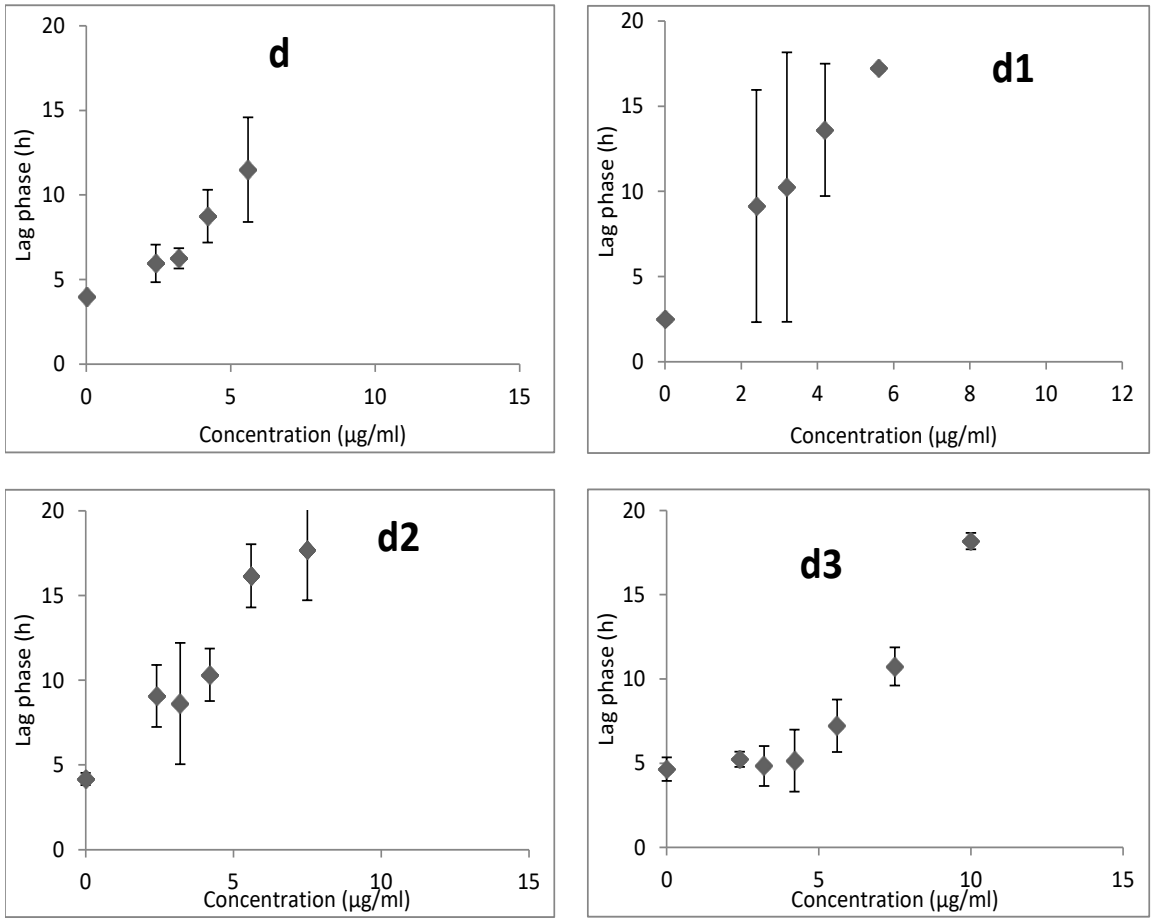

Figure 11. Lag phase values of *L.monocytogenes* in the presence of 0 , 17.59,35.17,70.34 casein protein respectively of (a,a1,a2,a3) nutmeg,(b,b1,b2,b3) cedar wood, (c,c1,c2,c3) Egyptian geranium,(d,d1,d2,d3)copaiba

4.4. Discussion

The antibacterial activity of the essential oils is based on their ability to interfere with the normal cellular activity of the bacteria hence leading to their complete destruction. According to the results that were obtained in the experiment, it was observed that there were two phases of bacterial growth which include; the lambda (λ) and the maximum growth phase (μmax). The increase in the lambda (λ) values of the *L. monocytogenes* was directed attributed to the increase in the concentrations of the essential oils. These results were in agreement with those obtained from the study conducted by Belda-Galbis et al. (2014),which study the antibacterial effects of the carvacrol essential oils.

According to Rolfe et al. (2012), the lag phase of the bacterial growth involves the equilibration process, which is regulated by different fact factors, which enables the bacterial cells to easily adapt and begin to utilize the resources which are provided for in the new environmental condition. In this case, the new environmental conditions were introduced following the addition of the essential oils and different concentrations of the casein proteins.

The steady increase in the lambda (λ) values of the *L. monocytogenes* was a clear indication that the essential oils had the ability to destroy the *L. monocytogenes*. According to the results obtained during the experiment, the lambda (λ) values varied from one essential oil to the other, a clear indication that the essential oils had different abilities to penetrate into the cellular contents of the *L. monocytogenes*.

The addition of the 17.59, 35.17 and 70.34 μg/ml concentrations of the essential oils significantly affected the ability of the different essential oils to destroy the *L.*

monocytogenes cells. Refer to **Figure 3** , higher values of the lambda (λ) were obtained in the complete absence of the casein proteins and presence of 17.59 µg/ml concentration of the casein proteins. Following the addition of the 35. 17 and 70.34 µg/ml concentrations of the casein proteins, there was further decrease in the effectiveness of the essential oils against the *L. monocytogenes*. This was a clear indication that the effectiveness of the essential oils against the *L. monocytogenes* increased on the addition of the 17.59 µg/ml concentration of the casein proteins while on the addition of high concentrations of the casein proteins, the effectiveness of the essential oils discriminately decreased.

The study results agree with the findings of the study conducted by Tserennadmid et al. (2010) on the antibacterial effects of the essential oils and their interactions with the bacterial cells in the presence of different types of food staffs. According to the study, it was determined that the foodstuffs which have high protein and fat contents have the ability to protect the bacteria which grow in them from the antibacterial effects of the essential oils and other natural antimicrobials.

The main factor which led to the decrease in the effectiveness of the activities of the essential oils against the *L. monocytogenes* can be linked to the fact that the food matrix, which in this case is the casein proteins, had the ability of creating the physical hurdles, which prevent maximum penetration of the essential oils into the cellular parts of the bacterial cells. This will make the essential oils less available to adequately act on the *L. monocytogenes* cells (Belda-Galbis et al., 2014).

The availability of conducive environment such as nutrients will facilitate the growth of bacteria cells. These nutrients should always be at an appropriate proportion

since they can have some negative effects on the bacterial growth when they are too low or too high as compared to the optimum level. According to the results that were obtained from the experiment, it was observed that the values of maximum growth rate (μmax) decreased with the increase in the concentration of the essential oils. This was a clear indication that the essential oils had inhibitory effects on the growth of the *L. monocytogenes* since they could interfere with the normal cellular functions of the *L.monocytogenes* hence leading to their eventual deaths.

These results are in agreement with the results obtained from the study conducted by Gutierrez et al. (2009) which indicated that different essential oils such as oregano, thyme, lemon balm, and marjoram developed inhibitory effects on the Listeria spp. through interfering with their cellular activities hence leading to a decrease in their cellular growth rates.

According to Nazzaro, Fratianni, De Martino, Coppola, and De Feo (2013), the essential oils are made up of a wide variety of secondary metabolites which play an important role in inhibiting and slowing down the bacterial growth. In that case, these essential oils have different modes of actions against the bacteria cells. Some of them target the cell membrane and cytoplasm while others would completely lead to change in the morphology of the bacterial cells. Another possible reason for the decrease in the rate of bacterial growth is the inhibition of the ATPase by the essential oils (Dubey & Sahu, 2014).

The rate of bacterial growth (μmax) was largely affected by the presence of different concentrations, 17.59, 35.17 and 70.34 μg/ml of casein proteins. According to the results obtained from the study that shown in **Figure 2** , the addition of 35.17 and

70.34 µg/ml, of the casein proteins led to increase rate of maximum growth than in the presence of the17.59 µg/ml of the casein proteins. It that case, it is justifiable to note that the lowest values of the *L. monocytogenes* maximum growth rates were observed in the presence of the 17.59 µg/ml concentration of the casein proteins.

This was a clear indication that the inhibitory effects of the essential oils against the *L. monocytogenes* decreased with the increase in the concentrations of the casein proteins. These results were in agreement with the results obtained from the study conducted by Volštátová, Havlík, Doskočil, Geigerová, and Rada (2015) which indicated that high concentration of the casein proteins largely affected the adhesion of the lactobacilli cells. It is also important to note that if the attachment of the bacterial cells is inhibited, their cellular growth will also be inhibited.

According to Machado, Nogueira, Pereira, Sousa, and Batista (2014), when there is an excess concentration of the food matrices such as proteins, there would be the formation of the protective layer around the bacterial cells, the *L. monocytogenes* as for this experiment, hence preventing the essential oils to come into direct contact with the bacterial cells. On low concentrations, 17.59 µg/ml of casein proteins, the *L. monocytogenes* were able to use up these proteins as a source of food, while at the same time, taking up the essential oils into their cellular systems hence leading to their destructions.

According to Vipra et al. (2013), the minimum inhibitory concentration (MIC) is the concentration at which the antibacterial agents such as the essential oils completely show their inhibitory effects against the growth of other organisms such as the bacterial growth. The essential oils do always represent a rich and unique resource of anti-bacterial

effects, which can be used in the encountering of the growing worldwide problems of bacterial resistance. The effectiveness of the antibacterial activity of the essential oil is directly related to its MIC value, which is a key factor in inhibiting the overall growth of the bacteria.

According to the results which were obtained from the experiment, it would be justifiable to note that the *L. monocytogenes* treated with different essential oils which were used in the experiment showed different lag phase (λ) and maximum growth rates (μmax) both in the absence and presence of different concentrations, 17.59, 35.17 and 70.34 μg/ml of the casein proteins. For example, an increase in the concentration of the essential oils led to the increase in the lambda (λ) values and a decrease in the maximum growth rate (μmax) of the *L. monocytogenes*.

Based on the fact that the addition of different concentrations, such as 17.59, 35.17 and 70.34 μg/ml, of the casein proteins, affected the lambda (λ) and maximum growth rates (μmax) of the *L. monocytogenes*, the minimum inhibitory concentrations of the different essential oils which were used in the experiment were also affected Figure **1**,**Figure 2, Figure 3**. Taking into consideration of the fact that the effectiveness of the essential oils against the *L.monocytogenes* bacteria were largely affected by the addition of the increasing concentrations of the casein proteins, it can be said that these concentrations of the casein proteins, 35.17 and 70.34 μg/ml, led to the increase in time taken for the essential oils to get into contact with the *L. monocytogenes*.

These results are in agreement with the findings from the study which was conducted by Mehrorosh et al. (2014) ,which indicated that the physical barriers which may exist between the essential oils and the target bacterial cells have negative effects on

the essential oils which are used in the destruction of the bacterial cells. This is because much of the concentration of the essential oils will be required to enable the essential oils to gain access to the cellular contents of the bacterial cells. The lowest minimum inhibitory concentration of the essential oils which were used in the experiment was obtained in the presence of 17.59 µg/ml concentration of the casein proteins than in the presence of any other, 35.17 and 70.34 µg/ml, and the concentration of the casein proteins Figure **1**.

This was based on the fact that on the addition of 17.59 µg/ml concentration of the casein proteins much of the essential oils were taken up by the *L. monocytogenes* cells while mixed with that concentration of the casein proteins. This reduced the time taken by the essential oils to come into contact with the *L. monocytogenes* bacterial cells. On the addition of the higher concentrations of the casein proteins, much time was needed by the essential oils to gain entry into the *L. monocytogenes* cells since these concentrations of proteins formed a physical barrier between the *L. monocytogenes* and the essential oils.

4.5. Conclusion

The increase in the concentration of the essential oils led to the increase lambda (λ) values and a decrease in the maximum growth phase (µmax) values. This was a clear indication that the essential oils were effective against the *L.monocytogenes*. The effectiveness of the essential oils against the bacterial cells depended on the ability of the essential oils to come into contact with the bacterial cells. On addition of the different concentrations of the casein proteins, 17.59, 35.17 and 70.34 µg/ml, the effectiveness of the essential oils against the *L. monocytogenes* was largely affected accordingly. In the

presence of the 17.59 µg/ml, the effectiveness of the essential oils against the *L.monocytogenes* increased accordingly.

This was based on that fact that the *L.monocytogenes* were able to take up the essential oils, which was dissolved in the 17.59 µg/ml of the casein proteins, hence leading to continuous direct contact between the *L. monocytogenes* cells and the essential oils. The effectiveness of the essential oils against the *Listeria monocytogenes* decrease on the addition of 35.17 and 70.34 µg/ml of casein proteins. This was based on the fact that the excess concentration of the casein proteins led to the formation of a physical barrier that prevents direct contact between the essential oils and the *L. monocytogenes* cells.

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Chapter V Recommendation and suggestion for future studies

The experiment only examined the effectiveness of individual essential oils against the *L. monocytogenes*, both in the absence of food matrix and in the presence of Different concentration of the casein proteins. To improve on the effectiveness of this study, a future research study about this experiment should, therefore, work on determining the effectiveness of a combination of two or more essential oils against the *Listeria monocytogenes* in the presence and absence of different concentration of casein proteins and also some other proteins such as soy protein and whey protein can be tested to see their activity on EO nanoemulsions.Also this study could be applied on different food matrix such as fiber,sodium chloride,sunflower oil,safflower oil.Finally, we can applied EO nanoemulsions against bacterial biofilm such *L.monocytogenes* and other food borne pathogen.