

FORMULATION, CHARACTERIZATION AND ANTIMICROBIAL EFFECT OF
CINNAMON OIL NANOEMULSIONS

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ABSTRACT

FORMULATION, CHARACTERIZATION AND ANTIMICROBIAL EFFECT OF CINNAMON OIL NANOEMULSIONS

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Activities of essential oils, such as antimicrobial, antioxidant and anticancer have been recognized for decades. To utilize the functionality of these materials in foods as natural additives instead of synthetic active materials, oil-in-water (O/W) nanoemulsions that are obtained by low and high-energy methods have been intensively explored in recent years. The purpose of this study was to formulate stable cinnamon oil nanoemulsions (NEs) having higher antimicrobial activity by using the low-energy approach: spontaneous emulsification (SE) method. To prepare the nanoemulsions, oil phase containing cinnamon oil (CO) and the carrier oil (coconut oil (CNO)) at various concentrations and surfactant (Tween 80) was titrated into an aqueous phase (distilled water) that was being stirred continuously. For antimicrobial activity, agar disc diffusion method with *E.coli* as the model microorganism was used. NEs were characterized by Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) techniques. Both DLS and TEM gave parallel results and mean particle size of the most stable NEs were found as ~100 nm for 6:4 (CO: CNO) oil phase composition. Effect of SE on antimicrobial activity was compared with cinnamon oil solutions that included same concentrations of CO and results showed

that antimicrobial activity increased 10-25% on the NEs prepared by spontaneous emulsification. NEs were also prepared by using two high energy homogenization methods: microfluidization and ultrasonication. When spontaneous emulsification, microfluidization and ultrasonication were compared at same CO%, at higher CO concentrations (6%, 8% and 10%), results were not significantly different for particle sizes. Moreover, for all CO concentrations, antimicrobial activity could not be enhanced by microfluidization towards *E. coli*. However, at higher CO concentrations (8% and 10%), enhanced antimicrobial activity were obtained by spontaneous emulsification and ultrasonication methods, and these results were not found to be significantly different ($p>0.05$). This study revealed that CNO could successfully be utilized as a carrier oil for preparing nanoemulsions and stable cinnamon oil nanoemulsions could be prepared at lower surfactant concentrations by the spontaneous emulsification method.

Keywords: Cinnamon oil, coconut oil, nanoemulsion, spontaneous emulsification, antimicrobial activity

ÖZ

TARÇIN YAĞI İÇEREN NANOEMÜLSİYONLARIN, FORMÜLASYONU, KARAKTERİZASYONU VE ANTİMİKROBİYAL ETKİSİ

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Esansiyel yağların antimikrobiyal, antioksidan ve antikanser gibi aktivitelere sahip olduğu uzun yıllardır bilinmektedir. Gıdalar içerisinde sentetik aktif maddelerin kullanımı yerine, doğal katkı maddeleri olarak esansiyel yağların bu özelliklerinden faydalanılması için, son yıllarda Y/S (su içinde yağ) emülsiyonlarının düşük enerjili ve yüksek enerjili metotlar ile elde edilmesi üzerine araştırmalar yoğun bir biçimde yürütülmektedir. Bu çalışmanın amacı, düşük enerjili metotlardan kendiliğinden emülsiyon oluşturma tekniği ile antimikrobiyal etkiye sahip tarçın yağı içeren dayanıklı nanoemülsiyonlar oluşturmaktır. Bu yöntemde; farklı konsantrasyonlarda tarçın yağı ve hindistan cevizi (taşıyıcı yağ) ve ayrıca sürfektan (Tween 80) içeren yağ fazı, su fazı içerisine titre edilerek manyetik karıştırıcıyla karıştırılmıştır. Antimikrobiyal aktiviteyi tespit etmek için, *E. coli* model organizma olarak seçilmiş, agar disk difüzyon metodu uygulanmıştır. Nanoemülsiyonların fiziksel karakterizasyonu için Dinamik Işık Saçılımı tekniği ve Transmisyon Elektron Mikroskobu teknikleri kullanılmıştır. Bu iki karakterizasyon yöntemi, 6:4 tarçın yağı:

hindistan cevizi yağı kombinasyonu içeren ve yüksek stabilite gösteren nanoemülsiyonlar için paralel sonuçlar vermiş, ortalama parçacık boyutu ~ 100 nm olarak saptanmıştır. Kendiliğinden emülsiyon oluşturma tekniği ile elde edilmiş nanoemülsiyonların antimikrobiyal etkisi, aynı konsantrasyonlarda tarçın yağı içeren solüsyonlar ile karşılaştırılmış, antimikrobiyal aktivitenin kendiliğinden emülsiyon oluşturma tekniği ile 10-25% arttığı gösterilmiştir. Nanoemülsiyonlar ayrıca yüksek enerjili metotlardan mikrofludizasyon ve ultrasonikasyon kullanılarak da hazırlanmıştır. Aynı tarçın yağı konsantrasyonlarında kendiliğinden emülsiyon oluşturma, mikrofludizasyon ve ultrasonikasyon ile elde edilmiş nanoemülsiyonlar karşılaştırıldığında, yüksek tarçın yağı konsantrasyonlarında (6%, 8%, 10%) parçacık boyutu için anlamlı bir farklılık elde edilememiştir. Ayrıca, mikrofludizasyon ile elde edilen nanoemülsiyonlarda *E. coli* ye karşı antimikrobiyal etki artırılmamıştır. Fakat, kendiliğinden emülsiyon oluşturma ve ultrasonikasyon metotlarıyla yüksek tarçın yağı konsantrasyonlarında (8%, 10%) artan antimikrobiyal etki elde edilmiş ve bu sonuçlar anlamlı bir farklılık göstermemiştir ($p>0.05$). Bu çalışma, hindistan cevizi yağının nanoemülsiyonların oluşturulmasında başarılı bir şekilde değerlendirilebileceğini ve kendiliğinden emülsiyon oluşturma metodu ile daha düşük konsantrasyonlarda sürfektan kullanılarak stabil nanoemülsiyonların hazırlanabileceğini göstermiştir.

Anahtar kelimeler: Tarçın yağı, hindistan cevizi yağı, nanoemülsiyon, kendiliğinden emülsiyon oluşturma, antimikrobiyal aktivite

To my family ...

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CHAPTER 1

INTRODUCTION

Food grade delivery systems have an important place to embed lipophilic functional components into food and beverages. These functional components can be flavors, colors, micro nutrients, antimicrobials etc. Different colloid based delivery systems such as emulsions, nanoemulsions and microemulsions are most particularly suitable for this purpose. Compositional, physicochemical and thermodynamic stability differences of these delivery systems enable the formation of colloidal systems which have different functional performances.

1.1 Nanoemulsion

Nanotechnology in food field leads to formation of great number of new products, while modifying the macroscale characteristics of foods such as texture, taste, processability, stability. Nanoemulsion technology forms an encapsulating system for functional materials thus degradation can be prevented and bioavailability of compounds are increased (Silva, Cerqueira, & Vicente, 2012). Nanoemulsions usually contains two immiscible liquids. One of these liquids is dispersed as small spherical droplets and depending on the type of the continuous phase, nanoemulsions are classified as either oil-in-water (O/W) or water-in-oil (W/O) (**Fig. 1.1**) (McClements, 2012).

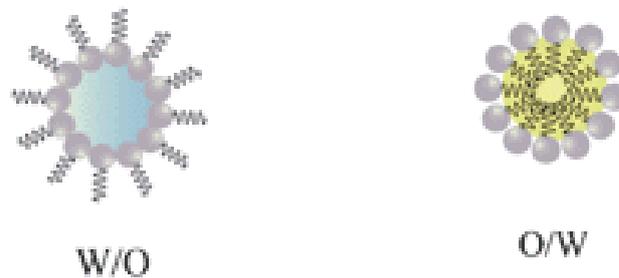


Figure 1.1 Oil and water micelles (McClements,2011)

Nanoemulsions are generally fabricated from oil phase (organic phase), aqueous phase, surfactant and possibly a co-surfactant (**Fig. 1.2**) (McClements, 2012).

Organic phase: Variety of nonpolar components such as triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids, flavor oils, essential oils, mineral oils, fat substitutes, waxes, oil-soluble vitamins, and lipophilic nutraceuticals (carotenoids, Co-enzyme Q) take part in the formulation of organic phase. Bulk physicochemical characteristics (polarity, water-solubility, interfacial tension, refractive index, viscosity, density, phase behavior, and chemical stability) of the oil phase affect the formation, stability and properties of nanoemulsions (McClements & Rao, 2011)

Aqueous phase: Primarily water constitutes the aqueous phase of nanoemulsions however variety of other chemical components can be present in the aqueous phase like acids, bases, minerals, proteins, carbohydrates and co- solvents. Formation, stability and physicochemical properties of nanoemulsions are directly affected from the composition, since the components affect the polarity, interfacial tension, refractive index, rheology, density, phase behavior, pH, and ionic strength of the aqueous phase (McClements & Rao, 2011).

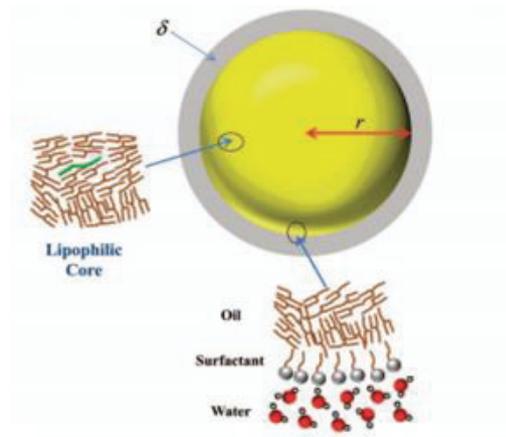


Figure 1.2 Schematic representation of core- shell structure of particles in O/W nanoemulsion system (adapted from McClements, 2012)

Particle size is one of the most important features of nanoemulsions. Nanoemulsions have droplet sizes ranging between 20 nm to 500 nm, however to differentiate nanoemulsions from conventional emulsions, in literature, there is no identified distinct size range (Conxita Solans & Solé, 2012). Stability, optical property and rheology of the nanoemulsion are mainly affected from the size of the droplets. Achieving a particle size in nanometer range with high uniformity (monodispersed system) is the main goal of nanoemulsion studies. A measure for the size distribution is known as the *Polydispersity Index* (PDI). When the PDI is smaller than 0.200, monodispersed size distribution is generally observed. If multiple size particles are involved in an emulsion, emulsions are defined as polydispersed (multimodal system). Large particles and large size distributions indicate the instability of the formulation. After formation of nanoemulsions, during storage some internal and external forces such as gravitational, thermal and interfacial stresses cause physical and chemical changes over time. Instability can come out of different forms such as creaming, sedimentation, flocculation, Ostwald ripening and coalescence (**Fig. 1.3**).

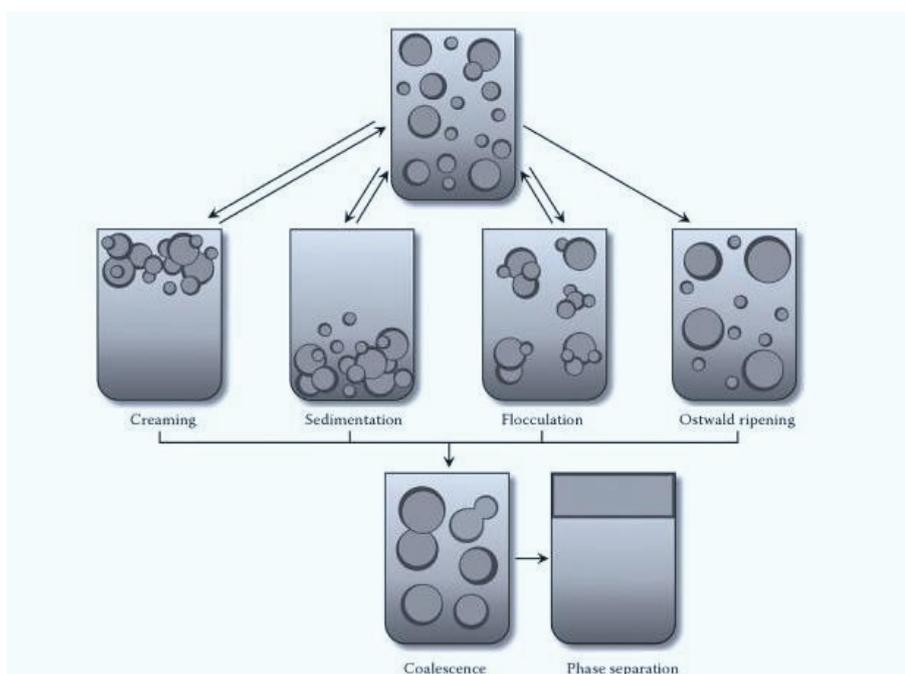


Figure 1.3 Destabilization mechanisms of nanoemulsions (adapted from Tiwari & Tiwari, 2013)

When the density of droplets is less than the continuous phase, droplets condensate at the nanoemulsions upper surface. This situation is known as creaming. In case of sedimentation, more dense droplets are pulled to the bottom of the nanoemulsions due to gravity. If reversible aggregation takes place, it is named as flocculation. When the dispersed phase is partly soluble in continuous phase, Ostwald Ripening (OR) occurs because small droplets which has high internal pressure, dispersed into continuous phase and due to the mass transport of dispersed material, larger particles form. Coalescence is the merging of small droplets into larger droplets and it results in complete phase separation (Tiwari & Tiwari, 2013).

Surface forces have effect on main interaction energies between emulsion droplets and thus on stabilization/destabilization mechanism. These forces are (**Table 1.1**):

Table 1.1 Significant forces in colloidal system (Kitchener & Mussellwhite, 1968)

Long Range Surface Forces	London- van der Waals attraction between particles
	Electrical double layer forces (Electrostatic and Steric Repulsion)
Short Range Surface Forces	Chemical bonding of molecules to surface groups (ionic, covalent or hydrogen bonds)
	Physical attachment of molecules (Dipole interaction)
	Hydrophobic bonding (Two non-hydrated species in aqueous environment)

It is known that short range surface forces have influence on the structure of the interface whereas long range forces determine whether an emulsion coagulates (Kitchener & Mussellwhite, 1968)

To prevent phase separation of an emulsion, surfactants are used. Surfactants enhance the stability of emulsion since they form a protective layer to prohibit aggregation. Surfactants reduce interfacial tension and Laplace pressure that occurs between oil and water molecules. Surfactant molecules include one or more functional groups that are not only strongly attracted to the bulk medium and also have little attraction with the medium. Surfactants can be considered under 3 big categories. These are protein, polysaccharide and small molecule surfactants (McClements, 2005):

1- Protein emulsifiers: Some food proteins such as soybean protein isolate, whey protein isolate, β -lactoglobulin are highly used in food industry as a surfactant because their nutritional values are high and generally recognized as safe (He et al., 2011). Proteins have amphiphilic characters and they stabilize emulsions via electrostatic repulsion, steric hindrance and generation of osmotic pressure at the outer surface of adsorbed protein molecules (Jones & McClements, 2010; McClements, 2005) .

2- Polysaccharide emulsifiers: Gum arabic (*Acacia senegal*), modified starches, modified celluloses, some kinds of pectin, and some galactomannans are the mostly used polysaccharide emulsifiers in food systems (Dickinson, 2009). Depending on their functional groups, polysaccharides are classified as anionic, cationic and nonionic. When monomer composition of polysaccharides are compared with proteins, polysaccharides are more uniform and this provide particular physicochemical and functional properties like solubility, viscosity enhancement, binding properties, gelation.

3- Small molecule surfactants: Small molecule surfactants have both hydrophilic (head group) and hydrophobic parts. These surfactants can be examined under 4 subgroup considering their hydrophilic functional groups in aqueous media. They are anionic, cationic, nonionic and zwitterionic (Chatterjee & Gupta, 2002).

- i. **Anionic surfactants:** These surfactants bear a negative charge. Anions of alkali metal salts of fatty acids, anions of long chain sulfonates, sulfates and phosphates are included in this group (Chatterjee & Gupta, 2002).
- ii. **Cationic surfactants:** These surfactants bear a positive charge. Generally consist of amine salts or ammonium and pyridinium compounds (Chatterjee & Gupta, 2002).

- iii. **Nonionic surfactants:** These surfactants commonly refers to polyoxyethylene compounds however sugar esters, amine oxides and fatty alkanolamides are also included in this group (Chatterjee & Gupta, 2002).
- iv. **Zwitterionic surfactants:** These surfactants have positive and negative charges in the hydrophilic part of the molecule. Lecithin (long chain phosphonyl cholines) belong to this group (Chatterjee & Gupta, 2002).

Kinetic stability of o/w emulsions usually increased by small molecule surfactants. To form nanoemulsions ; small molecule surfactants, proteins and polysaccharides can be used as surface active agents (McClements, 2012).

In this study, Tween 80 (Polysorbate 80) is used as a surfactant (**Fig.1.4**). Tween 80 has a single tail and is used in foods and pharmaceutical products commonly (Athas et al., 2014). Tween 80 is derived from polyethoxylated sorbitan and oleic acid. Polyethers (also known as polyoxyethylene group) is the hydrophilic group and oleic acid is lipophilic group.

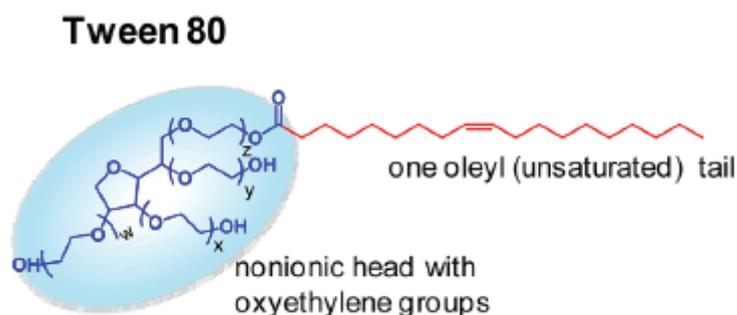


Figure 1.4 The molecular structure of Tween 80 (Hydrophobic tail in red and Hydrophilic head in blue)(Athas et al., 2014)

Nanoemulsions (NEs) are thermodynamically unstable but kinetically stable systems. To form a stable nanoemulsion, surfactant to oil ratio (SOR) and the amount of aqueous phase are very important factors. When preparation method, composition and component of the system is appropriately selected, nanoemulsions with high kinetic stability can be obtained. Without using a surfactant, nanoemulsion having a high kinetic stability is not ensured. Molar volume, geometry and polarity of the oil molecules; nature of the head and tail groups of the surfactant molecules and environmental conditions such as temperature, ionic strength, pH affects the maximum amount of oil incorporated in the system. If excess oil is added to the nanoemulsion system, micelles become saturated with oil and additional oil droplets cause to merging of oil droplets; in the same way, if the total amount of oil in the system is insufficient all of the oil molecules are solubilized and form surfactant micelles dispersed in aqueous phase. At this point, critical micelle concentration (CMC) of surfactant at which surfactant molecules start to form micelles, should be determined (McClements, 2012).

In aqueous media, surfactants are soluble to some degree and when surfactants reach a sufficient concentration, they aggregate to form micelles. Water is in contact with the hydrophilic head group of the surfactant and the hydrophobic region is buried in the inner part of the surfactant micelle. This concentration is called as CMC and to obtain CMC, change in surface tension as a function of surfactant concentration is plotted (**Fig.1.5**).

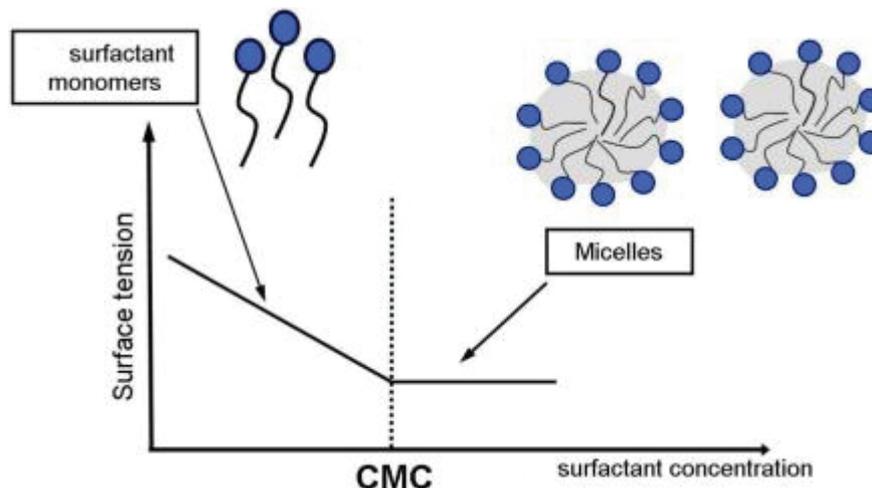


Figure 1.5 Schematic Diagram of the Critical Micelle Concentration (adapted from Pacwa-Płociniczak, Płaza, Piotrowska-Seget, & Cameotra, 2011)

To eliminate the problems based on surfactants, selection of the appropriate surfactant for the system is also essential. Surfactants which have high HLB (Hydrophilic-Lipophilic Balance) value (greater than 10) favors the formation of O/W nanoemulsions (Ghosh, Mukherjee, & Chandrasekaran, 2014). According to Bera et al. (Bera & Mandal, 2012), average diameter (Z- average) of O/W nanoemulsion particles decreases with increasing HLB value of non-ionic surfactants. Because of being more safe and biocompatible, non-ionic surfactants are preferred for food systems, drug delivery systems.. etc. and effects of factors such as pH and ionic strength are minimized (Chime, Kenechukwu, & Attama, 2014 ; Rao & McClements, 2011). Thermodynamic instability of nanoemulsions result from free energy of separate phases and colloidal dispersions. For nanoemulsions, free energy of separated phases is lower than the free energy of colloidal dispersion (**Fig.1.6**).

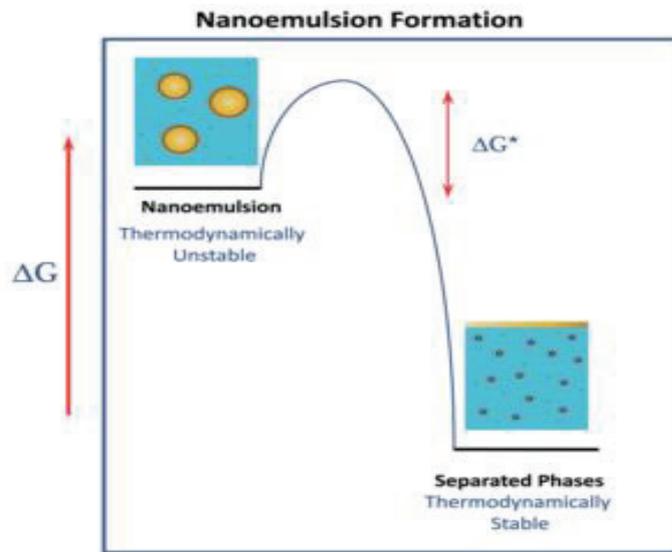


Figure 1.6 Schematic Diagram of the free energy of nanoemulsion systems (adapted from McClements, 2012)

The free energy of colloidal dispersion formation on droplet size is calculated using:

$$\Delta G_{\text{formation}} = \Delta G_I - T \Delta S_{\text{configuration}} \quad (\text{Eq. 1.1})$$

Equation 1.1 shows that nanoemulsions are thermodynamically unfavorable since systems always tend to have the lowest free energy. Here ΔG_I is interfacial free energy term and $T\Delta S_{\text{configuration}}$ is configuration entropy term. The interfacial free energy term opposes the formation of the colloidal dispersion. Due to increasing interfacial area with decreasing particle size, ΔG_I term is always positive. However, $T\Delta S_{\text{configuration}}$ is favorable to the formation of the dispersion because with decreasing particle size, number of different ways of droplet organization increase.

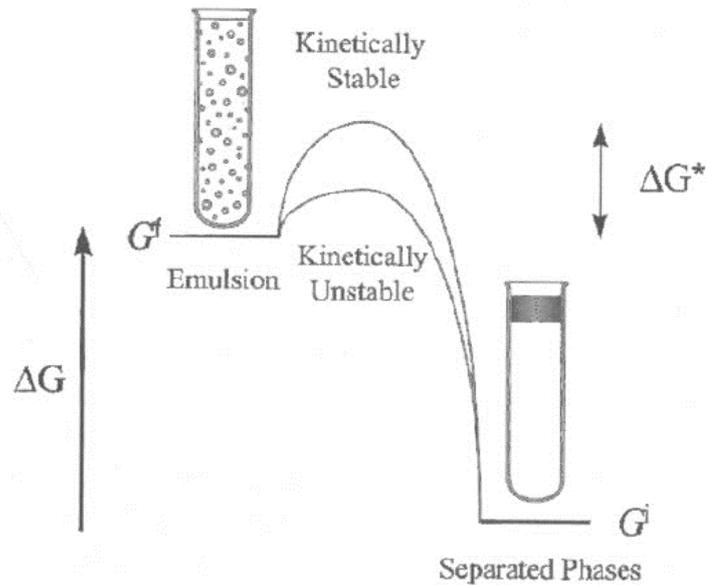


Figure 1.7 Schematic Diagram of Kinetically Stable Nanoemulsions (McClements, 2005).

Because of the free energy barrier, ΔG^* , between the two states, system remain kinetically stable (metastable) for a long period of time (for months or even years). Before reaching the most favorable state (thermodynamically stable), this activation energy must be overcome (Fig.1.7). Thermodynamically unstable nanoemulsion systems may exist in a metastable state thus be kinetically stable. Different metastable states exist for one emulsion system and until reaching the most thermodynamically stable states, emulsion moves from one metastable states to another. To create an activation energy in emulsion, emulsifiers or texture modifiers should be used (McClements, 2005).

In previous studies, development of nanoemulsions including bioactive agents (eugenol (Ghosh et al., 2014), carvacrol (Chang, McLandsborough, & McClements, 2013), thymol (Xue, Davidson, & Zhong, 2013) etc.) and flavor oil (thyme oil (Ziani, Chang, McLandsborough, & McClements, 2011), orange oil (Chang & McClements, 2014), grape seed oil (Davidov-Pardo & McClements, 2015)...etc.) were formulated. In these studies, composition and preparation method (Rao & McClements, 2011b), factors influencing the particle size (Ostertag, Weiss, & McClements, 2012), effect of surfactant type (Donsì, Annunziata, Vincensi, & Ferrari, 2012), influence of ripening inhibitors (Chang, McLandsborough, & McClements, 2012) and also antimicrobial (Ghosh et al., 2014) and antioxidant (Sessa, Tsao, Liu, Ferrari, & Donsì, 2011) properties of the nanoemulsions were investigated.

1.2 Microemulsions

Microemulsions (MEs) are thermodynamically stable systems because the free energy of separate phases is higher than the free energy of colloidal dispersion. Microemulsions include same constituents with nanoemulsions (oil phase, aqueous phase and surfactant). Preparation method of microemulsions is easier than nanoemulsions however high surfactant concentration is required. When compared to the wavelength of light, microemulsions have very small droplet size ($r < 25$) therefore, they are transparent or slightly turbid (Rao & McClements, 2011a ; McClements, 2012 ; Rao & McClements, 2011b) .

1.3 Nanoemulsion Formation

Fabrication of nanoemulsions is basically achieved either by high energy or low energy methods (Acosta, 2009 ; Leong, Wooster, Kentish, & Ashokkumar, 2009 ; Tadros, Izquierdo, Esquena, & Solans, 2004). Mechanical devices are used for high energy methods such as high- pressure homogenizers (Quintanilla-Carvajal et al., 2010), ultrasound generators (Maa & Hsu, 1999) and high speed devices like Ultra-Turrax (Anton, Benoit, & Saulnier, 2008). By using mechanical devices, generation of intensive disruptive forces lead to formation of oil droplets while breaking up the water and oil phases. For low energy methods, phase behavior and properties of ingredients have an important place because they utilize the stored energy to lead the generation of emulsion droplets in nanometric scale. Emulsification takes place while affecting the hydrophilic- lipophilic balance of the system by changing the parameters like temperature and composition (Chime et al., 2014). Membrane emulsification (Sanguansri & Augustin, 2006), spontaneous emulsification (Bouchemal, Briançon, Perrier, & Fessi, 2004), solvent displacement (Yin, Chu, Kobayashi, & Nakajima, 2009), emulsion inversion point (Sadler, Rondon-Gonzalez, Acrement, Choplin, & Marie, 2010) and phase inversion point (Shinoda & Saito, 1969) are low energy methods of emulsification. Low energy emulsification methods are more energy efficient since only simple stirring is needed , besides that usually smaller droplet size is obtained compared to high energy methods (Conxita Solans & Solé, 2012). Moreover, this method has the advantage to be used industrially and suitable for encapsulating the fragile active components (nonaggressive feature) (Anton & Vandamme, 2009). On the other hand, low-energy systems have some potential disadvantages, such as the oil and surfactant types are limited to form stable nanoemulsions and relatively high surfactant-to-oil ratios (SOR) are needed (Chang et al., 2013).

1.3.1 High Energy Methods

1.3.1.1 High Pressure Homogenizer

High pressure homogenizer (Microfluidizer) applies high pressure to force macro-emulsions in order to pass throughout narrow gaps (**Fig. 1.8**)

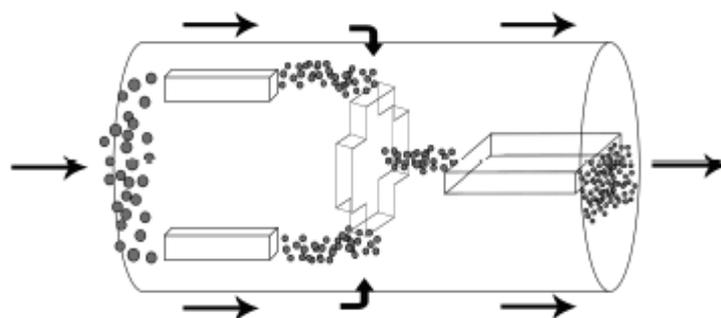


Figure 1.8 Schematic representation of working principles of microfluidizer (Kırtıl & Öztop, 2014)

During the process, velocity increases dramatically and as a consequence, shear, turbulent and cavitation forces are generated in the interaction chamber of the instrument. These generated forces induce the formation of nano-scaled droplets (Anton et al., 2008). For example, for designing nanoemulsion based delivery system of natural antimicrobials, high pressure homogenization with 5 passes at 300 MPa led to formation of droplets having a mean particle size between 100nm to 200nm with polydispersity index between 0.1 and 0.2 (Donsi et al., 2012). In another study, resveratrol encapsulation in grape seed oil nanoemulsion was conducted by microfluidization for 3 passes at 12,000 psi and nanoemulsions having 45 nm mean droplet diameter were obtained (Davidov-Pardo & McClements, 2015).

1.3.1.2 Ultrasonic Homogenization

In an ultrasonic system, emulsification is achieved by using a probe. Physical shear is provided by ultrasonic waves and by means of acoustic cavitation forces, nanoemulsion formation occurs (Fig. 1.9). However, this emulsification has limitations to be used in industrial scale (Chime et al., 2014; Leong et al., 2009). According to research of Leong et.al (Leong et al., 2009) , transparent O/W nanoemulsions with average diameter nearly as 40 nm was obtained by using water, sunflower oil and tween 80/span 80 emulsifier combination. According to another study, to investigate its bacterial activity, cinnamon oil nanoemulsions are formed by ultrasonic emulsification. In this study, nanoemulsion included water, Tween 80 and cinnamon oil and 20 kHz sonicator was used for 30 minutes. As a result of the study, nanoemulsions with particle size of 65 nm and showing bactericidal activity against *B. cereus*, were obtained (Ghosh, Saranya, Mukherjee, & Chandrasekaran, 2013).

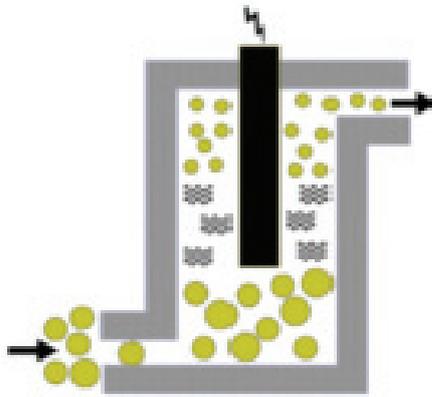


Figure 1.9 Schematic illustration of ultrasound homogenizer (Maan, Schroën, & Boom, 2011)

1.3.2 Low Energy Methods

1.3.2.1 Membrane Emulsification

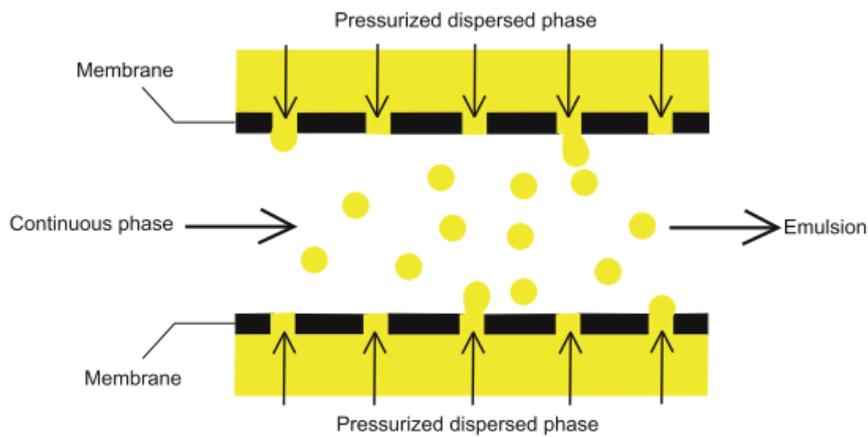


Figure 1.10 Schematic illustration of cross flow membrane emulsification (Maan et al., 2011)

By means of the pores of the membrane, formation of the droplets occur at the pore openings and then particles slide into cross flowing continuous phase (Fig. 1.10). With this low energy method, monodispersed (narrow size distribution range) emulsions are obtained at lower mechanical stress. When compared to high energy methods, this process requires much less surfactant. However, being not feasible for intense emulsions (>10%) and the low flux of dispersed phase are the main disadvantages of membrane emulsification (Silva et al., 2012 ; Maan et al., 2011).

1.3.2.2 Emulsion Inversion Point

At constant temperature, compositional changes occur in this method. System is diluted with oil or water thus formation of intended structures and at the end kinetically stable nanoemulsions take place. **Fig.1.11** shows schematic representation of the EPI method. For emulsification, aqueous phase was titrated into organic phase that includes oil and hydrophilic surfactant. During this process, W/O emulsion, O/W/O multiple emulsion and O/W emulsion form respectively. Finally, at critical water concentration, phase inversion occur and oil particles are produced. In figure 1.11, compositional changes, water-to-oil ratio (WOR) is represented by the x-axis and changes in the formulation of the system, hydrophilic- lipophilic deviation (HLD) is represented by the y-axis. (Silva et al., 2012 ; Ostertag et al., 2012) .

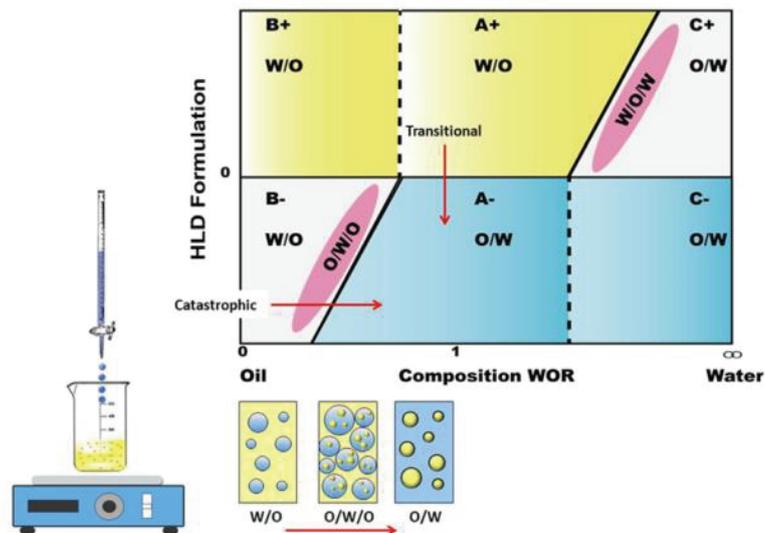


Figure 1.11 Schematic representation of the emulsion phase inversion method (Ostertag et al., 2012).

1.3.2.3 Phase Inversion Point

In this method, arrangements of the surfactant (non –ionic small molecule surfactant) to the oil- water interface alter through changing the temperature or by adding salts and applying external flow. Temperature change is mostly used and this method is also called as phase inversion temperature (PIT). In accordance with the researches, it was found that non-ionic surfactants show more hydrophilic behavior with increasing temperature and their chemical configuration change due to the changes in the curvature of surfactant thus phase inversion is promoted by new physical arrangements. This phenomena is associated with Critical Packing Parameters (CPP) of the surfactants (**Fig.1.12**)

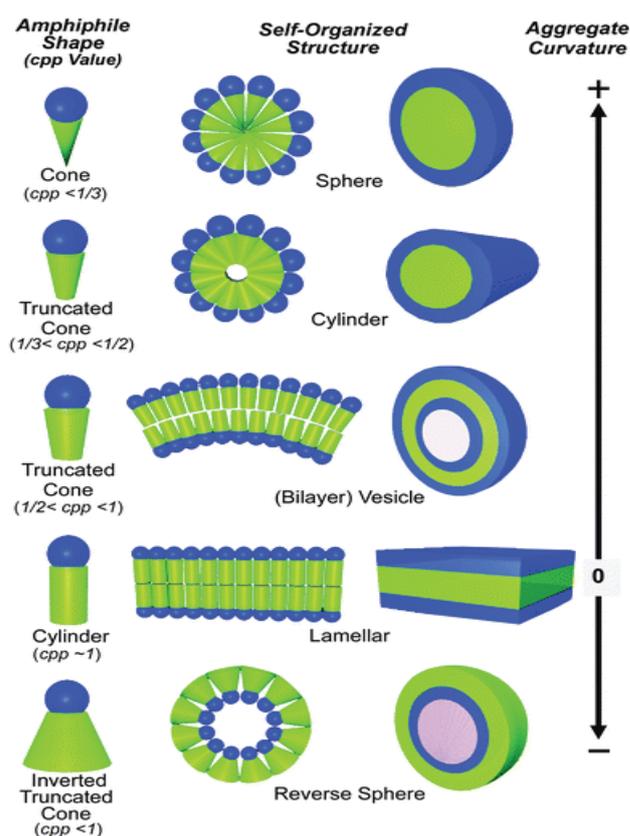


Figure 1.12 Molecular structure of amphiphilic molecules (Ramanathan et al., 2013)

In the case of $CPP > 1$, surfactants are in tendency to form W/O emulsions because hydrophobic tail of surfactant is larger than the hydrophilic head group. Therefore, solubility of surfactant in oil increases, spontaneous curvature of surfactant becomes concave (inverted micelles) and w/o emulsions are formed. If the $CPP = 1$, emulsion system breaks down and coalescence occurs. In the system bi-continuous or liquid crystalline structures are formed. At the $CPP < 1$, solubility of surfactant in water increases due to larger hydrophilic head group. As a result, convex spontaneous curvature is formed and this structure favors the formation of O/W emulsions (T. F. Tadros, 2009).

1.3.2.4 Solvent Displacement Method

In this method, oil phase (contain lipophilic functional compounds) is first mixed with water miscible organic solvent (acetone, ethanol, ethyl methyl ketone). Aqueous phase contains surfactants and after the oil phase is poured into the aqueous phase, instantaneous nanoemulsion formation is promoted by rapid diffusion of organic solvent from oil phase to aqueous phase. At the end of the process, organic solvent is removed from nanoemulsion thereby reducing pressure (vacuum evaporation)(Fig. 1.13) (Silva et al., 2012 ; Chime et al., 2014).

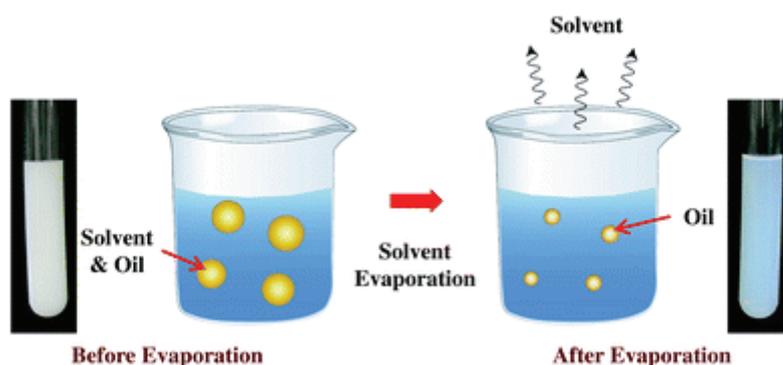


Figure 1.13 Schematic representation of the solvent displacement method (McClements & Xiao, 2012).

1.3.2.5 Spontaneous Emulsification

Spontaneous emulsification (SE) is a widely used technique to form nano-scaled particles. Examples include: development of new emulsions for agricultural applications, drug delivery systems, improvement of new detergents, development of functional foods, production of lubricant oils for specific uses (López-Montilla, Herrera-Morales, Pandey, & Shah, 2002). Only simple stirring is sufficient for spontaneous emulsification at room temperature. Without causing the change in the curvature of the surfactant molecule, surfactant molecule movement from the dispersed phase to continuous phase lead to formation of nanoemulsions (**Fig.1.14**) (Conxita Solans & Solé, 2012). SOR, surfactant type, initial surfactant location and oil type influence the size of the droplets produced by SE method.

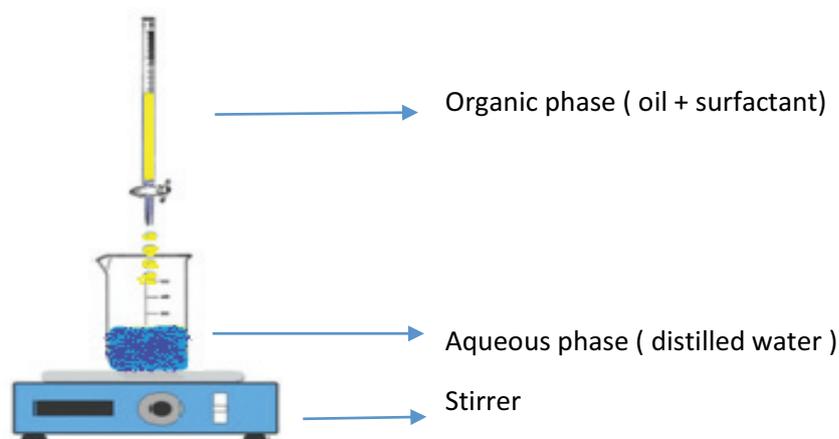


Figure 1.14 Schematic Diagram of the spontaneous emulsification method (adapted from (Ostertag et al., 2012) .

High amount of surfactant is required to stabilize the droplets formed. Insufficient surfactant amount in the system does not form protective coating. As a result, particles collide each other and droplet aggregation is observed (Komaiko & McClements, 2015). Requirement of the proper surfactant amount to obtain smaller particle size may be related to phase behavior of the surfactant- oil-water (SOW) system that was used to form nanoemulsions because formation of ultrafine droplets by using spontaneous emulsification method occurs with only certain SOW compositions for each surfactant, oil and water combination. If the amount of surfactant is too high, it again leads to large droplet formation and phase separation because surfactants begin to form micelles. For this reason, generally “U shaped” curve (Fig.1.15) is reported for particle size distribution graphs (Rao & McClements, 2011b).

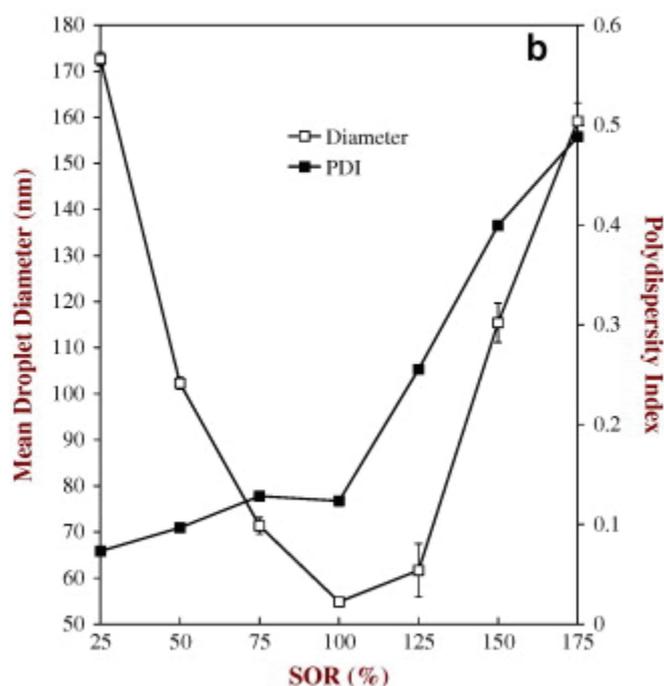


Figure 1.15 Graphical representation of “U shaped” curve. In this graph SOR (Surfactant to oil ratio) shows the proportion between surfactant and oil phase. (Saber, Fang, & McClements, 2013).

For nanoemulsion formation, affinity of surfactant to hydrophobic phase is an important factor. If surfactants are hydrophilic to a high degree, diffusion occurs rapidly (basis of spontaneous emulsification process to obtain O/W nanoemulsions) however it should not be too hydrophilic because it dissolves properly in the oil phase (Anton & Vandamme, 2009). When HLB value of surfactant is too low, surfactant has high affinity for oil than water. For this reason, it is suitable to form reverse micelles in oil and it stabilizes W/O emulsions. If HLB value of the surfactants is moderate, surfactant has equal affinity both for oil and water and surfactant is suitable for formation of bicontinuous microemulsions or liquid crystalline phases. On the other hand, if HLB value of surfactant become too high, surfactant has higher affinity for water than oil. Therefore, it tends to form micelles in water and it stabilizes O/W emulsions. HLB values of Tween 80 is 15.0 and it is very useful to obtain small particle size for O/W nanoemulsions (Ostertag et al., 2012).

For formation of small droplets by SE method, surfactant should not be in the aqueous phase initially. Surfactant movement from organic phase to aqueous phase drives the formation of nano-scaled particles by SE mechanism (Komaiko & McClements, 2015).

Varied oil types have different physicochemical properties as viscosity, density, interfacial tension which impact on the self-emulsification. For example, with LCT (canola oil peanut oil olive oil) and mineral oils, large droplets are formed (z-average $< 0,6 \mu\text{m}$) but while using MCT and flavor oils (orange oil, lemon oil), smallest particles (Ostertag et al., 2012).

1.4 Antimicrobial Activity of Nanoemulsions

Although a great variety of preservation techniques are available, in the food industry food spoilage and poisoning by microbes is a leading concern. Food spoilage and food-borne illness originate from giant population of microorganisms such as *Escherichia coli*, *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella Typhi*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Penicillium* sp., *Aspergillus niger*, *Rhizopus nigricans*, *Aspergillus flavus* etc (Ghosh et al., 2013).

Escherichia coli is a facultatively anaerobic, Gram – negative, rod shape bacteria and take part in Enterobacteriaceae family that was generally found in soil and water, cause flavor and textural defects in foods. This organisms also live in the normal intestinal flora of humans and other warm blooded animals, but some cause disease in humans. In terms of their virulence properties, pathogenic mechanisms, clinical syndromes and O:H serogroups, there are most importantly at least 6 classes of *E. coli* that cause food-borne gastrointestinal disease in humans. These are:

Enteropathogenic *E. coli* (EPEC) that is associated with infantile diarrhea, Enterotoxigenic *E. coli* (ETEC) that is associated with traveler’s diarrhea and infant diarrhea, Enteroinvasive *E. coli* (EIEC) that causes dysentery like shigellosis, Enteroaggregative *E. coli* (EAaggEC) that causes persistent diarrhea, Diffusely Adherent *E. coli* (DAEC), Enterohemorrhagic *E. coli* (EHEC) that are associated with bloody diarrhea and hemolytic uremic syndrome (HUS) (Donnelly, 2014; Morabito, 2014)

To prevent foodborne microorganisms and to reduce the usage of synthetic and antimicrobial compounds in foods through natural ways, essential oils (EOs) are considered as alternative natural antimicrobial additives. However they have low water solubility, high volatility and strong odour. EOs are aromatic hydrophilic liquid products, therefore O/W nano and microemulsions have been thought to be efficient delivery systems (Liang et al., 2012). Liang et al. reported that nanoemulsions of peppermint oil (PO) could be formulated by using medium chain triglycerides (MCT) and modified starch (food grade polymer) while using water as the aqueous media. In

this study, high pressure homogenizer was used for emulsification and antimicrobial activity of the nanoemulsion was tested by using minimum inhibitory concentration method (MIC) against two Gram-positive bacteria: *Listeria monocytogenes* and *Staphylococcus aureus*. When emulsions with PO and bulk PO was compared, peppermint oil time-kill plots showed that these nanoemulsions demonstrated improved long term antimicrobial activity (Liang et al., 2012). Furthermore, in another study to increase the bioactivity of compounds by nanoemulsions, D-Limonene nanoemulsions were formed by high pressure homogenization. In the formulation, D-Limonene, sunflower oil, Tween 20 and glycerol monooleate were blended. Antimicrobial activity of these nanoemulsions were investigated against *E. coli*, *L. delbrueckii* and *S. cerevisiae*. For all microorganisms, MIC reduced from 25 g/l to 10 g/l (Donsi, Annunziata, Sessa, & Ferrari, 2011).

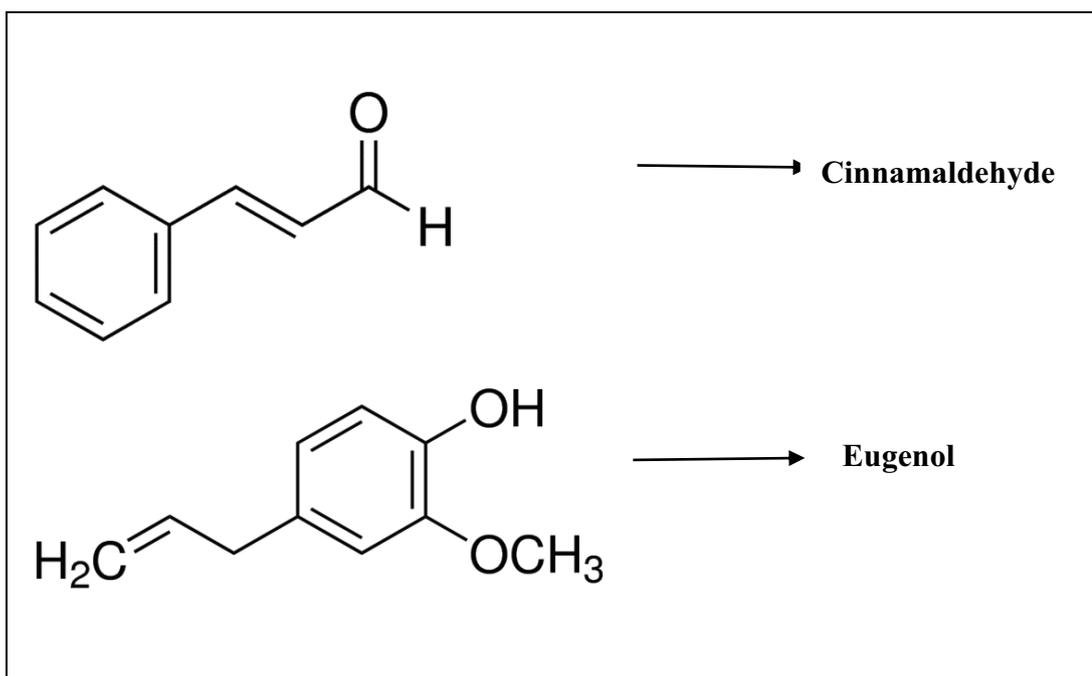
Mode of action of nanoemulsions towards microorganisms are explained based on the interaction with the oil phase and cells. Lipid containing cell membranes of microorganisms are damaged by nanoemulsion particles due to active ingredients because of their hydrophobic nature. Lipophilic character of the emulsions causes accumulation on the cell membrane. Moreover, electrostatic attractions between emulsion and pathogen as well as energy release that was trapped within the emulsion cause to the cell lysis (Chime et al., 2014 & Donsi, Annunziata, Vincensi, & Ferrari, 2012).

1.5 Cinnamon oil

Cinnamon as a spice was used for medicinal purposes and flavoring from ancient times to the present. Its botanical name is *Cinnamomum* and has 250 species such as *Cinnamomum verum* (syn. *C. zeylanicum*) and *Cinnamomum cassia* (Chinese cinnamon). By distillation of bark and leaves of cinnamon tree, cinnamon oil is obtained (Peter, 2006).

In this study cinnamon leaf oil (*C. zeylanicum*) was used. *C. zeylanicum* contains high amount of Eugenol (4-allyl-2-methoxyphenol) and cinnamaldehyde (3-Phenyl-2-propenal) (Ghosh et al., 2013).

Table 1.2 Main bioactive components in cinnamon leaf oil (*C.zeylanicum*) (from Sigma –Aldrich website)



These active constituents show antimicrobial activity against important food pathogens such as *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Helicobacter pylori* and *Salmonella Typhi*. Through the phospholipid bilayer of bacterial cell membrane, penetration of eugenol occurs and it causes the alteration of membrane structures and accordingly permeability changes. For this reason, intracellular constituents of cell leak and cell death occurs (Ghosh et al., 2014). Phenolic compounds also disrupt the structure of cell membrane while interchanging its hydroxyl group with other ions like potassium.

Moreover, phenolic compounds act as a protonophore while carrying protons through lipid bilayers therefore they dissipate the proton motive force. In addition, phenolic compounds affect the permeability of cell membrane and cause leakage of some substances such as amino acids, ATP, ions and nucleic acids (Donsi et al., 2012). In another study, membrane damage of *E. coli* O157:H7 was analyzed by fatty acid profiles (GC analysis of FAMES) after exposure to Eugenol (phenolic compound) and Cinnamaldehyde (aliphatic aldehyde). Changes in rod morphology were observed by scanning electron microscope (SEM). When the cells were treated with cinnamaldehyde, reduction in unsaturated fatty acid (UFAs) profile of *E. coli* was observed. Moreover, SEM analysis showed that cinnamaldehyde caused the structural alterations in the membrane. Big holes and white spots were observed on the cellular wall. Besides, it was investigated that, Eugenol caused cell wall deterioration and high degree of cell lysis. However, with GC analysis, it was found that Eugenol had slight effect on the change of fatty acid profile of *E. coli* and the reason of this case was explained as: 'Eugenol had hydroxyl groups and this group binds to proteins. There is a different mechanism of action which affected the membrane protein (Asqua et al., 2007).

1.6 Carrier oil

Addition of neutral hydrophobic co-solvents (MCT, corn oil, sunflower oil) into oil phase acts as carrier oil for functional ingredients such as essential oils (cinnamon oil, thyme oil), β -carotene, α -Tocopherol (Silva et al., 2012).

The main mechanism causing nanoemulsion breakdown is sedimentation or creaming with Ostwald ripening (OR). Polydispersity in emulsions and also solubility differences between large and small particles lead to destabilization of nanoemulsion via OR (molecular diffusion). Compositional ripening effect can be generated against

OR using a second component with relatively high molecular weight and very low aqueous solubility. Moreover, addition of a second surfactant which has same alkyl chain length and higher degree of ethoxylation than primary surfactant can decrease the OR rate (C Solans, Izquierdo, Nolla, Azemar, & Garciacelma, 2005). These type of components are also known as ripening inhibitors. Incorporation of a second oil in the emulsion system causes compositional differences between large and small particles throughout OR process. This phenomenon can be explained more detailed with entropic stabilization. With the addition of a second oil in the continuous phase, the entropy of mixing provides chemical potential against OR and counterbalances the driving force for OR. Soluble oil becomes more mobile between droplets when nanoemulsion undergoes OR. In time, while the amount of larger droplets increase in the soluble oil, insoluble oil is enriched with smaller droplets. Therefore, osmotic pressure develops between small and big droplets, and soluble oil drives back to the smaller droplets. Thus, OR can be slowed down (Bagchi, Bagchi, Moriyama, & Shahidi, 2013).

Type of carrier oil is an important factor to form a stable nanoemulsion while using different methods and oil compositions. For instances, by using a homogenizer, O/W beverage emulsions that were prepared by long chain triglycerides (canola or corn oil), soybean soluble polysaccharides (SSPS), citric acid and orange peel oil showed better stability than emulsions prepared with medium chain triglycerides (MCTs) (Zhao, Liu, Ma, Yuan, & Gao, 2015). This phenomena was explained with chemical structure of carrier oils. It was thought that carrier oils with LCT have more suitable chemical structure for hydrogen bonds and polymer entanglement of SSPS. When hydrocarbon chain length increased, oil – water interfacial tension rises so MCT stabilized emulsions were more unstable (Zhao et al., 2015). Besides that in another study, with spontaneous emulsification (SE), stable carvacrol nanoemulsions were obtained by MCT and reason was explained as changing physicochemical properties (viscosity, interfacial tension, phase behavior) with different carrier oils because of differences in molecular characteristics (chain length, unsaturation) (Chang et al., 2013).

1.6.1 Coconut oil

Coconut oil (CNO) is obtained from kernels of seeds of *Cocos nucifera* (Coconut). It is insoluble in water and has melting point of 21-27 °C. It is used in cosmetics and pharmaceuticals as solvent, superfatting agent, clouding agent, detergent, wetting agent and emulsifier; in foods as coating agent, emulsifier, formulation aid, and texturizer (Ash & Ash, 2013)

Coconut oil could be used as a carrier oil in nanoemulsion systems since it is composed of high amounts of Medium Chain Fatty Acids (MCFAs) (> 50 wt. % of fatty acids) (Marten, Pfeuffer, & Schrezenmeir, 2006).

1.7 Identification and Characterization of Nanoemulsions

Analytical techniques (separation, characterization and imaging techniques) are used for identification and characterization of nanoemulsions. Dynamic light scattering (DLS), zeta potential, differential scanning calorimetry, and nuclear magnetic resonance are the commonly used characterization techniques for nanoemulsions. Depending on the matrix of analyzed nanoemulsion, microscopy can be used as a direct imaging technique. Some of these techniques are: Transmission Electron Microscopy (TEM), Atomic Force Microscopy, Scanning Electron Microscopy (Silva et al., 2012).

1.7.1 Dynamic Light Scattering (DLS)

DLS (Photon correlation spectroscopy) is a rapid determination technique for droplet size (hydrodynamic radius of particle size), polydispersity and zeta potential measurements. Particles do Brownian motion in relation of their particle size and analysis of the fluctuations in the intensity of scattering is measured by DLS (Chime et al., 2014, Silva et al., 2012). This technique depends on the measurement of the translational diffusion coefficient (D). Particle – particle interaction is eliminated in dilute emulsions and by using the Stokes- Einstein equation (Eq. 1.2), size of particles can be calculated (McClements, 2005; Ghosh et al., 2014):

$$D = \frac{kT}{6\eta R} \quad (\text{Eq. 1.2})$$

Here D is the translational diffusion coefficient, η is the viscosity of the continuous phase, k is the Boltzmann's constant, and T is the absolute temperature.

This method gives z-average particle diameter. Broadness of the size distribution (polydispersity index (PDI)) obtained from the cumulative analysis of dynamic light is also measured by DLS. PDI gives information about nanoemulsion homogeneity and quality as well storage stability (Chime et al., 2014, Silva et al., 2012). If PDI is smaller than 0.1, this shows very good nanodispersity and quality (Anton & Vandamme, 2009). While using this method, to avoid from multiple scattering effects, samples should be diluted (Ziani et al., 2011; Qian, Decker, Xiao, & McClements, 2012). According to the research of Chang et al. (2013), surfactant concentration effect on particle size was measured by DLS. In the study, carvacrol nanoemulsions were formed by spontaneous emulsification method. Formulation of the nanoemulsion included 25% carvacrol + 75% MCT (10% of total system) and TWEEN 80 at different concentrations (5, 10, 15, and 20 wt %). Decreasing mean particle sizes with increasing surfactant concentration was observed. For 5, 10, 15, and 20 wt %

surfactant concentrations, mean particle diameters were found as 168, 55, 34, and 25 nm respectively (Chang et al., 2013). According to another research (Zhao et al., 2015), effect of carrier oil on particle size was detected by using DLS method for investigation of orange oil beverage emulsions. In this study, canola oil, corn oil and MCT were used as carrier oils and mean particle size was obtained as 332.3, 370.5, 449.2 nm respectively for these carrier oils.

1.7.2 Transmission Electron Microscopy

Dispersed phase's higher resolution images are obtained by TEM following the analysis by digital image processing. Qualitative and quantitative information (size and size distributions) can be obtained from the images. For nanoemulsions, TEM is usually used to confirm the results obtained by DLS. The main disadvantage of using TEM for nanoemulsions is: during preparation process, structure of the sample may change and electron beam of TEM can cause to damage on sample (Silva et al., 2012; Chime et al., 2014).

1.7.3 Differential Scanning Calorimeter (DSC)

Thermal transitions in samples are identified during heating by DSC. For this method, a reference is used and it should have a well-defined heat capacity at the temperature range of scanning. Phase transitions such as melting of crystalline regions in emulsions can be analyzed with this technique (the solid fat proportions and ice crystals proportion) (Silva et al., 2012). According to the research of Thanasukarn et al. (2004), it was found that emulsion stability was affected from fat crystallization which was also associated with the surfactant used. Crystallization temperature of the surfactants was also detected by using DSC (Thanasukarn, Pongsawatmanit, & McClements, 2004).

1.7.4 Nuclear Magnetic Resonance (NMR)

NMR is a complex analytical tool. Quantitative and structural analysis studies of the compounds in a sample can be achieved both for solid and liquid states. It can be used as a complementary measurement. NMR application for nanoemulsion characterization has been rarely used (Silva et al., 2012). In a study of an amorphous drug nanosuspensions, the effect of addition of a second insoluble component in the continuous phase against Ostwald Ripening was monitored by NMR spectroscopy (Lindfors et al., 2006). It was known that inhibition can be achieved when the homogeneous composite mixture of the drug and inhibitor is obtained. At the end of the study, it was found that NMR was a convenient technique to understand whether a homogeneous mixture of the drug and the inhibitor was obtained or not.

1.8 Objectives of This Study

The hypothesis in this study can be phrased as in the following:

‘Antimicrobial activity of cinnamon oil can be increased by using nanoemulsification methods.’

Based on this hypothesis, objectives of the study could be defined as:

- Investigating the antimicrobial activity of cinnamon oil through nanoemulsions prepared by spontaneous emulsification;
- Formulating stable cinnamon oil nanoemulsions with spontaneous emulsification by using coconut oil as a carrier oil

To test the hypothesis, different emulsification methods were used including, spontaneous emulsification, microfluidization and ultrasonic homogenization. To characterize the nanoemulsions; particle size, transmission electron microscopy and disc diffusion test for antimicrobial activity were determined.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

This study concentrated on the use of cinnamon oil. However, preliminary experiments were conducted to find the best material for formulating nanoemulsions. As will be explained later, cinnamon oil having the highest antimicrobial activity was used as the main active agent in nanoemulsion preparation. List of the all bioactive compounds and essential oils that were examined in terms of antimicrobial activity are listed below:

- Cinnamon oil (from Sigma-Aldrich)
- Rosemary oil (from Sigma-Aldrich)
- Linalyl acetate (from Sigma-Aldrich)
- α – terpinene (from Sigma-Aldrich)
- Orange oil (from local supermarket -Turkey)
- Peppermint oil (from local supermarket -Turkey)
- Basil oil (from local supermarket-Turkey)
- Lemon oil (from local supermarket-Turkey)
- Camomile oil (from local supermarket-Turkey)
- Thyme oil(from local supermarket-Turkey)
- Fenugreek oil (from local supermarket-Turkey)

Coconut oil which was used as a carrier oil and essential from making nanoemulsions obtained from ‘www.hammadeler.com’. Ethanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Filter papers were obtained from Whatman (Maidstone, UK). A nonionic surfactant (Tween 80) was obtained from Merck chemicals (Darmstadt,

Germany). For (BHI) agar preparation, Agar Bacteriological (Agar No.1), Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) was purchased from OXOID (Basingstoke, Hampshire, England). Brain heart infusion broth (Bury, Lancashire, UK) was purchased from OXOID. For McFarland Standard preparation, Barium Chloride was purchased from Sigma- Aldrich (St. Louis, MO, USA) and Sulfuric acid was purchased from Merck chemicals (Darmstadt, Germany). For antimicrobial test, ATCC25922 *E. coli* strain was kindly provided by Food Safety Laboratory in Food Engineering Department at METU. Antimicrobial susceptibility test disc, Tetracycline (TE) was purchased from OXOID (Basingstoke, Hampshire, England). Distilled water was used in all experiments

2.2 Low Energy Method

2.2.1 Nanoemulsion Preparation by Spontaneous Emulsification

Spontaneous emulsification was carried out by titration of organic phase containing Tween 80 and different amount of cinnamon oil and coconut oil into aqueous phase while the system was continuously stirred at 750 rpm with magnetic stirrer at ambient temperature (≈ 25 °C). Experiments were performed using standardized conditions: 10 wt % oil (cinnamon + coconut), 10 wt % surfactant (Tween 80) and 80 wt % aqueous phase (distillate water). To conduct particle size measurement and antimicrobial tests, emulsions that included 2%, 4%, 5%, 6%, 8% and 10% cinnamon oil were prepared. In these samples, firstly oils were mixed together during 30 minutes and then surfactant was added and organic phase was mixed for another 30 minutes. The resulting mixture was titrated into aqueous phase at a rate of 1 ml /min.

2.3 High Energy Methods

2.3.1 Sample Preparation by Silent Crusher

To investigate the different cinnamon oil concentration on antimicrobial activity (80%, 60%, 40%, 20%, 10%, 8%, 5%, 0.5 %) , samples were prepared by using silent crusher at 15,000 rpm for 1 min (Heidolph Instruments GmbH & Co. KG, Germany). Dilution of the samples was achieved using ethanol.

2.3.2 Pretreatment with Ultra-Turrax Homogenizer for Nanoemulsion Formation

Pre-homogenization of O/W nanoemulsions were done by mixing oil phase (cinnamon oil + coconut oil + Tween 80) and aqueous phase (distillate water) with Ultra-Turrax (WiseTis Homogenizer, Witeg Labortechnik GmbH, Germany) at 10000 rpm for 2 min.

2.3.3 Nanoemulsion formation by Microfluidization

To investigate the effect of microfluidization on particle size and antimicrobial activity, pre-homogenized nanoemulsions containing 10 wt % oil, (2:8, 4:6, 5:5, 6:4, 8:2, 10:0 , cinnamon oil :coconut oil) , 10 wt % surfactant (Tween 80) and 80 wt % aqueous phase (distillate water) was subjected to high pressure microfluidization (Nano Disperser - NLM 100, South Korea). The samples were treated at ~ 827 bar for 3 passes. After passing through the microfluidizer the sample was collected to conduct particle size measurements and antimicrobial tests.

2.3.4 Nanoemulsion formation by Ultrasonication

To examine the effect of ultrasonication on particle size and antimicrobial activity of nanoemulsions, first, oil mixtures (2:8, 4:6, 5:5, 6:4, 8:2, 10:0 ,cinnamon oil :coconut oil) were obtained following 30 minutes mixing with magnetic stirrer . After that, surfactant was added to the mixtures and obtained organic phase was mixed for another 30 minutes. Nanoemulsion was formed when organic phase (10 wt% (cinnamon oil + coconut oil) + 10 wt% Tween 80) and aqueous phase (distillate water) mixture was sonicated using a ultrasonicator (BandelinSonoplus HD 3100, Bandelin electronic GmbH & Co. KG, Berlin Germany)(sonotrode: TT13) for 10 min at 75 W.

2.4 Determination of the Critical Micelle Concentration (CMC) of Tween 80

To test if the surfactant used form micelles and inhibit nanoemulsion formation, critical micelle concentration of Tween 80 was determined. Experiments were conducted at METU Central Lab Facilities. Tween 80 – water mixture containing 2%, 4%, 6%,8%, 10%,12%, 14%,16%,18% and 20% Tween 80 was titrated into oil phase with 6:4 CO to CNO ratio by using tensiometer (Attension Theta , Biolin Scientific) to find surface tension between oil - water interface.

2.5 Determination of Fatty Acid Composition of Coconut Oil

Experiment was conducted at TUBITAK Marmara Research Center .While applying the ISO 12966-2:2011 method, the fatty acid composition was determined by using Perkin Elmer gas chromatography system (Auto system GLX, Shelton, U.S.A.) that included a flame ionization detector (FID). Fused silica capillary column SPTM –2380 (100 m length × 0.25 mm with a 0.25 µm film thickness) which was obtained from Supelco (Bellefonte, U.S.A.) was used for the chromatographic separation of fatty acid methyl esters (FAMES)

2.6 Characterization of Nanoemulsions

2.6.1 Mean Particle Size Measurements

Experiments were conducted at METU Central Lab Facilities. Dynamic light scattering (MALVERN Nano ZS90, Worcestershire, UK) was used to determine the particle size distribution and to measure the mean particle diameter (z-averages). By using intensity time fluctuations of laser beam (633 nm) that is scattered from sample with 173° , the mean particle size of samples were determined. On an average, 15 run was done for each individual measurement. Samples were diluted before the measurement to avoid multiple scattering effect.

2.6.2 Transmission Electron Microscopy (TEM)

Experiments were conducted at METU Central Lab Facilities. Transmission Electron Microscopy (FEI Tecnai G2 Spirit BioTwin CTEM, Oregon USA) was used to examine the morphology and size of nanoparticles. Samples were prepared while transferring diluted solution to a freshly glow discharged TEM copper grid (300 mesh copper Formvar / Carbon) and after samples were allowed to dry at room temperature.

2.7 Antimicrobial Test

2.7.1 Agar Disc Diffusion Method

Before agar disc diffusion test, *E. coli* was inoculated on BHI agar with a loop from the stock culture. It was incubated at 37 °C for 20-24 hours (ET 120 Oven, Şimşek Laborteknik, Turkey). Following incubation, several colonies were selected and suspended in MHB. After incubating again in the incubator at 37°C for 2 hours, turbidity of the suspensions was controlled with 0.5 McFarland standard by using white paper with black lines. If suspension showed same transparency with McFarland, it was ready for inoculation on MHA for agar disc diffusion test. Inoculation was performed using a cotton swab. Entire plate was covered by streaking back and forth from edge to edge. Swabbing procedure was repeated 3 times while rotating plate 60°.

Standard 6 mm paper discs that were obtained from filter paper were used for disc diffusion tests. 20 µl of the active compound containing nanoemulsion was put on a disc. After 30 minutes the discs were placed on the inoculated plate while pressing each disc down firmly.

When the incubation was completed, zone diameters around the discs were measured. In order to control the accuracy of inhibition, antimicrobial disc such as Tetracycline (TE 30) were used as positive control and these zone diameters were compared with Clinical Laboratory Standards Institute (CLSI). Throughout this procedure, validation of the antimicrobial tests was conducted.

Zone diameters were measured holding the plates on black nonreflecting surface. Measurements were done by using a rule.

Table 2.1 Pre-screen Test Results of Antimicrobial Activity of Pure Essential Oils and Bioactive Compounds

Bioactive Material	Average Inhibition Zone Diameter (mm)
Cinnamon oil	17
Rosemary oil	10.25
Linalyl acetate	–
α – terpinene	–
Orange oil	–
Peppermint oil	–
Basil oil	–
Lemon oil	–
Camomile oil	–
Thyme oil	–
Fenugreek oil	–

2.8 Experimental Design

Considering the methods, factors and characterization techniques stated previously; the factors and corresponding levels could be summarized in the following table:

Table 2.2 Factors and Levels

Factors	Levels
Homogenization Methods	Spontaneous Emulsification (SE) Microfluidization Ultrasound
CO concentration in solution	0.5% , 6%, 8%, 10%, 20%, 40%, 60%, 80%, 100%
Surfactant type in nanoemulsion (NE)	Tween 80
Total Oil concentration in NE	10%
Tween 80 concentration in NE	20%, 10%, 5%
Aqueous phase concentration in NE	70%, 80%, 85%
Carrier oil (CO) concentration in NEs that were obtained by SE, Microfluidization, Ultrasonication	2%, 4%, 6%, 8%, 10%

2.9 Statistical Analysis

Using freshly prepared samples, all experiments were carried out two or three times and results were reported as the mean value. Analysis of Variance (ANOVA) was performed by using Minitab (ver.16.2.0.0, Minitab Inc., United Kingdom) with Tukey's test.

CHAPTER 3

RESULTS AND DISCUSSION

Fatty Acid Composition of Coconut Oil

As mentioned in Chapter 1, presence of medium chain triglycerides is essential for nanoemulsion formation using spontaneous emulsification. Medium chain triglyceride mixtures (MCTs) that are sold commercially, are usually used for this purpose (Chang et al., 2013; Saberi et al., 2013). MCTs have small particle size (28 -78 nm) when compared with LCTs (Canola oil) (215 -233 nm) therefore they are highly preferred in the formulation of nanoemulsions (McClements & Rao, 2011). In this study rather than purchasing an expensive MCT mixture, coconut oil which was known to include higher amounts of MCT was used. Coconut oil roughly includes 40% long chain triglycerides (LCT) and 60% MCTs (Marina, Che Man, Nazimah, & Amin, 2009). To find out the MCT content of the coconut oil used in the study, fatty acid composition analysis was conducted at TUBITAK Marmara Research Center. Results are given in **Table 3.1**.

Chain length of fatty acid molecules of MCTs varies from 6 to 12 carbons (Marten et al., 2006). As it is seen in the table; caproic acid, caprylic acid, capric acid and lauric acid are MCTs that are present in the coconut oil. Results showed that the coconut oil used contains 63.28% MCT. Results were comparable with the Codex Alimentarius standards (2013) and literature (Marina et al., 2009).

In this study, coconut oil was used as carrier oil (second oil) and it was shown that it had ripening inhibitor effect against Oswald Ripening (OR). Incorporation of a second oil in the emulsion system causes compositional differences between large and small particles throughout OR process. When nanoemulsion undergoes OR, slightly soluble oil become more mobile between droplets. With the addition of a second oil phase in

the continuous phase, chemical potential against OR is created by the entropy of mixing and this counterbalances the driving force for OR. In time, while the amount of larger droplets increase in the soluble oil, insoluble oil is enriched with smaller droplets. This creates osmotic pressure between small and big droplets, and soluble oil drives back to the smaller droplets because of the osmotic force. Thus OR can be slowed down and more stable nanoemulsions can be formed (Bagchi et al., 2013). Coconut oil acted as the 2nd oil phase in this study and as will be seen in later sections provided great stability to nanoemulsions.

Table 3.1 Fatty Acid Composition of the Coconut Oil Used in the Study

Compounds	Percentage (%)
Oleic acid (C18:1)	5.32
Myristic acid (C14:0)	14.70
Arachidic acid (C20:0)	0.08
Gadoleic acid (C20:1)	0.03
Behenic acid (C22:0)	0.01
Lignoceric acid (C24:0)	0.02
Palmitic acid (C16:0)	8.22
Palmitoleic acid (C16:1)	0.01
Heptadecanoic acid (C17:0)	0.01
Stearic acid (C18:0)	3.02
Linoleic acid (C18:2)	0.91
Pentadecanoic acid (C15:0)	0.01
Caproic acid (C6:0)	0.65
Tricosanoic acid (C23:0)	0.01
Caprylic acid (C8:0)	7.99
Capric acid (C10:0)	6.44
Lauric acid (C12:0)	48.20

3.2 Spontaneous Emulsification

3.2.1 Effect of Different Oil Combinations on the Mean Particle Size of Nanoemulsion

The first factor studied on the nanoemulsions was the effect of oil phase composition on the characteristics of the emulsions. Oil phase included cinnamon oil (CO) and coconut oil (CNO) with different mass ratios (CO: CNO = 2:8, 4:6, 6:4, 8:2, 10:0). For this analysis, total oil ratio of the emulsions was kept at 10% while surfactant (Tween 80) and aqueous phase were 10% and 80% respectively. Effect of oil composition on polydispersity index (PdI) and mean particle size of nanoemulsions are shown in Figure 3.1

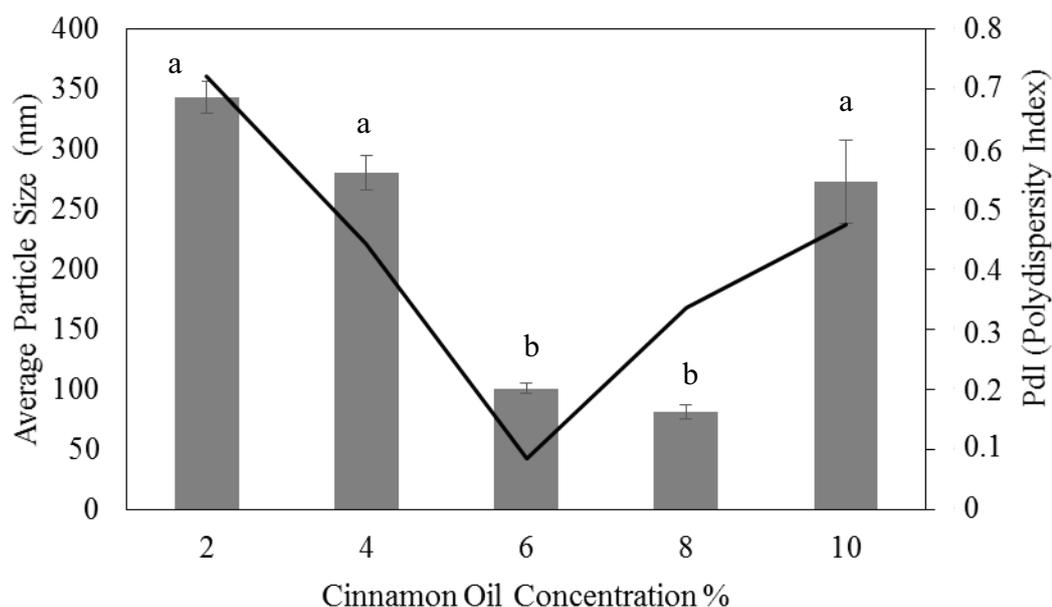


Figure 3.1 Effect of Oil Phase Composition on Particle Size: (■) and Effect of Oil Phase Composition on PdI: (—) . Different letters represent significant difference ($p \leq 0.05$)

According to the results, smaller droplets were obtained, 101 nm and 81 nm respectively for 6% and 8% CO concentrations in the organic phase. ANOVA results showed that there was no significant difference between these two concentrations. However PdIs at the 2 concentrations were different. The mean particle diameter increased ($d \approx 343$ nm) at a CO: CNO ratio of 2:8 and milky white cream layer was observed on the top of the emulsion in a short time. In addition to that nanoemulsions were highly unstable and phase separation was observed at cinnamon oil concentrations around 10% (**Figure 3.2**).

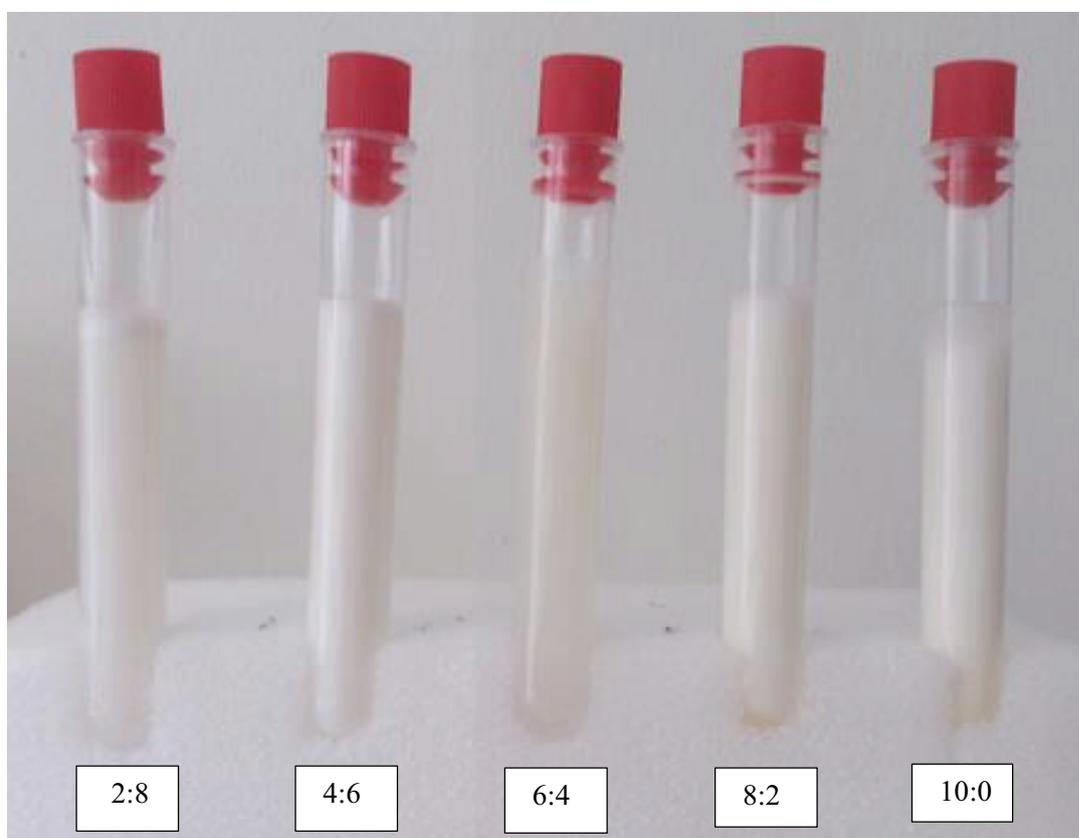


Figure 3.2 Effect of CO:CNO Ratio on Physical Stability of Nanoemulsions. From left to right the photograph shows CO: CNO ratio in the system of 2:8, 4:6, 6:4, 8:2, and 10:0 respectively.

Identification of oil phase composition is significant for not only efficient emulsion formation, but also for good long term emulsion stability. Since initial droplet size which has direct effect on viscosity, interfacial tension, interfacial dynamics, and phase behavior all depend on oil phase composition. Moreover, oil phase composition influences the water solubility of oil molecules and polarity of emulsions while affecting the droplet growth (Davidov-Pardo & McClements, 2015).

Our results indicated that by using spontaneous emulsification method, there was an optimal oil phase composition to obtain more stable, small droplets containing nanoemulsions. Although to form nanoemulsions with small droplets is possible by use of essential oil, Ostwald ripening (OR) is a problem due to water solubility properties (Julian McClements, Henson, Popplewell, Decker, & Jun Choi, 2012). OR can be inhibited by addition of the appropriate amount of highly hydrophobic materials (corn oil, MCT) into the oil phase. Those ripening inhibitors affects the initial size of the droplets and afterwards affects the stability of these droplets against growth (Chang et al., 2012). As expected, addition of a second oil in the organic phase, decreased the droplet size to a certain extent (Davidov-Pardo & McClements, 2015). This phenomenon was explained in detail with entropic stabilization. In our experiments, coconut oil was used as carrier oil and it was obvious that it had a ripening inhibitor effect since stable nanoemulsions could not be formed with cinnamon oil alone. Moreover, as a second oil sunflower oil (LCT) was used in the formulation of nanoemulsions, however quick phase separation was observed. LCTs have larger dimensions when compared with flavor oils and MCTs. For this reason, initially occurring big particles can cause the less stability (Qian et al., 2012).

While conducting particle size measurements through Dynamic Light Scattering (DLS), dilution of the samples are required. Importance of dilution in DLS measurements will be explained in a latter section. Although smallest particle size was obtained with 8:2 (CO: CNO) ratio, phase separation was observed for undiluted samples in a short period. Inhomogeneity was also observed when the PDI values were examined (Chang et al., 2013). However, when the ratio of CO: CNO become 6:4 w/w, statistically same particle size, but narrower size distribution was observed.

A similar case was also observed in the study of Chang et al. (2013) where carvacrol and MCT were present in the oil phase at the same concentration (10% w/w). Although, smallest particles were obtained for 25% carvacrol containing nanoemulsions in oil phase, it showed less stability during one month storage time for its diluted and undiluted samples. At the beginning, nanoemulsions which contained 20% carvacrol, had high particle size; after 30 days storage, it demonstrated high stability and its particle size was smaller than 25% carvacrol containing emulsions. It was seen from the particle size distribution plot that at 8:2 CO to CNO ratio, two different peaks began to form (Fig. 3.3). This situation was an indicator of inhomogeneity. Likewise for 10:0, 4:6 and 2:8 CO to CNO ratios wide range particle size distribution was observed. Only for 6:4 ratio, one peak was observed.

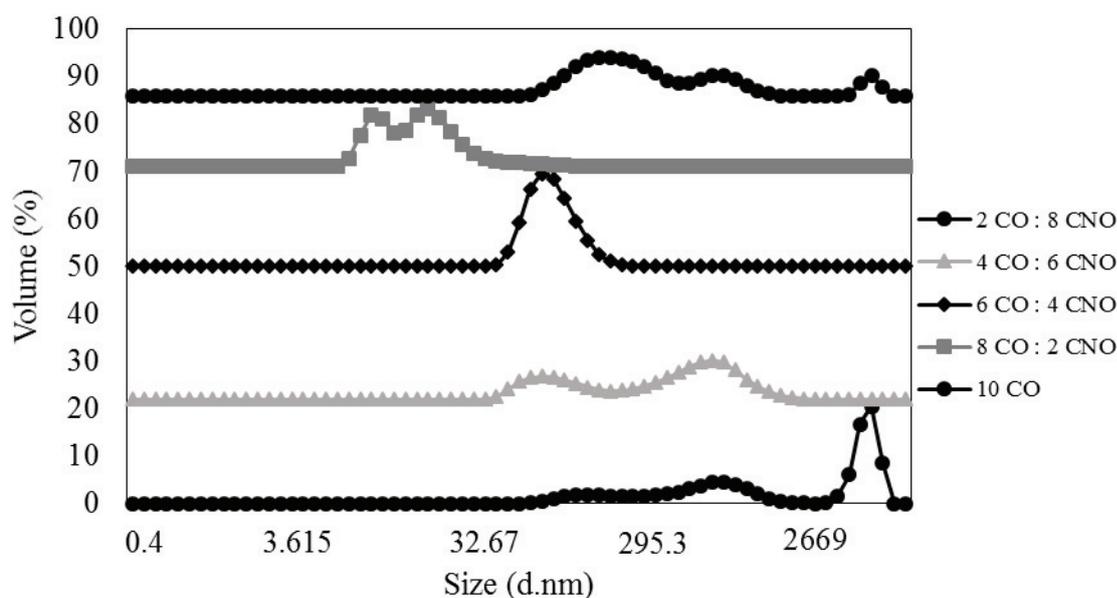


Figure 3.3 Size Distribution Graph of Different Oil Combinations.

At 6:4 (CO: CNO) ratio, physical stability for 30 days was highly provided and this level was used for rest of the experiments. Also, this concentration was thought to be more acceptable to be used in food systems.

3.3 Effect of Surfactant to Oil Ratio (SOR) on the Mean Particle Size of Nanoemulsions

Finding the minimum amount of surfactant that formed stable nanoemulsions which has small droplet size is very significant because of the safety, taste and economic reasons. In these experiments, effect of surfactant concentration on particle size of nanoemulsions was examined for three different concentrations (SOR: 0.5, SOR: 1 and SOR: 2) while the total amount of oil phase was held constant as 10 wt. % (cinnamon oil: coconut oil 6:4 (w/w)). **Figure 3.4** showed that the mean particle diameter as measured by DLS decreased with increasing amount of surfactant.

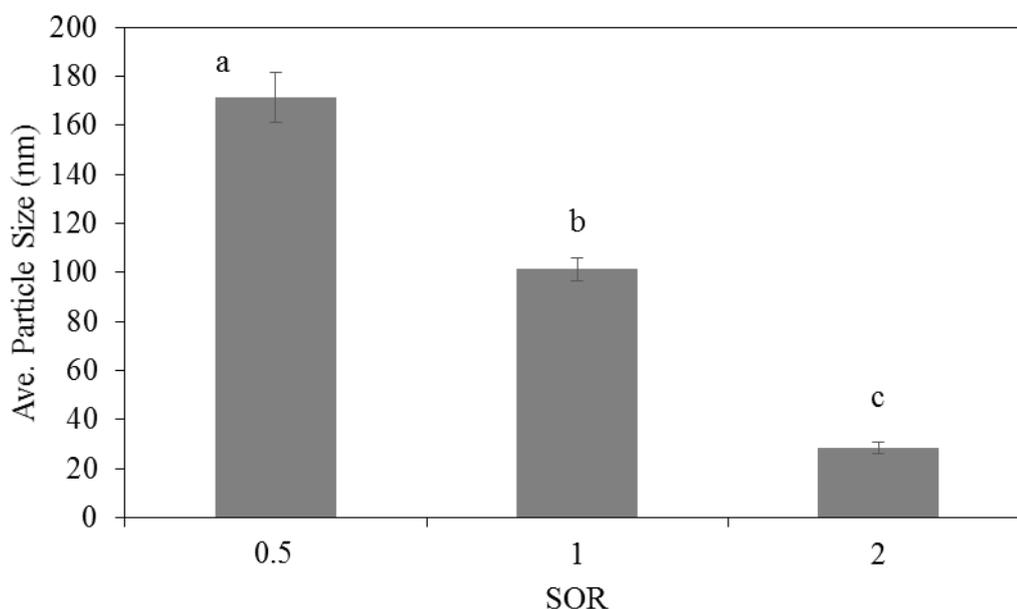


Figure 3.4 Effect of SOR (Surfactant to Oil Ratio) on Particle Size of Sample. Different letters represent significant difference ($p \leq 0.05$)

The mean particle diameters were about 171nm, 101 nm and 29 nm for 5 %, 10 % and 20% surfactant concentrations, respectively. With increasing SOR, emulsions tends to be less turbid, because small droplets are formed and light scattering reduced (**Fig. 3.5**).



Figure 3.5 Effect of SOR on Nanoemulsions. From left to right the photograph shows SOR in the system of 0.5, 1, 2 respectively.

Surfactants form a coating layer around the surface of oil droplets and with decreasing droplet size, the specific surface area increases and to stabilize the droplets larger surfactant concentrations are required (Ostertag et al., 2012). Required surfactant amount to obtain smaller particle size can be associated with phase behavior of the surfactant-oil-water (SOW) system that was used to form nanoemulsions, since formation of ultrafine droplets by using spontaneous emulsification method occurs with only certain SOW compositions for each surfactant, oil and water combination (Komaiko & McClements, 2015). Although, increasing surfactant amount reduces droplet size, this case is observed up to a point. When very high amount of surfactant is used, it can lead to droplet formation due to formation of micelles at Critical Micelle Concentration (CMC). That is why, CMC of the used surfactant in our study was also determined. On the other hand, if surfactant amount is not enough, proper SOW state is not formed and emulsions are prone to formation of large droplets (Komaiko & McClements, 2015).

At the end of the study, usage of SOR: 1 was considered appropriate for other experiments since nanoemulsions with ≈ 100 nm mean particle size could be obtained with SOR: 1. Rather than obtaining smaller particle size with very high amount of surfactant, stable nanoemulsions were tried to be formulated at 10% Tween 80 concentrations by SE because more cost effective and safe.

3.4 Determination of Critical Micelle Concentration (CMC) of Tween 80

To obtain CMC, the surface tension versus the logarithm of concentration plot was used. Concentration at the inflection point of the curve shows CMC (Menger, Shi , & Rizvi, 2010). For CMC measurement surface tension of the surfactants at different concentrations was measured at METU Central Laboratories. In the present study, point of CMC was not reached even at 20 % Tween 80 concentration (**Appendix B**). The result indicated that up to 20 % concentration, surfactant could be used without causing the formation of micelles.

3.5 Stability of Cinnamon oil Nanoemulsions

For practical applications, stability of emulsions is very important. For this study, kinetic stability of nanoemulsions was investigated by measuring droplet size in a four week period. Nanoemulsion system with SOR: 1 and consisted of 10 % oil (CO: CNO 6:4 (w/w)) phase was used for this purpose.

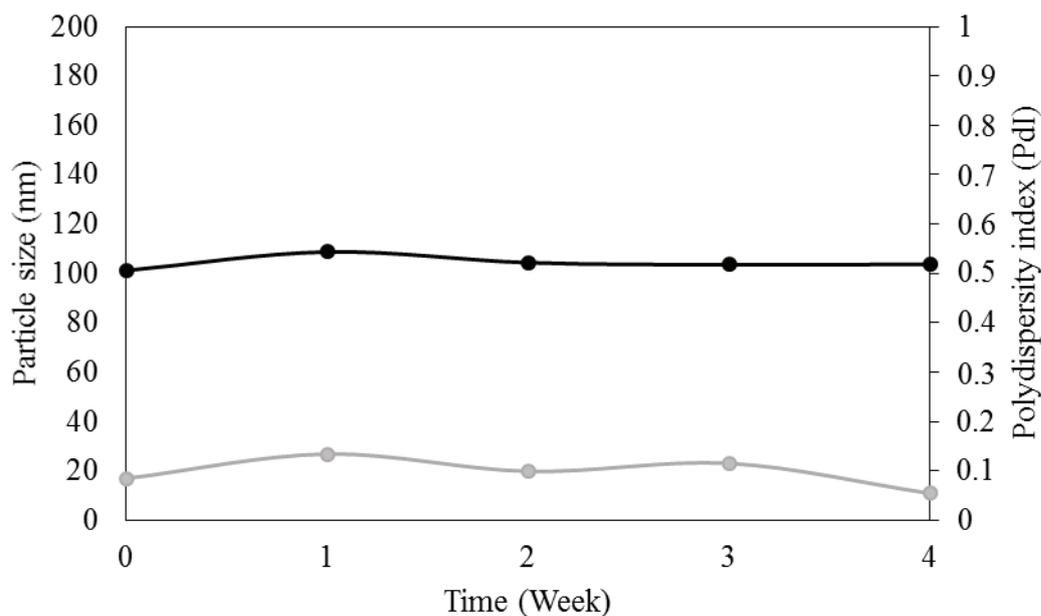


Figure 3.6 Stability of Cinnamon Oil Nanoemulsions During 4 Weeks.

Particle size: (—) and PdI: (—)

Figure 3.6 showed that mean particle diameter slightly increased from 101 nm to 103 nm during storage for 30 days at ~ 25 °C. ANOVA results show that, there is no significant difference for the mean particle size and PdI of the nanoemulsions during 1 month period ($p > 0.05$). These results confirmed the stability of our formulation. PdI gives information about the width of the droplet size distribution. In this study, PdI remained below 0.15 during a month storage time. This situation reflects their relative

homogeneity. When the PDI is smaller than 0.2, it shows generally a monodispersed size distribution (Tiwari & Tiwari, 2013). To reach the most favorable state (thermodynamically stable), activation energy of the system must be overcome. Because of the free energy barrier, ΔG^* , between two states, system remain kinetically stable (metastable) for a long period of time (for months or even years) (McClements, 2005). These experiment results indicated that height of energy barriers between nanoemulsion and separated states was high and these nanoemulsions showed long-term stability (McClements, 2012).

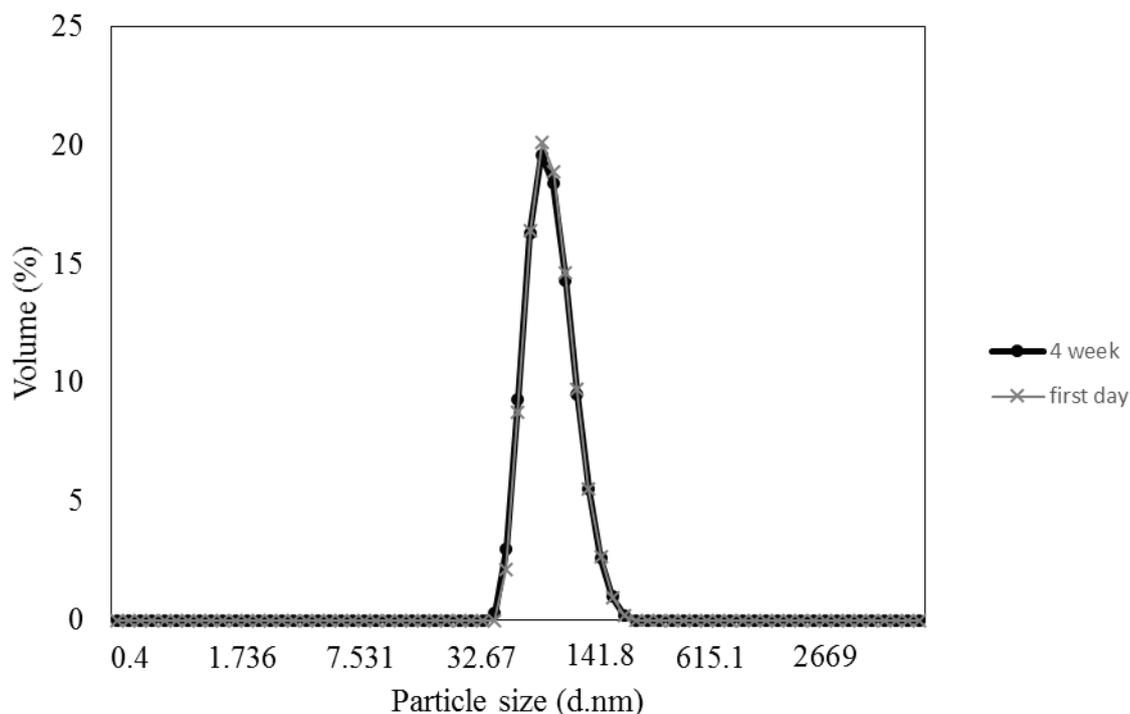


Figure 3.7 Size Distribution Graph of First Day and 30th Day of Nanoemulsion

Fig. 3.7 also indicated that no significant change was observed in mean particle size during one month storage. Size distribution was still almost same after 4 weeks.

3.6 Transmission Electron Microscopy Results

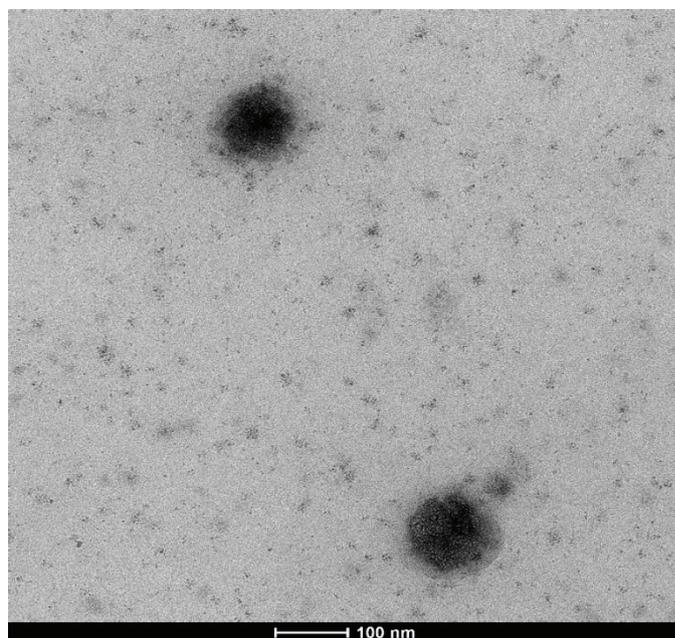
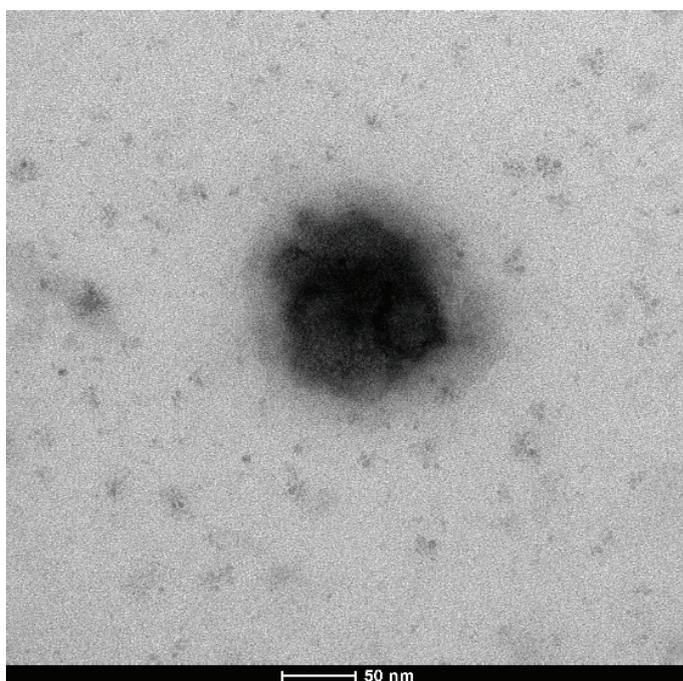


Figure 3.8 Transmission Electron Microscopy Bright Field Images of Nanoemulsion

To confirm the particle shape and measured particle size in accordance with Malvern Nano ZS90 DLS equipment, Transmission electron microscopy (CTEM) analysis was also carried out (Bouchemal et al., 2004). Transmission electron micrographs of cinnamon oil nanoemulsion with 6:4 cinnamon oil to coconut oil ratio, is shown in Figure 3.8. Results indicated that emulsion droplets were spherical in shape and the droplet size was in nanometric range. Moreover, size of particles in images (≈ 100 nm) were almost same with the results of dynamic light scattering (Fig.3.8).

3.7 High Pressure Homogenization (Microfluidization)

3.7.1 Effect of Oil Phase Composition on the Mean Particle Size of the Nanoemulsion

For this study; by using microfluidization, effect of oil phase composition on the characteristics of emulsion were investigated. As in the spontaneous emulsification, oil phase composition included cinnamon oil and coconut oil with different mass ratios (cinnamon oil: coconut oil = 2:8, 4:6, 6:4, 8:2, 10:0). System components were kept constant as 10 % oil phase, 10 % surfactant (Tween 80) and 80 % aqueous phase.

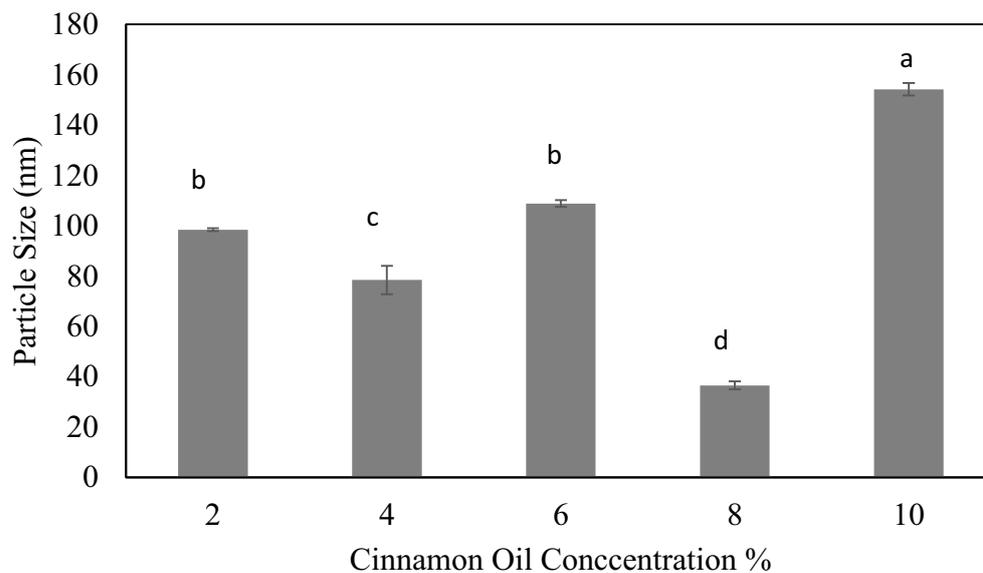


Figure 3.9 Effect of Oil Combination on Particle Size. Different letters represent significant difference ($p \leq 0.05$)

Similarly, for 8:2 CO: CNO oil ratio, smaller mean particle size was obtained but this result was significantly different than others (**Fig.3.9**) ($p < 0.05$). Generally, U shape curve (Chang et al., 2013 ; Chang & McClements, 2014) is obtained for the graph of particle size vs. concentration, however there were no any regular trend for decreasing and increasing of particle size of droplets.

3.8 Ultrasonication

3.8.1 Effect of Oil Phase Composition on the Mean Particle Size of the Nanoemulsion

For this study; by using an ultrasonic homogenizer, effect of oil phase composition on the characteristics of nanoemulsion were also investigated. Oil phase composition included cinnamon oil and coconut oil with different mass ratios (cinnamon oil: coconut oil = 2:8, 4:6, 6:4, 8:2, 10:0). System components were kept constant as 10 % oil phase, 10 % surfactant (Tween 80) and 80 % aqueous phase.

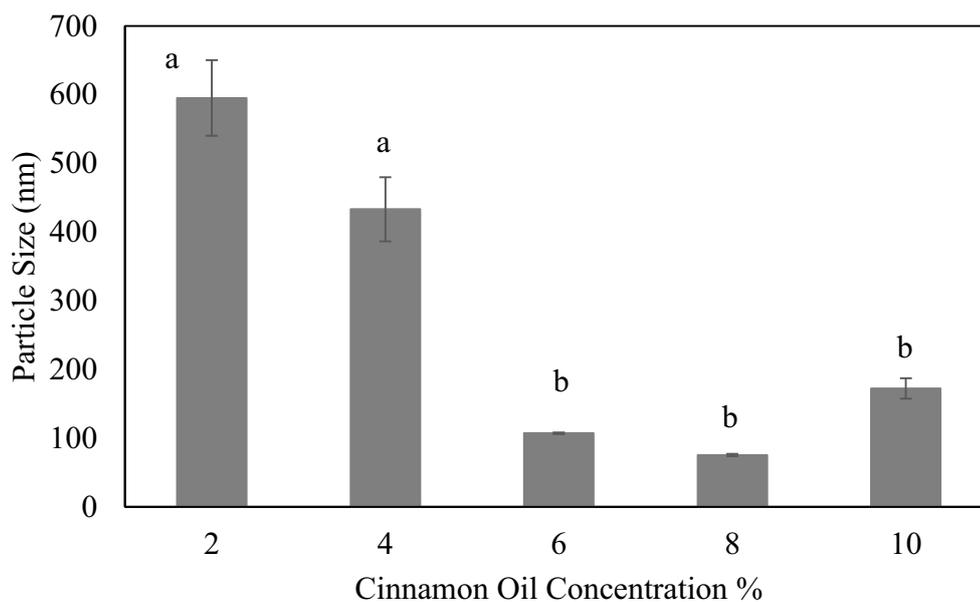


Figure 3.10 Effect of oil combination on particle size. Different letters represent significant difference ($p \leq 0.05$)

There were no significant difference between 6%, 8% and 10% CO concentrations in terms of mean particle size and smallest particle size (mean particle diameter) was obtained at 8% CO concentration as 75.4 nm (**Fig. 3.10**). However, formulation have a great effect on these results. In the study of Ghosh et al. (2013), almost same experiment procedure with this study was applied. Cinnamon oil nanoemulsions that included 6 wt % CO, Tween 80 (1:1 CO: Surfactant) and distilled water, were obtained by using ultrasonicator at 750 W during 10 minute (Ghosh et al., 2013). Higher energy was applied in this study however carrier oil was not used. This nanoemulsion had ~410 nm mean droplet size, although 6wt % CO containing nanoemulsion in this thesis study has ~107 nm. This big differences can be originated from effect of carrier oil in the system. Moreover, besides the concentrations of the individual components in nanoemulsion formulation , ultrasonication time and power could have effect on the particle size of final emulsion (Mahdi Jafari, He, & Bhandari, 2006). For this reason, if different process time and power was applied to system, different particle size results could be obtained with same formulations.

3.8.2 Comparison of Ultrasonication, Microfluidization and Spontaneous Emulsification for the Mean Particle Size

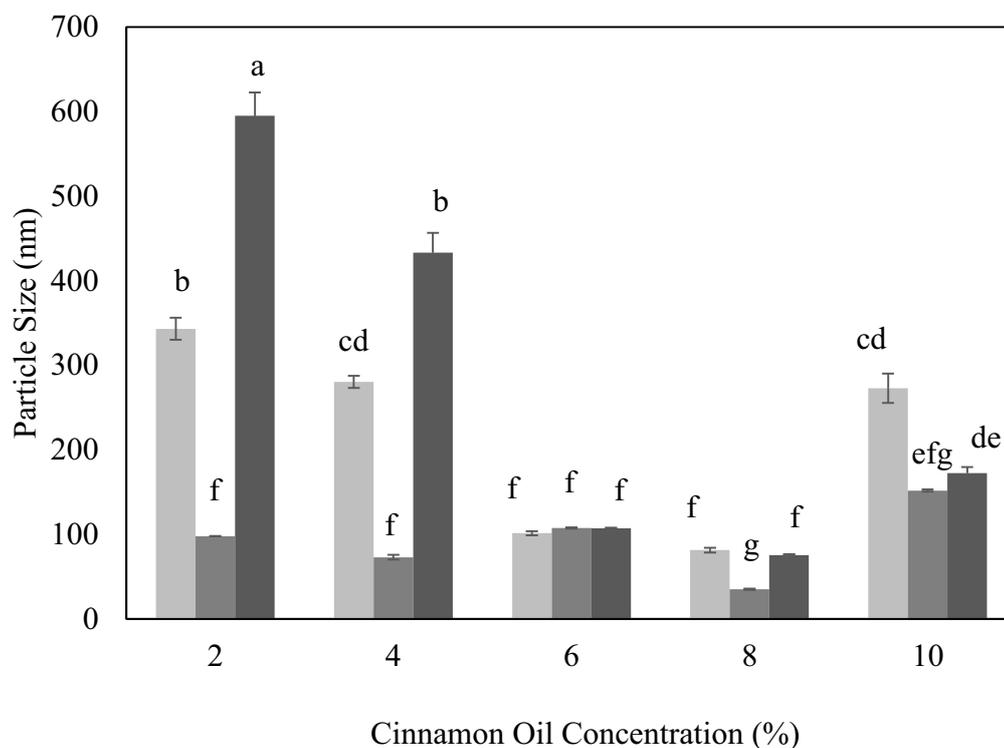


Figure 3.11 Effect of Ultrasonication, Microfluidization and SE on particle size. Ultrasonication: (■), Microfluidization: (■) and SE: (■) . ($p \leq 0.05$)

To understand the change in particle size between nanoemulsions that prepared with spontaneous emulsification, microfluidization and ultrasonication processes, obtained results were analyzed. According to ANOVA results, at lower CO % (2% and 4 %) concentrations, significantly different results were obtained and minimum value was achieved by microfluidization. However, at higher CO concentrations (6%, 8% and 10%), results were not significantly different, when three methods are compared at same CO % (Fig.3.11).

3.9 EXTRA SECTION: Importance of Correct Dilution on Particle Size

Detection of Nanoemulsions by Using Dynamic Light Scattering

DLS working principle is based on the Brownian motion of the particles. Due to relatively small particle size, Brownian motion dominates the gravitational force, thus nanoemulsion has high stability (Silva et al., 2012). If the emulsion shows instability, this means that inhomogeneity appears in the samples and this situation is detected with DLS through the particle random motion. This measurement depends on the detection of the translational diffusion coefficient (D) following the substitution on Stokes- Einstein equation (McClements, 2005; Ghosh et al., 2014). However, for correct measurement, particle – particle interactions should be eliminated by dilution. Especially for opaque nanoemulsions and high concentrated samples, correct dilution factor should be found. To detect this factor, mean particle size of gradually diluted samples should be measured. As a result, obtained mean particle size vs. concentration graphs should be plotted. With decreasing concentration, mean particle size increases to some extent and after that mean particle size value almost becomes fixed for nanoemulsions.

In this study, it was observed that from 1/5 dilution to 1/100 dilution, mean particle size increased and after 1/100 dilution mean particle size remained constant for 1/200, 1/500 and 1/1000 dilution. For this reason, in this study DLS measurements were conducted at 1/100 dilution. Effect of dilution on particle size difference can be seen in the **Figure 3.12**. With increasing dilution, mean particle size increased. The reason was that if adequate dilution was not done, particles would be in interaction with others. In other words, they would affect the random motion of each other's (McClements, 2005; Ghosh et al., 2014).

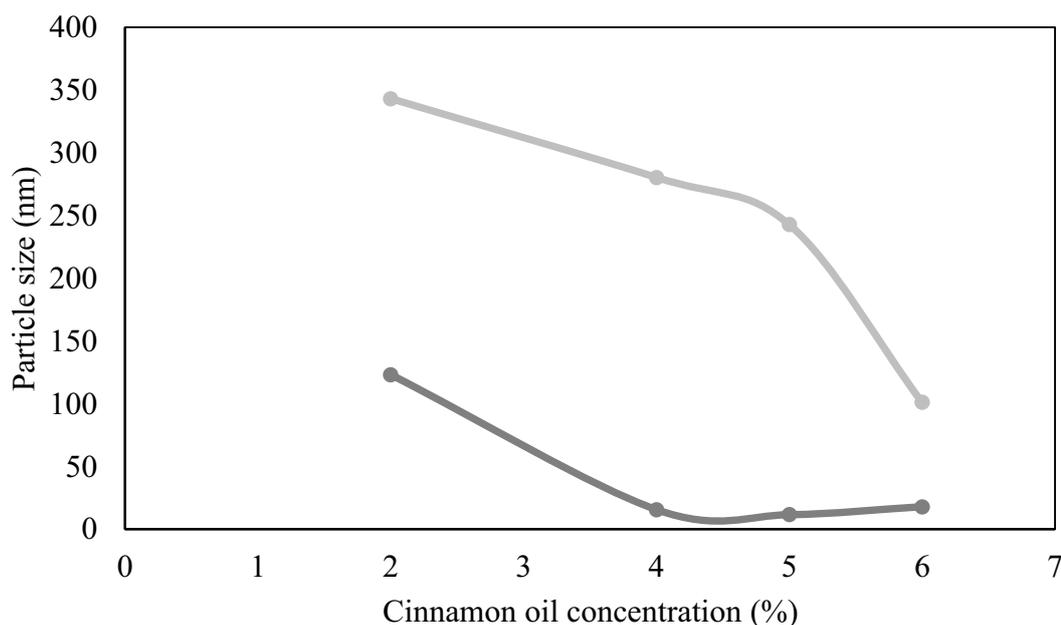


Figure 3.12 Effect of Dilution on Particle Size. 1/5: (■), 1/100 : (■).

3.10 Effects of Essential Oils and Bioactive Compounds on Antimicrobial Activity towards *E. coli*

3.10.1 Screening of Pure Essential Oils and Bioactive Compounds Based on Their Antimicrobial Activity

At the beginning of the study, effect of antimicrobial activity of some essential oils (100% concentrations) – cinnamon oil, rosemary oil , orange oil , peppermint oil , basil oil , lemon oil, camomile oil , thyme oil, fenugreek oil and some bioactive compounds (100% concentrations) – linalyl acetate and α – terpinene were investigated against *E. coli* by agar disc diffusion method (**Table 2.1**). Aim of these experiments was to screen the pure oil types or bioactive compounds for selecting the ones having higher antimicrobial effect against *E. coli*. Results showed that, antimicrobial effect was only

detected for cinnamon (CO) and rosemary oil (RO). For other oils and active materials, no clear zone was observed on the plates.

Disc diffusion experiment results of pure cinnamon oil and rosemary oil are given in Figure 3.13

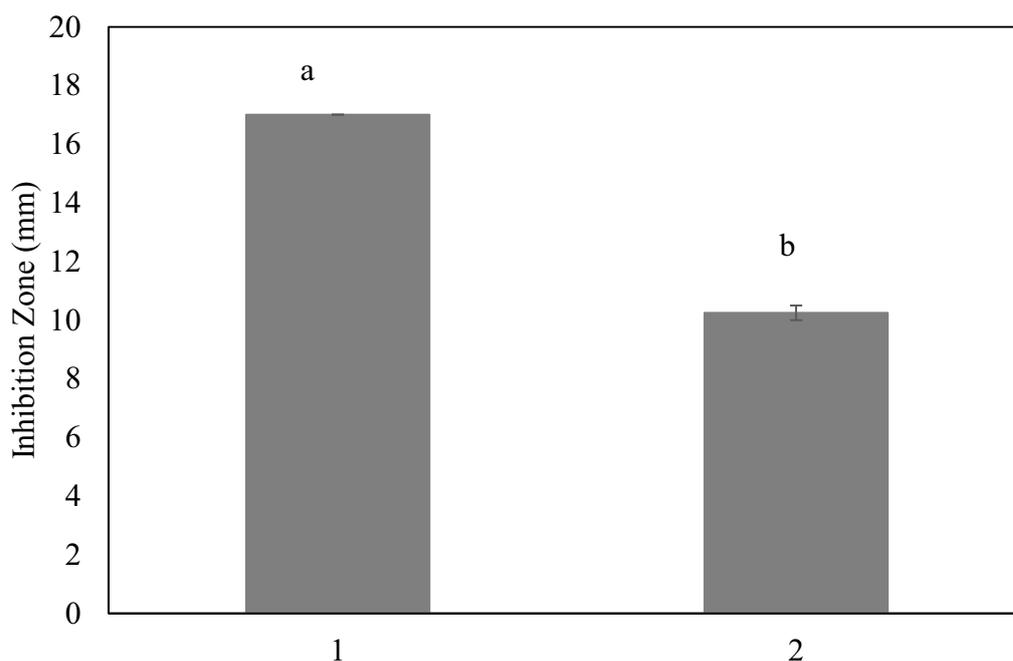


Figure 3.13 Antimicrobial Activity of Cinnamon Oil (CO) (1) and Rosemary Oil (RO)(2)

ANOVA results showed that there was significant difference between antimicrobial activities of cinnamon oil and rosemary oil towards *E. coli* ($p \leq 0.05$).

According to this results, CO was found to have higher antimicrobial effect than RO. With pure CO, 17mm zone was obtained (Fig.3.14), whereas with pure RO, 10.25 mm zone was recorded.

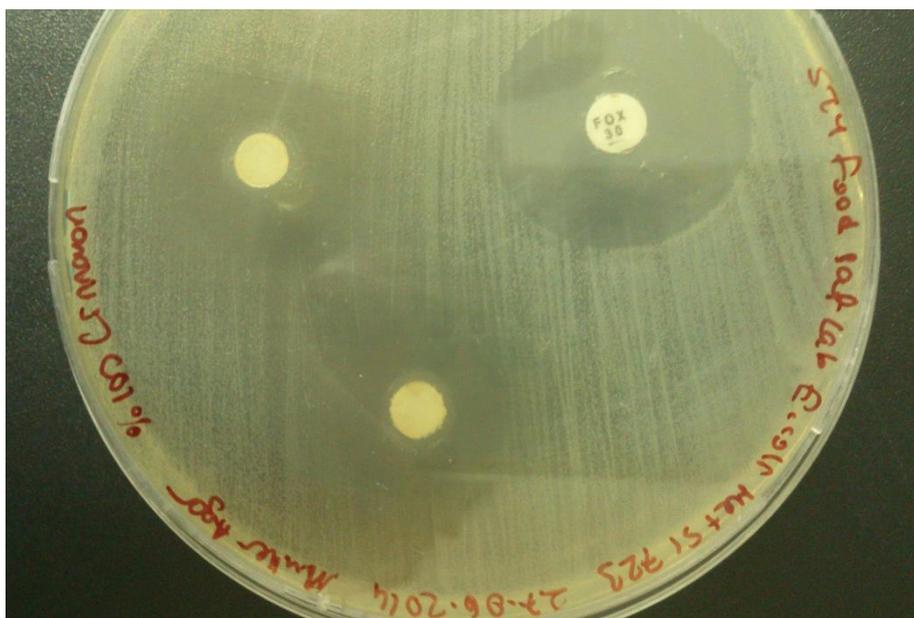


Figure 3.14 Antimicrobial Effect of 100% CO on *E. coli*

The difference of the antimicrobial activity can be originated in composition, structure and functional group variation of these essential oils. While CO is mainly composed of eugenol, cinnamaldehyde, β -caryophyllene, eugenol acetate, limonene (Tzortzakidis, 2009); RO include borneol, limonene, verbenone, camphor, α -pinene, 1,8-cineole in high quantity (Ozin, Ukic, Amojlik, & Ovin, 2007). This antimicrobial activity differences also can be resulted from the effect of these materials towards microorganisms used in experiments. For example, Nanasombat et al. (2011) reported that strongest antibacterial effect of cinnamon oil was obtained against *B. cereus* when compared with *E. coli* (Nanasombat & Wimuttigol, 2011).

In this study, ATCC25922 *E. coli* strain was used for antimicrobial tests. However it is known that, different strains of bacteria can cause different results. For instance, in the study of Ruparelia et al. (2008), to investigate the antimicrobial activity of copper and silver nanoparticle, 4 strains of *E. coli* (MTCC 443, MTCC 739, MTCC 1302, MTCC 1687) were used. At the end of the study it was found that most sensitive strain to silver and copper nanoparticles was MTCC 443 and among all *E. coli* strains,

MTCC 739 and MTCC 1687 showed least sensitivity (Ruparelia, Chatterjee, Duttagupta, & Mukherji, 2008)

In this study, agar disc diffusion method was used for the antimicrobial activity tests. However different antimicrobial test procedures can lead to different results. For example, in the study of Nanasombat et al. (2011), direct addition of active materials on filter paper without any drying procedure was performed. Filter papers (6mm) were placed on the agar (sabouraud dextrose agar) and 10 ml of 100% essential oils were added to filter paper immediately. For this test, diameter of the inhibition zone obtained was 24.5 mm for cinnamon oil against *E.coli* (Nanasombat & Wimuttigol, 2011). If compared, the amount of cinnamon oil was higher than the one used in this study. In addition to that, since cinnamon oil was directly put on filter paper and not kept for drying, this procedure incapacitated the absorption of all essential oil by filter paper and allowed the direct diffusion of non-absorbed oil. That's why, high antimicrobial effect was observed.

In another study to determine antimicrobial and antioxidant effect of rosemary essential oil (Ozin et al., 2007) , n-hexane was used as solvent to dilute RO and the hole plate agar diffusion method was used for antimicrobial susceptibility test. 22 mm inhibition zone was measured for 20% RO and 25 mm inhibition zone was measured for 50% RO. Although, RO was used at low concentrations, high antimicrobial effects compared to our study was observed. In this method, filter paper was not used and agar surface were punched to put the tested essential oil in it. This procedure might also have affected the diffusion mechanism.

In addition to these studies, Afsharzadeh et al. (2013), investigated the microorganism sensitivity in diffusion methods (the disc diffusion, hole plate, and cylinder agar diffusion and agar dilution methods). Most antimicrobial resistant bacteria against extracts of conifer was found as *E. coli* among the other three: *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*. In accordance with this study, weak antimicrobial effect was found against *E.coli* by hole plate agar diffusion

method, although it demonstrated complete resistance in disc diffusion method. The reason of this was explained with low extract diffusion and inhibition of active material diffusion by precipitation of insoluble materials (Afsharzadeh, Naderinasab, & Tayarani, 2013).

3.10.2 Effects of Essential Oil Mixture on Antimicrobial Activity

In order to investigate the utilization of 2 essential oils in combination, oils having high antimicrobial activity (cinnamon oil and rosemary oil) were mixed at 1:1 ratio.

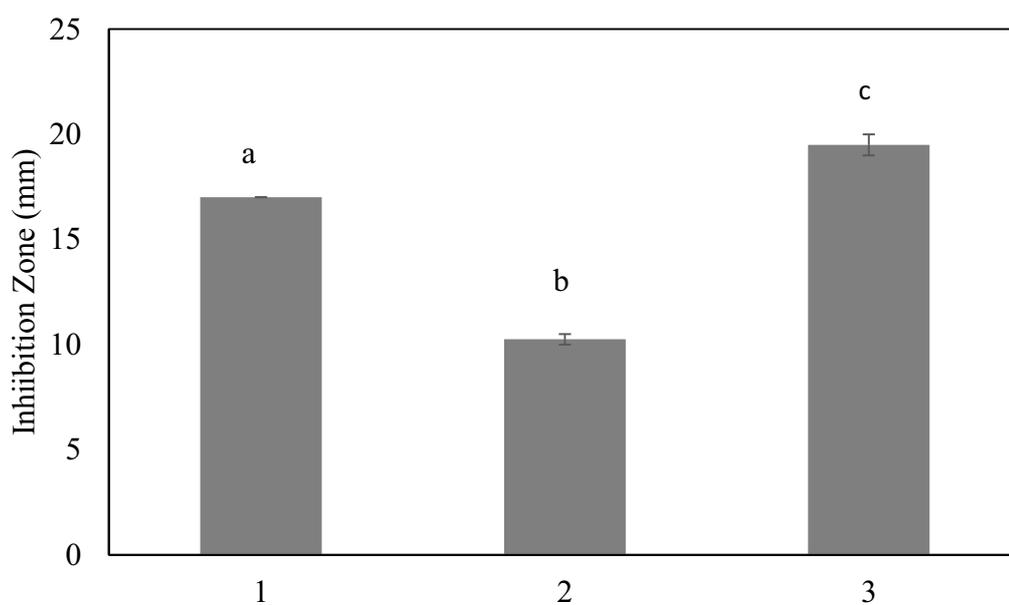


Figure 3.15 Antimicrobial Activity of Pure Oils and Combination of Oils (1:1)
(1:CO , 2: RO, 3: Combination of oils)

Results showed that antimicrobial activity of CO, RO and combination of these oils were found significant different from each other, ($p \leq 0.05$) (Fig. 3.15).

When the sum of the individual effects is equal to the sum of the combined effect, the resulting case is called additive antibacterial activity and if absence of interaction exists it is referred as indifferent (Bassolé & Juliani, 2012) . For this reason, the effect was between additive and indifferent in this experiment (Fig. 3.16)

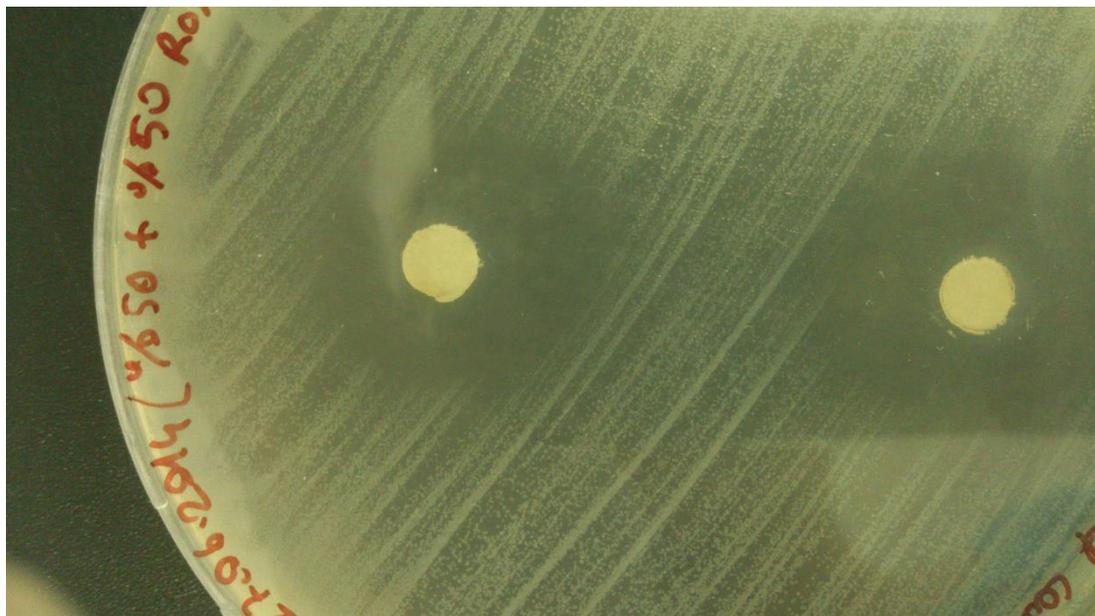


Figure 3.16 Antimicrobial Effect of CO – RO Mixture (1:1) on *E. coli*

Combination of either single essential oils or purified active materials have effect on multiple biochemical processes in bacteria. According to the research of Bassolé et al. (2012), phenolic monoterpenes and phenylpropanoids (eugenol) showed strong antimicrobial effect when in combination with other components that increased the bioactivity of the mixture. It was also found that combination of phenolics (thymol and carvacrol) with eugenol demonstrated synergistic effect against *E. coli* strains (Bassolé & Juliani, 2012).

These results showed that higher antimicrobial effects could be obtained with combinations of different oils. However, this mixture altered in odour and aroma that would be unavoidable. Therefore, it was decided to continue to next experiments only by using cinnamon oil.

3.10.3 Effect of Cinnamon Oil Concentration on Antimicrobial Activity of Solutions

In this part of the study, antimicrobial effect was examined for 9 different concentrations (100%, 80%, 60%, 40%, 20%, 10%, 8%, 6% and 0.5 %).

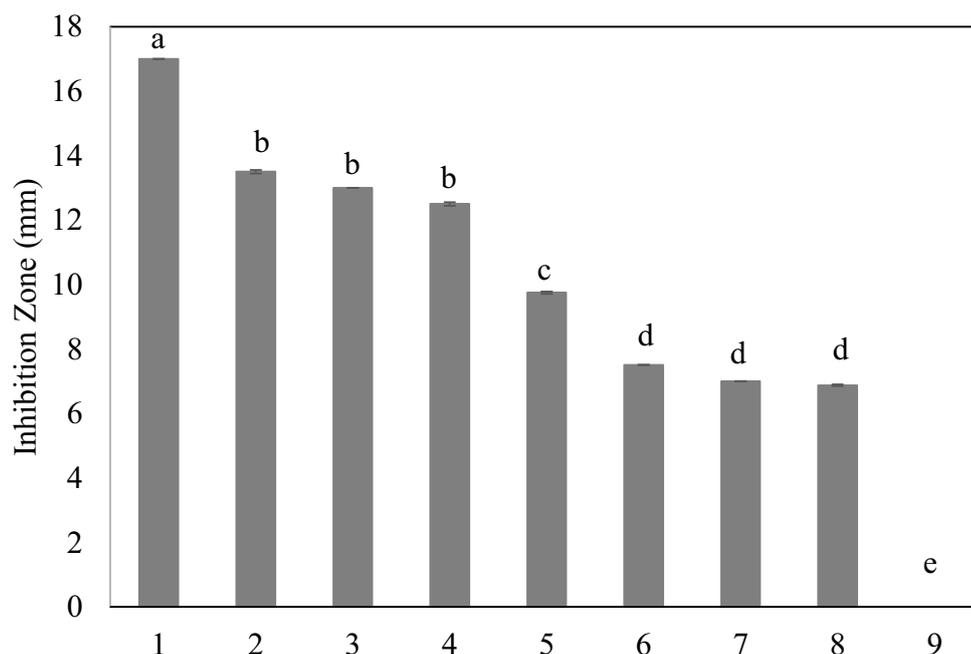


Figure 3.17 Antimicrobial Activity of CO at Different Concentrations (1: 100% , 2: 80% , 3: 60% , 4:40% , 5:20% , 6:10% , 7:8% , 8:6% , 9:0,5%) Different letters represent significant difference ($p \leq 0.05$)

According to ANOVA results, there was no significant difference between samples containing 80%, 60% and 40% CO. Similarly, significant difference was not observed between 10%, 8% and 6% CO including samples (**Fig. 3.17**).

Used solvent to dilute the samples expressed no antimicrobial activity in this experiment. Moreover, there was an increasing antimicrobial activity with increasing concentration of cinnamon oil. Similar results were obtained in other studies. Royo et

al. (2010) used filter paper test by agar diffusion method against *Salmonella* Enteritidis and *Staphylococcus aureus* to test the antimicrobial activity of oregano and sage essential oils. In this study, increasing antimicrobial activity was tested with increasing concentration of essential oils (Royo, Fernández-Pan, & Maté, 2010). In another study, antibacterial and antifungal potentials of leaves of *Cassia fistula* were investigated by agar disc diffusion method and linear increase of antibacterial and antifungal activity was observed with increase in concentration of the extracts (Bhalodia, Nariya, & Shukla, 2011). However, Figure 3.17 showed that there was not a significant increasing trend for antimicrobial activity as cinnamon oil concentration increased. This case can be explained that active materials present antimicrobial effects above some threshold values because some CO concentrations caused to formation of similar antimicrobial activity and solely above some values, increasing activity was observed.

3.10.4 Effect of Different Cinnamon Oil Concentration on Antimicrobial Activity of Nanoemulsions Obtained by Spontaneous Emulsification

Antimicrobial effect was examined for 5 different concentrations (10%, 8%, 6%, 4%, 2%). Antimicrobial activities of the nanoemulsions at different CO concentrations are given in Figure 3.18

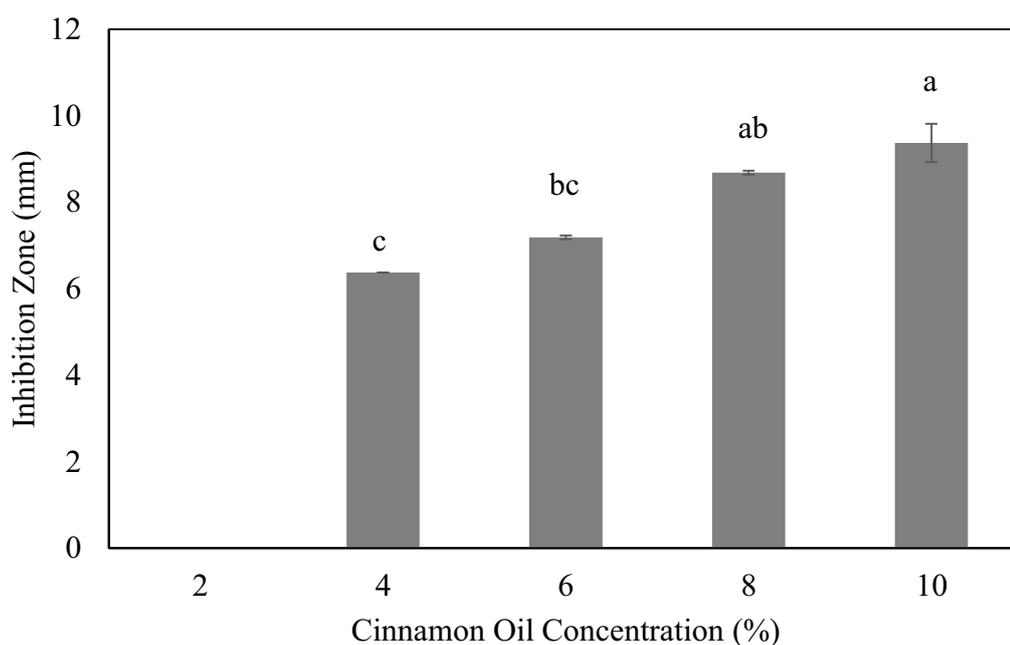


Figure 3.18 Effect of CO Concentration on Antimicrobial Activity of Nanoemulsions Obtained by Spontaneous Emulsification (SE). Different letters represent significant difference ($p \leq 0.05$)

2% cinnamon oil concentration did not show any antimicrobial activity against *E. coli* in all tests. With increasing active agent concentration, increasing antimicrobial activity was observed. Average inhibition zones were recorded as 6.3, 7.2, 8.6 and 9.3 mm for 4%, 6%, 8% and 10% CO concentrations, respectively (Fig. 3.18). Similar

results were obtained in other research studies. In the studies of inhibitory effect of carvacrol in nanoemulsions against yeasts, Chang et al. (2013) reported that with increasing carvacrol concentration in lipid phase of nanoemulsions, antimicrobial effect increased towards *Saccharomyces cerevisiae*. However, while 10%, 15%, 20% and 25% carvacrol concentrations in lipid phase showed similar effect, antimicrobial activity increased for 30% and % 40 carvacrol concentrations (Chang et al., 2013).

3.10.5 Effect of SOR on Antimicrobial Activity of Nanoemulsions

In these experiments, effect of surfactant concentration on antimicrobial activity of nanoemulsions was examined for 2 different concentrations of surfactant (SOR: 1 and SOR: 2) by SE method while the total amount of oil phase was held constant as 10 wt % (cinnamon oil: coconut oil 6:4 (w/w)) (Figure 3.19)

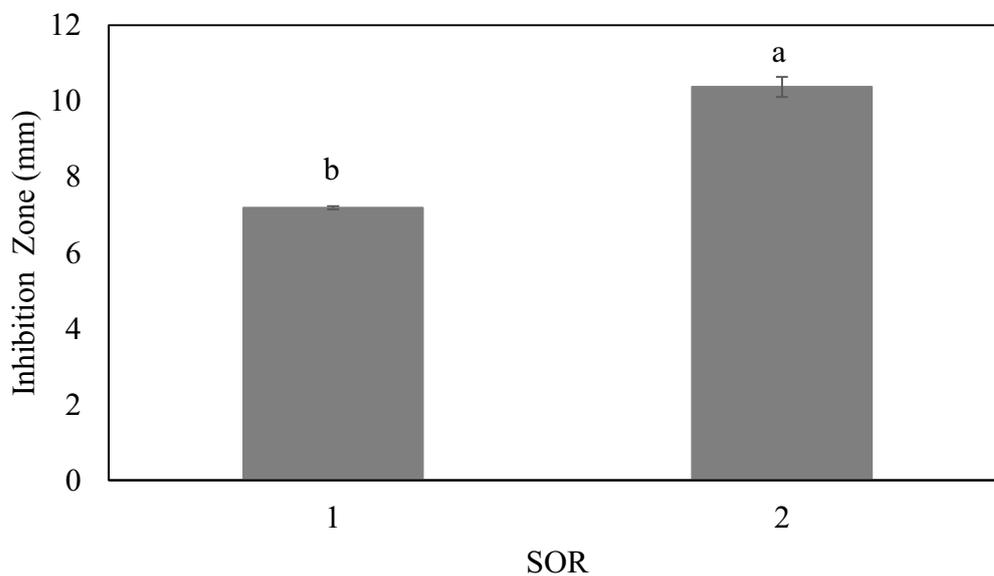


Figure 3.19 Effect of SOR on Antimicrobial Activity of Nanoemulsions.

As a result, average inhibition zones were 7.2 mm for SOR: 1 (Fig. 3.20) and 10.4 mm for SOR: 2 (Fig. 3.21). According to ANOVA results, these results were significantly different ($p \leq 0.05$)

With increasing surfactant amount, antimicrobial activity of nanoemulsions increased at the rate of 44.4%. The mean particle diameters were about 101 nm and 29 nm for SOR: 1 and SOR: 2, respectively (Fig. 3.4). This antimicrobial activity raising was because of droplet size decreasing. Particle size reduction causes increase in the surface area of droplets therefore interaction possibility with bacteria increases. As a result, antimicrobial activity increases (Ghosh et al., 2013). However, particle diameter decreasing rate did not same with increasing inhibition rate. Retaining active compounds powerfully in protective layer of high amount of surfactant can be source of this situation (Donsi et al., 2012).



Figure 3.20 Antimicrobial Activity of Nanoemulsion with SOR:1



Figure 3.21 Antimicrobial Activity of Nanoemulsion with SOR:2

3.10.6 Comparison of Antimicrobial Activity of Cinnamon Oil Solutions and Cinnamon Oil Nanoemulsions obtained by SE

To understand the change in antimicrobial activity between a cinnamon oil solution (cinnamon oil+ ethanol) and cinnamon oil nanoemulsion (cinnamon oil + coconut oil + Tween 80+ distilled water) prepared by SE method, agar disc diffusion method was conducted for the same concentrations of cinnamon oil.

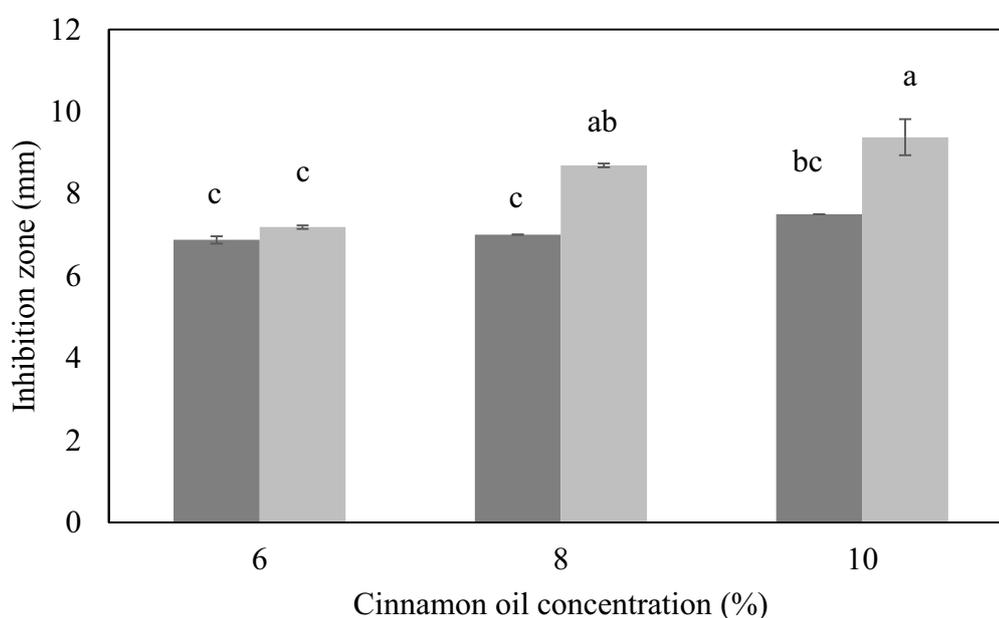


Figure 3.22 Effect of CO Concentration on Antimicrobial Activity.

Solution: (■) and Nanoemulsion:(■) Different letters represent significant difference ($p \leq 0.05$)

Different sample preparation methods affected the inhibition zones significant for 8% and 10% CO concentrations. There was approximately 24% increase for 8% CO and

25% increase for 10% CO concentration. Increase was not significant at 6% CO (Fig.3.22).

Because of increasing bioactivity of the substances with nanoemulsification process, increased antimicrobial activity is observed (Liang et al., 2012). For example, with formation of D- Limonene nanoemulsion, antimicrobial effect increased against *E.coli*, *L. delbrueckii* and *S. cerevisiae*. For this nanoemulsion system blending of D- Limonene, sunflower oil, Tween 20 and glycerol monooleate was used and for these microorganisms, minimum inhibitory concentration (MIC) was reduced from 25 g/l to 10 g/l (Donsi et al., 2011). Moreover, antimicrobial activity of pure peppermint oil was increased with nanoemulsion formation by using peppermint oil (PO), MCT, modified starch (food grade polymer) and water. When emulsions with PO and bulk PO was compared by MIC method, peppermint oil time- kill plots show that these nanoemulsions showed improved long term antimicrobial activity against *Listeria monocytogenes* and *Staphylococcus aureus* (Liang et al., 2012)

3.10.7 Effect of Different Cinnamon Oil Concentration on Antimicrobial Activity of Nanoemulsions obtained by Microfluidization

Antimicrobial effect was examined for 5 different concentrations (10%, 8%, 6%, 4%, 2%). Antimicrobial activities of nanoemulsions that were obtained by microfluidization are shown in Figure 3.23.

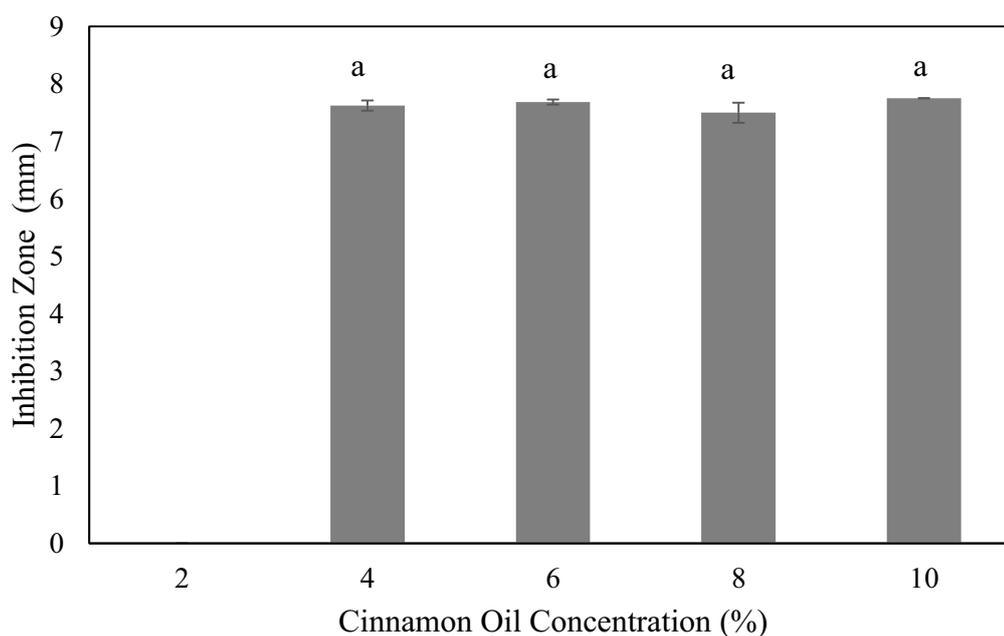


Figure 3.23 Effect of CO Concentration on Antimicrobial Activity of Nanoemulsion Obtained by Microfluidization. Different letters represent significant difference ($p \leq 0.05$)

As a result, average inhibition zones were 7.6, 7.7, 7.5, 7.7 for 4%, 6%, 8%, 10% CO concentrations, respectively and according to ANOVA results, there were no significant difference between all results for this study ($p > 0.05$). 2% cinnamon oil concentration again did not show any antimicrobial activity against *E. coli* in all tests.

These results were expected because there was no increasing antimicrobial activity with increasing active agent concentration. Similar results were also observed in the study of Royo et al. (2010). Nanoemulsions that contain oregano essential oils were prepared with high pressure homogenizer. However, there were no differences identified between nanoemulsions contain 40 and 80 g.kg⁻¹ oregano oil. Same inhibition zones were obtained against *S. Enteritidis* , in spite of increasing active component concentration (Royo et al., 2010). It is highly probable that temperature or high shear during microfluidization have destroyed components that responsible for the antimicrobial effect.

3.10.8 Comparison of Spontaneous Emulsification and Microfluidization for Antimicrobial Activity

To understand the antimicrobial activity differences between cinnamon oil nanoemulsions (cinnamon oil + coconut oil + Tween 80+ distilled water) that prepared by SE method and microfluidization, agar disc diffusion method was conducted for the same concentrations of cinnamon oil.

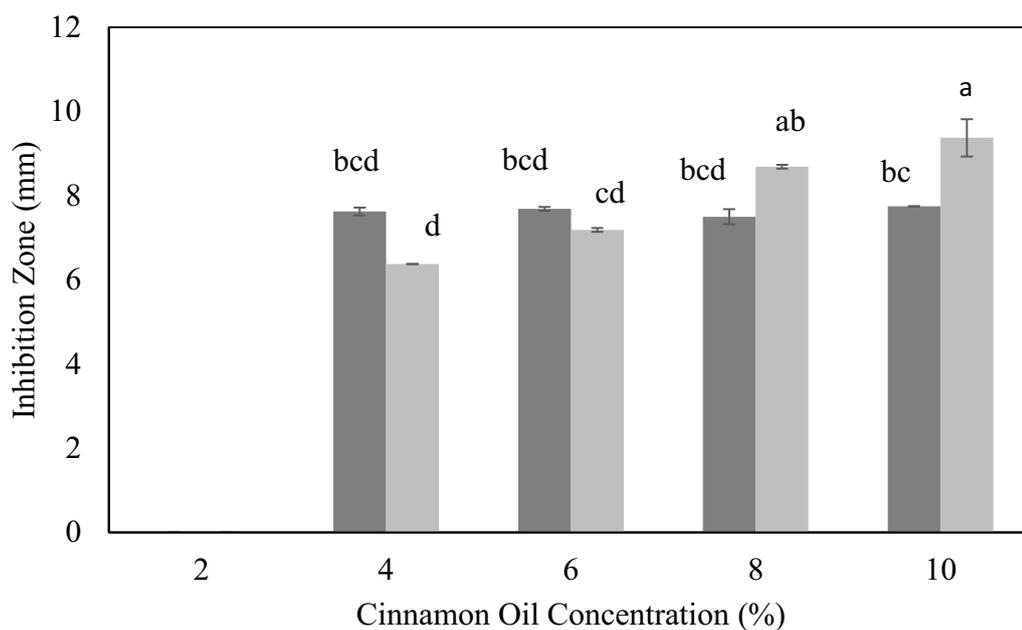


Figure 3.24 Effect of Nanoemulsification Method and CO Concentration on Antimicrobial Activity. Microfluidization (■) and SE(■)

According to ANOVA results, when microfluidization was used, for all cinnamon oil concentrations no significant difference was detected ($p > 0.05$) (**Fig.3.24**). Antimicrobial activity was almost same for all combinations. Likewise, when microfluidization and spontaneous emulsification (SE) were compared for 4%, 6% and 8% CO concentrations, there were no significant difference on the size of inhibition zones. As expected, increased active material amount, increased the inhibition by SE. With SE, at higher concentrations of active compounds, higher antimicrobial activity was obtained. Subjecting high mechanical stress can cause volatile antimicrobial agent loss therefore nearly same antimicrobial activity was observed as a result of microfluidization although increasing concentration of active compounds. However, low energy methods such as spontaneous emulsification is mostly favored to cover fragile active materials (Anton & Vandamme, 2009) and while not disrupting active materials so much, increased antimicrobial activity was observed by SE.

3.10.9 Effect of Different Cinnamon Oil Concentrations on Antimicrobial Activity of Nanoemulsions Obtained by Ultrasonication

Antimicrobial effect was examined for 5 different concentrations (10%, 8%, 6%, 4%, 2%). Antimicrobial activities of nanoemulsions that were obtained by ultrasonication are shown in Figure 3.25.

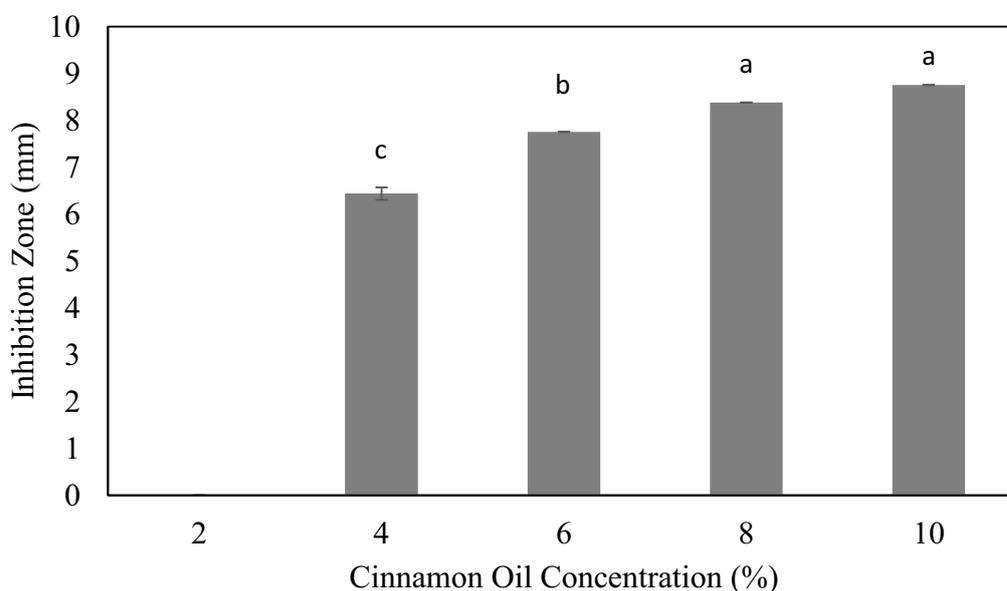


Figure 3.25 Effect of CO Concentration on Antimicrobial Activity of Nanoemulsion Obtained by Ultrasonication. Different letters represent significant difference ($p \leq 0.05$)

Average inhibition zones were 6.4, 7.7, 8.3, 8.7 mm for 4%, 6%, 8%, 10% CO concentrations, respectively. According to ANOVA results, there were no significant difference between 8% - 10% CO concentrations respectively. 2% cinnamon oil concentration again did not show any antimicrobial activity against *E. coli* in all tests.

Slightly increasing antimicrobial effect was observed with increasing CO concentrations by ultrasonic homogenization (Fig. 3.25)

3.10.10 Comparison of Spontaneous Emulsification and Ultrasonication for Antimicrobial Activity

To understand the antimicrobial activity differences between cinnamon oil nanoemulsions (cinnamon oil + coconut oil + Tween 80+ distilled water) that were prepared by SE method and ultrasonication, agar disc diffusion method was conducted for the same concentrations of cinnamon oil.

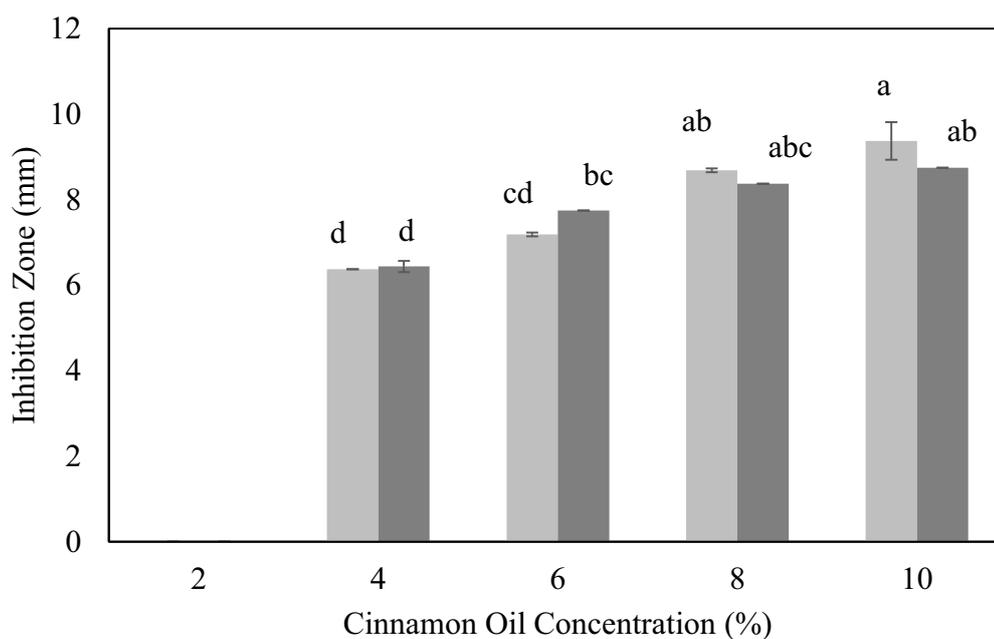


Figure 3.26 Effect of Nanoemulsification Method and CO Concentration on Antimicrobial Activity. Ultrasonication (■) and SE(■) Different letters represent significant difference ($p \leq 0.05$)

According to ANOVA results, there were no significant difference for antimicrobial activity, when two method was compared at same CO concentrations. With increasing CO concentrations, increased antimicrobial activity was observed for two methods (**Fig. 3.26**). At 6% and 8% CO concentrations, similar particle size was obtained (**Fig. 3.11**) and same antimicrobial effect was observed. However, although ultrasonication process formed smaller particles at 8% and 10% CO concentrations, antimicrobial activity became similar for both of these methods. Because of heat production during ultrasonication experiments, volatile compounds degraded and this volatile material loss can cause antimicrobial activity decrease (Capelo-Martínez, 2009)

3.10.11 Comparison of Spontaneous Emulsification and Ultrasonication for Antimicrobial Activity

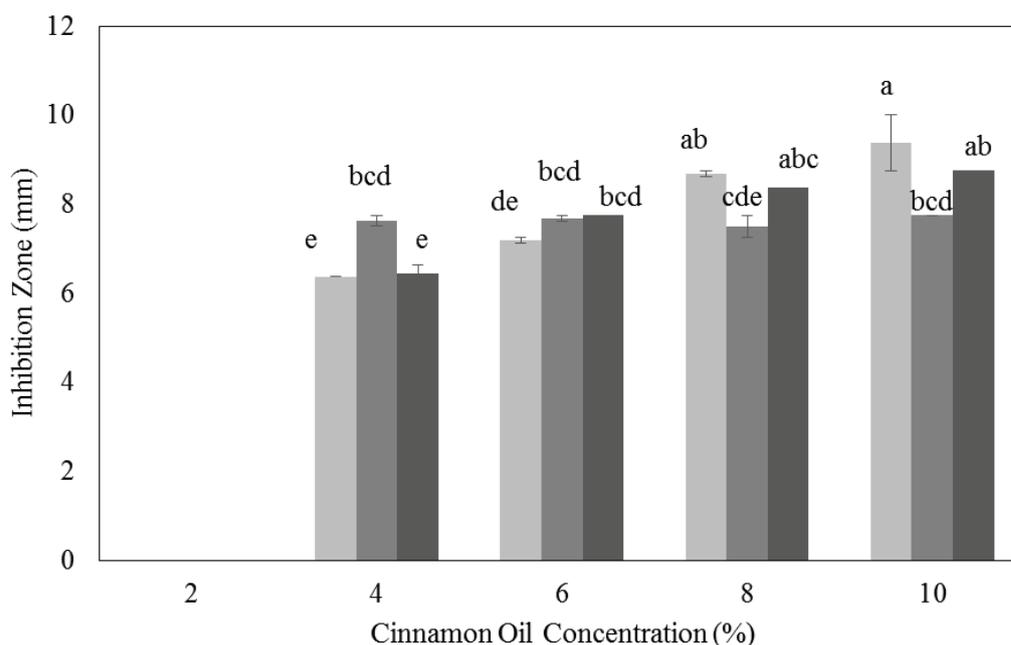


Figure 3.27 Effect of Ultrasonication, Microfluidization and SE on particle size. Ultrasonication: (■), Microfluidization: (■) and SE: (■)

To understand the change in antimicrobial activity between nanoemulsions that prepared with spontaneous emulsification, microfluidization and ultrasonication processes, obtained results were analyzed. 2% CO concentration did not show any antimicrobial activity against *E. coli* in all tests. According to ANOVA results, when microfluidization was used, there were no significantly different results for all cinnamon oil concentrations ($p > 0.05$). Moreover, when spontaneous emulsification and ultrasonication were compared at same CO%, there were no significant difference for antimicrobial activity. At higher CO concentrations (8% and 10%), nanoemulsions that obtained by SE and ultrasonication, exhibited higher antimicrobial activity.

CHAPTER 4

CONCLUSION

Essential oils have strong antibacterial, antifungal and antioxidant properties. For this reason, in recent years the use of essential oils has been widespread instead of synthetic chemical compounds. Oil-in- water (O/W) nanoemulsions systems are most commonly used as colloidal delivery system for entrapping these functional components into the aqueous based food, beverage ,cosmetic, pharmaceutical and chemical products. Cinnamon oil (CO) contains highly active compounds and to utilize these materials, nanoemulsions can be formulated. In this study, to understand the effect of cinnamon oil in nanoemulsion systems; formulation, characterization and antimicrobial activity tests were conducted. Two important outcomes of the study were found as:

- Obtaining stable cinnamon oil nanoemulsion by using spontaneous emulsification method at lower surfactant concentrations when compared with the studies in literature;
- Utilizing coconut oil as a second oil (carrier oil) in the system rather than using an expensive MCT mixture.

Fatty acid analysis of coconut oil showed that MCTs amount was very high and coconut oil (CNO) was found to be effective as a carrier oil.

Different oil combinations and concentrations were examined with spontaneous emulsification (SE). Smaller mean particle sizes were obtained for 6:4 and 8:2 CO-CNO ratios. However, when the size distribution graph was taken into consideration and tendency to phase separation was observed, the ratio of 6:4 was believed to be more appropriate for stability experiments (*e.g.*, stability test). Highly stable nanoemulsions were obtained with spontaneous emulsification at 6:4 CO-CNO ratio with the aid of coconut oil. No phase separation was observed at the end of 4 weeks

period and kinetic stability of this nanoemulsion was confirmed. Without using coconut oil, stable nanoemulsions were not formed. Both DLS and TEM gave parallel results and mean particle size of the stable NEs are found ~100 nm for 6:4 (cinnamon oil: coconut oil) combination.

Effect of SE on antimicrobial activity against *E. coli* were found to be enhanced compared to solutions that include cinnamon oil at same concentrations. Results showed that antimicrobial activity increased 10- 25% by spontaneous emulsification. NEs were also prepared by using microfluidization and ultrasonication. However, at higher CO concentrations (6%, 8% and 10%), results were not significantly different, when three methods are compared at same CO %. Moreover, antimicrobial activity could not be enhanced by microfluidization towards *E. coli* for all CO concentrations. At higher CO concentrations (8% and 10%), enhanced antimicrobial activity were obtained by spontaneous emulsification and ultrasonication methods and the results were not found to be significantly different ($p>0.05$). However, considering the cost of the process and the test volumes studied, spontaneous emulsification was found to be more appropriate for this nanoemulsion system.

The result of this study showed that these nanoemulsions did not inhibit the *E. coli* completely with agar disc diffusion method however they showed antimicrobial effect to some extent. The use of essential oil loaded nanoemulsions in food applications can be promising because they show critical effects on final antimicrobial activity of the products while meeting consumer demands with natural products which are free of synthetic additives. Moreover, even though complete inhibition is not ensured, these type of formulations can be used to enhance the antimicrobial effect in drugs with antibiotic and EOs combination. Therefore, antibiotic usage can be reduced with obtaining synergistic effect.

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APPENDIX A

STATISTICAL ANALYSES

Table A.1 One way ANOVA for Effect of cinnamon oil concentration on particle size of nanoemulsion prepared by spontaneous emulsification method .

Source	DF	SS	MS	F	P
CO %	4	109651	27413	42.35	0.000
Error	5	3237	647		
Total	9	112888			

CO (%)	Mean	Grouping
2	343.15	A
4	280.40	A
10	272.70	A
6	101.21	B
8	81.39	B

Table A.2 One way ANOVA for Effect of different oil combinations on Pdl of nanoemulsion

Source	DF	SS	MS	F	P
CO %	4	0.4263	0.1066	8.16	0.020
Error	5	0.0653	0.0131		
Total	9	0.4915			

<u>CO (%)</u>	<u>Mean</u>	<u>Grouping</u>
2	0.7215	A
10	0.4735	A B
4	0.4425	A B
8	0.3365	A B
6	0.0850	B

Table A.3 One way ANOVA for Effect of SOR on the particle size of sample

<u>Source</u>	<u>DF</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>P</u>
SOR	2	20409.7	10204.8	113.80	0.001
Error	3	269.0	89.7		
Total	5	20678.7			

<u>SOR</u>	<u>Mean</u>	<u>Grouping</u>
0.5	171.38	A
1.0	101.21	B
2.0	28.52	C

Table A.4 One way ANOVA for Effect of cinnamon oil concentration on particle size of nanoemulsion obtained by microfluidizer

Source	DF	SS	MS	F	P
CO %	4	14914.9	3728.7	217.40	0.000
Error	5	85.8	17.2		
Total	9	15000.7			

CO (%)	Mean	Grouping
10	151.70	A
6	107.45	B
2	97.84	B
4	72.78	C
8	34.94	D

Table A.5 One way ANOVA for Effect of cinnamon oil concentration on particle size of nanoemulsion obtained by ultrasonicator

Source	DF	SS	MS	F	P
CO %	4	411865	102966	47.49	0.000
Error	5	10840	2168		
Total	9	422705			

CO (%)	Mean	Grouping
2	595.00	A
4	433.20	A
10	172.30	B
6	107.20	B
8	75.47	B

Table A.6 Two way ANOVA for Comparison of ultrasonication, microfluidization and spontaneous emulsification for particle size

Source	DF	Seq SS	Adj SS	Adj MS	F	P
E type	2	175160	175160	87580	92.74	0.000
Concentration	4	314084	314084	78521	83.15	0.000
E type*Concentration	8	222295	222295	27787	29.42	0.000
Error	15	14165	14165	944		
Total	29	725705				

Emulsification type	CO Concentration	Mean	Grouping
U	2	595.0	A
U	4	433.2	B
SE	2	343.1	B C
SE	4	280.4	C D
SE	10	272.7	C D E
U	10	172.3	D E F
HE	10	151.7	E F G
HE	6	107.3	F G
U	6	107.2	F G
SE	6	101.3	F G
HE	2	97.8	F G
SE	8	81.4	F G
U	8	75.5	F G
HE	4	72.8	F G
HE	8	34.9	G

Table A.7 One way ANOVA for Antimicrobial activity of cinnamon oil and Rosemary oil at 100% concentrations against *E. coli* ATCC 25922

Source	DF	SS	MS	F	P
Cinnamon & Rosemary oil	1	45.5625	45.5625	729.00	0.001
Error	2	0.1250	0.0625		
Total	3	45.6875			

Oil Type	Mean	Grouping
CO	17.0000	A
RO	10.2500	B

Table A.8 One way ANOVA for Antimicrobial activity of cinnamon oil (pure) , Rosemary oil (pure) and their combinations (1:1) against *E. coli* ATCC 25922

Source	DF	SS	MS	F	P
CO&RO& Combination	2	91.583	45.792	219.80	0.001
Error	3	0.625	0.208		
Total	5	92.208			

<u>Oil Type</u>	<u>Mean</u>	<u>Grouping</u>
Combination	19.500	A
CO	17.000	B
RO	10.250	C

Table A.9 One way ANOVA for Antimicrobial activity of cinnamon oil solutions at different concentrations against *E. coli* ATCC 25922

<u>Source</u>	<u>DF</u>	<u>SS</u>	<u>MS</u>	<u>F</u>	<u>P</u>
CO %	8	401.319	50.165	390.47	0.000
Error	9	1.156	0.128		
Total	17	402.476			

<u>CO (%)</u>	<u>Mean</u>	<u>Grouping</u>
100.0	17.000	A
80.0	13.500	B
60.0	13.000	B
40.0	12.500	B
20.0	9.750	C
10.0	7.500	D
8.0	7.000	D
6.0	6.875	D
0.05	0.000	E

Table A.10 One way ANOVA for Effect of cinnamon oil concentration on antimicrobial activity of nanoemulsion obtained by spontaneous emulsification

Source	DF	SS	MS	F	P
CO %	3	11.258	3.753	18.84	0.008
Error	4	0.797	0.199		
Total	7	12.055			

CO (%)	Mean	Grouping
10	9.3750	A
8	8.6875	A B
6	7.1875	B C
4	6.3750	C

Table A.11 One way ANOVA for Effect of SOR on antimicrobial activity of nanoemulsions

Source	DF	SS	MS	F	P
SOR	1	10.160	10.160	70.30	0.014
Error	2	0.289	0.145		
Total	3	10.449			

SOR	Mean	Grouping
2	10.3750	A
1	7.1875	B

Table A.12 Two way ANOVA for Comparison of Nanoemulsions Obtained by Spontaneous Emulsification and Solutions for Antimicrobial Activity

Source	DF	Seq SS	Adj SS	Adj MS	F	P
E type	1	5.0052	5.0052	5.0052	36.26	0.001
Concentration	2	3.9870	3.9870	1.9935	14.44	0.005
E type*Concentration	2	1.4557	1.4557	0.7279	5.27	0.048
Error	6	0.8281	0.8281	0.1380		
Total	11	11.2760				

Emulsification type	CO Concentration	Mean	Grouping
SE	10	9.4	A
SE	8	8.7	A B
S	10	7.5	B C
SE	6	7.2	C
S	8	7.0	C
S	6	6.9	C

Table A.13 One way ANOVA for Effect of cinnamon oil concentration on antimicrobial activity of nanoemulsion obtained by microfluidization

Source	DF	SS	MS	F	P
CO %	3	0.19336	0.06445	6.60	0.050
Error	4	0.03906	0.00977		
Total	7	0.23242			

CO (%)	Mean	Grouping
10	7.75000	A
4	7.75000	A
8	7.68750	A
6	7.37500	A

Table A.14 Two way ANOVA for Comparison of Spontaneous Emulsification and Microfluidization for antimicrobial activity

Source	DF	Seq SS	Adj SS	Adj MS	F	P
E type	1	0.2822	0.2822	0.2822	2.35	0.164
Concentration	3	5.7451	5.7451	1.9150	15.94	0.001
E type*Concentration	3	5.5811	5.5811	1.8604	15.49	0.001
Error	8	0.9609	0.9609	0.1201		
Total	15	12.5693				

Emulsification Type	CO Concentration	Mean	Grouping
SE	10	9.4	A
SE	8	8.7	A B
MF	10	7.8	B C
MF	6	7.7	B C D
MF	4	7.6	B C D
MF	8	7.5	B C D
SE	6	7.2	C D
SE	4	6.4	D

Table A.15 One way ANOVA for Effect of cinnamon oil concentration on antimicrobial activity of nanoemulsion obtained by ultrasonication

Source	DF	SS	MS	F	P
CO %	3	6.1777	2.0592	117.15	0.000
Error	4	0.0703	0.0176		
Total	7	6.2480			

CO (%)	Mean	Grouping
10	8.7500	A
8	8.3750	A
6	7.7500	B
4	6.4375	C

Table A.16 Two way ANOVA for Comparison of Spontaneous Emulsification and Ultrasonication for antimicrobial activity

Source	DF	Seq SS	Adj SS	Adj MS	F	P
E type	3	16.6514	16.6514	5.5505	51.20	0.000
Concentration	1	0.0244	0.0244	0.0244	0.23	0.648
E type*Concentration	3	0.7842	0.7842	0.2614	2.41	0.142
Error	8	0.8672	0.8672	0.1084		
Total	15	18.3271				

Emulsification Type	CO Concentration (%)	Mean	Grouping
SE	10	9.4	A
U	10	8.8	A B
SE	8	8.7	A B
U	8	8.4	A B C
U	6	7.8	B C
SE	6	7.2	C D
U	4	6.4	D
SE	4	6.4	D

Table A.17 Two way ANOVA for Comparison of ultrasonication, microfluidization and spontaneous emulsification for antimicrobial activity

Source	DF	Seq SS	Adj SS	Adj MS	F	P
E type	2	0.2982	0.2982	0.1491	1.73	0.218
Concentration	3	11.2344	11.2344	3.7448	43.58	0.000
E type*Concentration	6	6.2695	6.2695	1.0449	12.16	0.000
Error	12	1.0313	1.0313	0.0859		
Total	23	18.8333				

Emulsification Type	CO Concentration (%)	Mean	Grouping
SE	10	9.4	A
U	10	8.8	A B
SE	8	8.7	A B
U	8	8.4	A B C
U	6	7.8	B C D
MF	10	7.8	B C D
MF	6	7.7	B C D
MF	4	7.6	B C D
MF	8	7.5	C D E
SE	6	7.2	D E
U	4	6.4	E
SE	4	6.4	E

APPENDIX B

CRITICAL MICELLE CONCENTRATION DETERMINATION

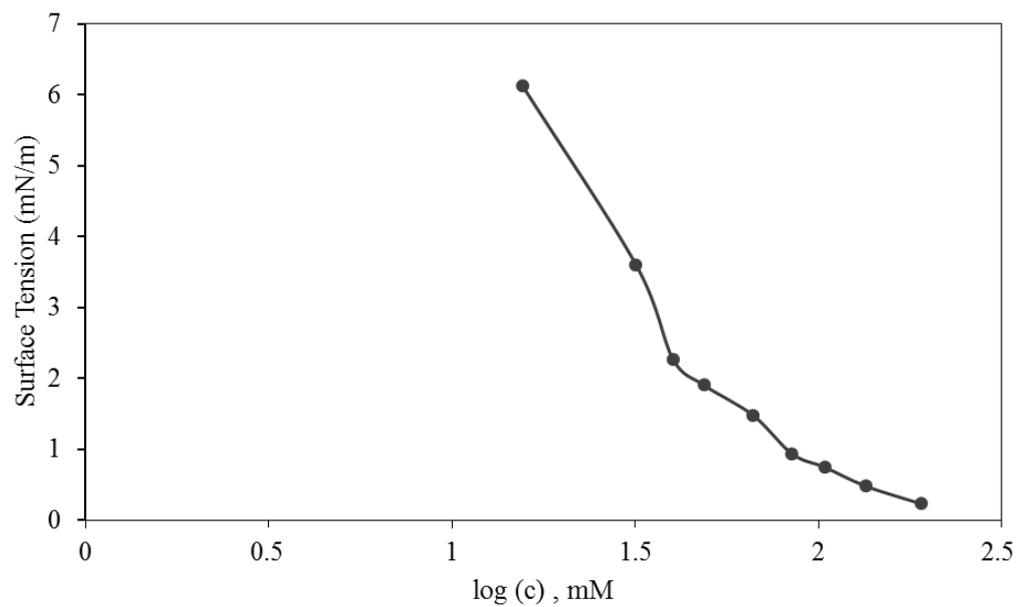


Figure B.1 Critical Micelle Concentration Determination