Discussion
Chapter 4

Discussion

Microemulsions and nanoemulsions have their potential advantages, over conventional emulsions, such as optical transparency and greater stability (McClements 2002a; McClemets 2002b). These potential advantages owe to their low droplet size i.e. <100 nm (McClements 2011; McClements 2012). Pioneer work on microemulsion was done in 1940s (Hoar and Schulman 1943) but reports on nanoemulsions are available since 1980s only (Benita et al 1986a; Benita et al 1986b; Levy and Benita 1989). Nanoemulsion formulation gained attention due to its promising application in delivery of water-insoluble/sparsely-soluble drugs (Benita et al 1986a; Benita et al 1986b; Levy and Benita 1989; Bradley 1998; Santos-Magalhaes et al 2000; Bourdon et al 2000; Jenning et al 2000). These nano-range delivery agents were formulated using vegetable oil such as soybean oil, etc (Wretlind 1981). However, there were not many reports on nanoemulsion formulation with plant essential oil. This aroused our interest to work on plant essential oil based microemulsion and nanoemulsion formulation; and its potential applications as antibacterial agent, antiseptic agent in wound healing and larvicidal agent for mosquito vector control.

4.1 MICROEMULSION FORMULATION

Formulation of microemulsion was optimized for different process parameters such as oil type, surfactant type, surfactant concentration and oil-surfactant mixing ratio to obtain microemulsion with lowest droplet size and greater kinetic stability. Table 4.1 shows the optimized process parameters for different microemulsion formulation.
Table 4.1. Optimized parameters of microemulsion formulated using different plant essential oils

<table>
<thead>
<tr>
<th>Optimized Parameters*</th>
<th>Basil oil</th>
<th>Cinnamon oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (nm)</td>
<td>11.49</td>
<td>5 ± 0.32</td>
</tr>
<tr>
<td>Oil : Surfactant mixing ratio (vol/vol)</td>
<td>1 : 5</td>
<td>1 : 4</td>
</tr>
<tr>
<td>Surfactant concentration (vol %)</td>
<td>30</td>
<td>24</td>
</tr>
</tbody>
</table>

*Surfactant = Tween20; Visual appearance = Transparent

4.1.1 EFFECT OF OIL TYPE ON EMULSION DROPLET SIZE

Microemulsion was formulated using basil oil, cinnamon oil, mustard oil and sesame oil by spontaneous emulsification. Basil oil and cinnamon oil formed thermodynamically stable microemulsion with droplet size of 11 nm and 5.7 nm respectively, whereas mustard oil and sesame oil did not form thermodynamically stable emulsions and the droplet size was also above 500 nm. Hence, detailed characterization was done for basil oil and cinnamon oil microemulsion only, and not for mustard oil and sesame oil emulsion.

Effect of oil type on emulsion droplet size may be due to low viscosity of the oil. Our results corroborated with Jafari et al (2008) and Qian and McClements (2011), that different oil system form emulsion with different droplet size, in these cases flavor oils form emulsion with reduced droplet size when compared to triglyceride oils. Kolb et al (2001) also have shown that increase in the viscosity of the oil system increases emulsion droplet size.

4.1.2 EFFECT OF SURFACTANT CONCENTRATION ON EMULSION DROPLET SIZE

Normally surfactants stabilize oil-in-water microemulsion. Brooks et al (1998) reported that surfactants with lower hydrophile-lipophile balance HLB value (3-6) favor
the formation of water-in-oil emulsion and surfactants with higher HLB value (8-18) favors the formation of oil-in-water emulsion. Tween20 was opted for making oil-in-microemulsion because of its high HLB value of 16.7, which favors formation of oil-in-water type of emulsion. Due to its high (HLB) value Tween20 molecules diffuse from the organic phase (oil and surfactant) to the aqueous phase and forms low droplet size emulsions. Also being a small molecule surfactant, Tween20 gets adsorbed onto the surface of emulsion droplet more quickly when compared to high molecular weight surfactants (e.g. polymers). Hence, it is more effective in minimizing droplet size than polymeric surfactants (Azeem et al 2009a; Azeem et al 2009b; Jafari et al 2007; Yuan et al 2008).

Increasing surfactant concentration from 6 % to 24 % with cinnamon oil system, droplet size reduced from 9127 nm to 5.7 nm respectively. Further increase in surfactant concentration to 30 %, showed no significant reduction in droplet size. The optimized surfactant concentration was found to be 24 % i.e. oil : surfactant ratio of 1:4 (vol/vol). In the case of basil oil microemulsion, optimized surfactant concentration was found to be 30 % (i.e. oil : surfactant ratio = 1:5 vol/vol) and the mean droplet size was 11 nm. The results for surfactant concentration to droplet size for cinnamon and basil oil system parallels the concept of Gutierrez et al (2008). Oil-surfactant ratio plays a significant role in determining droplet size of emulsion and droplet diameter could be minimized by increasing surfactant concentration.

In the present study, basil oil based microemulsion formulation having a droplet size of 11 nm and cinnamon oil based microemulsion having a droplet size of 5.79 nm were obtained without the addition of co-surfactant and all the constituents of emulsion were generally recognized as safe (GRAS) range.

4.1.3 EFFECT OF SURFACTANT CONCENTRATION ON VISUAL APPEARANCE AND TURBIDITY

Increase in surfactant concentration from 6 % to 24 % (vol/vol) using cinnamon oil (6 % vol/vol), resulted in reduction of turbidity ($\text{Abs}_{600\text{nm}}$) from 1.06 to 0.011
respectively. The CMF1 emulsion with 6 % surfactant looked turbid but the CMF4 formulation was optically transparent. In case of basil oil also similar observation was made. Gradual decrease in turbidity ($\text{Abs}_{600\text{nm}}$) from 0.873 to 0.001 was observed when the surfactant concentration was increased from 12 % (vol/vol) to 30 % (vol/vol) respectively. Hence, it can be concluded that surfactant concentration played an important role on the visual appearance of the microemulsion formulation. Increase in surfactant concentration reduces droplet size of microemulsion formulation. The reduced droplet size of emulsion formulation (5-20 nm in case of microemulsion formulation) are very small than the wavelength of light. When light waves passes through these emulsion formulations, the very low droplet size scatters the light waves very weakly and makes the emulsion system optically transparent. The results corroborated with the findings of McClements, (2012), (2012a), and (2012b), that increase in surfactant concentration minimize droplet diameter and also reduces turbidity of emulsion system.

Zhang et al (2008) and (2009) also considered the microemulsion to be stable and belong to a monophasic area in the phase diagram, only if the emulsion remained optically transparent and homogeneous after forceful vortexing using a vortex mixer.

4.1.4 EFFECT OF SURFACTANT CONCENTRATION ON VISCOSITY

With increase in surfactant concentration from 6 % to 24 % using cinnamon oil, viscosity of the emulsion increased from 2.0 cP to 25.6 cP correspondingly. In case of basil oil microemulsion, viscosity of the formulation was increased from 2.9 cP to 24.2 cP, when the surfactant concentration increased from 12 % to 30 % vol/vol.

These findings are in parallel with Eini et al (1976) that increase in emulsifier concentration, water molecules gets trapped in cross-linking emulsifier chains and resulted in increased viscosity of the emulsion. So, the hydrophilic tail portions of the surfactants get hydrated by the water molecules.
4.1.5 DROPLET MORPHOLOGY OF MICROEMULSION FORMULATION

The TEM (transmission electron microscopy) image of the formulation CMF4 was spherical in shape. Droplets of CMF4 microemulsion was in the range of 5-10 nm. This result matches with that of McClements (2012) that morphology of microemulsion droplets are spherical or ellipsoid or worm-like based on the molecular geometry of surfactants used to stabilizing microemulsion. This could be explained by the fact that interfacial tension of microemulsion is much lower; hence the droplets can form any of the above mentioned shape and structure.

4.2 NANOEMULSION FORMULATION

4.2.1 LOW ENERGY METHOD: SPONTANEOUS EMULSIFICATION

The colloidal dispersions formed using spontaneous emulsification can be either microemulsion or nanoemulsion. Based on the surfactant-to-oil ratio (SOR), emulsions are defined as microemulsion and nanoemulsion. Microemulsion is formed when the SOR is more than 1 and nanoemulsion is formed at low SOR (McClements 2012).

4.2.1.1 EFFECT OF SURFACTANT CONCENTRATION ON EMULSION DROPLET SIZE

Emulsion with droplet size >10 μm was formed when the surfactant-to-oil ratio was below 1, using cinnamon as oil phase. Using basil oil, the mean droplet size of nanoemulsion decreased with increasing surfactant i.e., droplet size reduced from 921 nm to 85 nm when the surfactant concentration increased from 1.2 % to 6 % (vol/vol) respectively. This parallels with Anton and Vandamme (2009) that Labrafil M1944 CS® (Oleoyl macrogolglycerides) based nanoemulsion stabilized by Cremophor ELP® (polyoxiethylated-35 castor oil, HLB ~ 12–14) decreased droplet size when the surfactant-to-oil ratio (SOR) increased from 20 % to 100 %. According to Lamaallam et al (2005), increased adsorption of surfactants onto the oil/water interface decreases
interfacial tension and facilitates the formation of droplets with reduced size. Larger amount of surfactant could have got diffused from the organic phase containing oil and surfactant to the aqueous phase, thereby formulating emulsion with small droplets (Anton and Vandamme 2009).

4.2.1.2 EFFECT OF STIRRING SPEED ON EMULSION DROPLET SIZE

Unlike microemulsions, nanoemulsions are thermodynamically unstable systems and they always require the input of energy to convert the phase separated components to a colloidal suspension. The minimum amount of energy required to formulate nanoemulsion should exceed the positive Gibbs free energy that increases the contact area between the aqueous and oil phases (McClements 2012).

Stirring speed also exhibited effect on emulsion droplet size. Increase in stirring speed of the magnetic stirrer facilitated formation of low droplet diameter i.e. droplet size reduced from 295 nm to 85 nm with corresponding increase of stirring speed from 200 rpm to 800 rpm.

Saberi et al (2013) also reported reduction in droplet size of nanoemulsion containing mixture of Vitamin E acetate and medium chain triglyceride oil (MIGLYOL812) as oil phase. Nanoemulsion with droplet diameter of 56 nm was obtained at 800 rpm with surfactant-to-emulsion (SER) ration of 10 %.

Rang et al (1998) explained the reduction in droplet size on the basis that the applied mechanical energy through the magnetic stirrer would have distributed the different constituent phases of emulsion suggesting the requirement of mild agitation of oil, water and surfactant during spontaneous emulsification.

4.2.1.3 EFFECT OF HOLDING TEMPERATURE ON EMULSION DROPLET SIZE

Effect of holding temperature on basil nanoemulsion droplet size was studied by keeping the organic phase in varying temperature prior to the addition to water.
Nanoemulsion droplet size decreased from 85 nm to 28 nm with corresponding increase in the holding temperature from 25 °C to 75 °C at a constant stirring speed of 800 rpm.

Saberi et al (2013) reported decrease in droplet size of Vitamin E acetate and medium chain triglyceride oil based nanoemulsion from 55 nm to 48 nm when the organic phase temperature was increased from 25 °C to 90 °C for 10 % of Tween80-to-oil ratio.

Sahin and Sumnu (2006) reported that decrease in viscosity of the oil phase with increase in temperature. This decrease in viscosity of oil phase i.e. the dispersed phase facilitates the break-up of oil/water interface resulting in formation of emulsion with smaller droplet size.

Israelachvili (2011) reported that the solubility and molecular geometry of non-ionic surfactants varies with varying temperature owing to the fact that increase in temperature results in the dehydration of the hydrophilic head-groups of surfactants.

Anton and Vandamme (2009) further reported that the alteration of solubility and molecular geometry of the non-ionic surfactants, with increase in temperature, enhances their oil-solubility and the interfacial tension at oil-water interface is reduced to the phase inversion temperature (PIT).

4.2.2 HIGH ENERGY METHOD: ULTRASONIC EMULSIFICATION

Nanoemulsions were formulated using different plant based essential oils such as basil oil, cinnamon oil, mustard oil and sesame oil by ultrasonic emulsification method. Nanoemulsion was optimized for different process parameters such as oil type, surfactant type, surfactant concentration, oil-surfactant mixing ratio and sonication time (Table 4.2).
Table 4.2. Optimized parameters of nanoemulsion formulated by ultrasonic emulsification using different plant essential oils

<table>
<thead>
<tr>
<th>Optimized Parameters*</th>
<th>Basil oil</th>
<th>Cinnamon oil</th>
<th>Mustard oil</th>
<th>Sesame oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (nm)</td>
<td>30</td>
<td>65</td>
<td>55</td>
<td>20</td>
</tr>
<tr>
<td>Sonication time</td>
<td>15</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Visual appearance</td>
<td>Transparent</td>
<td>Turbid</td>
<td>Transparent</td>
<td>Transparent</td>
</tr>
</tbody>
</table>

*Surfactant = Tween80; Oil : Surfactant mixing ratio (v/v) = 1:3; Sonicator power output = 750 Watt; Sonicator Frequency = 20 KHz

Nakabayashi et al (2011) reported that emulsion droplet size can be resolved by optimizing process parameters like emulsifier concentration, oil concentration, oil-emulsifier mixing ratio, emulsification time, energy input and viscosity of continuous phase.

4.2.2.1 EFFECT OF SURFACTANT CONCENTRATION ON EMULSION DROPLET SIZE

Nanoemulsion droplet size decreased with increase in surfactant concentration. Using 6 % of basil oil and emulsification time of 15 min, when the Tween80 concentration increased from 6 % to 18 %, corresponding decrease in droplet size from 41.15 nm to 29.6 nm was observed. Similar results were obtained with cinnamon oil, mustard oil and sesame oil. Keeping oil concentration (6 %) and emulsification time (30 min) constant, when the Tween80 concentration increased from 6 % to 18 %, droplet size decreased from 254 nm to 65 nm (in case of cinnamon oil), from 132.5 nm to 55 nm (in case of mustard oil), 95 nm to 20 nm (in case of sesame oil) respectively.

Qian and McClements (2011) reported small molecule surfactants like Tween80, get adsorbed onto the emulsion surface rapidly and reduce the interfacial tension at
oil/water interface. Hence, small molecule surfactants are more effective in minimizing droplet size when compared to high molecular surfactants like polymers.

Gullapalli and Sheth (1999) and Wang et al (2009) reported that HLB value is a rough guide for selecting the surfactants for stabilizing nanoemulsions. Tween80 was used as surfactant to stabilize the as it has a high hydrophilic-lipophilic balance (HLB-15) that favors the formation of oil-in-water emulsion.

Tween80, being a non-ionic surfactant, stabilizes nanoemulsion by steric stabilization. Grigoriev and Miller (2009) reported that the bulky molecular groups of surfactants directed towards the aqueous dispersion medium creates a steric barrier and provides emulsion stability.

Gutierrez et al (2008) reported that minimum droplet size can be achieved at low oil surfactant ratio. Anjali et al (2012) reported reduction in droplet size of neem oil nanoemulsion from 251 nm to 31 nm, when the surfactant concentration increased from 1.8 % to 18 % vol/vol. Also, Leong et al (2009) and Li et al (2012) reported reduction in droplet size of sunflower oil and D-limonene nanoemulsion respectively with increase in emulsifier concentration. Tang et al (2012) reported that when the concentration of emulsifier i.e. Polyoxy-35-castor oil (Cremophore EL) was increased, droplet size of aspirin nanoemulsion, formulated by using propylene glycol monolaurate Type II (Lauroglycol™90) as oil phase, was reduced.

Nanoemulsions are thermodynamically unstable systems and the total free energy required for nanoemulsion formation is positive. Thus, the process of nanoemulsion formation is non-spontaneous. Surfactants aid the formulation of nanoemulsion process by lowering interfacial tension at oil/water interface (Tadros et al 2004).

Oil concentration used in all the nanoemulsion formulations i.e. basil oil nanoemulsion, cinnamon oil nanoemulsion, mustard oil nanoemulsion and sesame oil nanoemulsion, was very low (6 % of total emulsion volume) and water was used as continuous phase. Hence, the process fits into Taylor’s prediction (Eq. 4.1) that, emulsion radius (r) is directly related with interfacial tension (Taylor, 1934):
Where $\epsilon$ is the interfacial tension, $\eta$ is the continuous phase viscosity and $\dot{\epsilon}$ is the shear rate.

Mustard oil nanoemulsion was optimized for surfactant concentration of 24%. At emulsification time of 30 min and 24% of surfactant concentration, mustard oil nanoemulsion with 25 nm (using Tween80 as surfactant) and 30.5 nm (using Tween20 as surfactant) respectively were formulated. When the concentration of surfactant was further increased to 30%, an increase in particle size was observed i.e. droplet size increased to 45 nm (using Tween80 as surfactant) and 52 nm (using Tween20 as surfactant) respectively. Similar results were observed by Kommuru et al (2001), Wang et al (2009) and Yoo et al (2010). This increase in droplet size is attributed to the fact that a highly viscous liquid crystalline phase forms beyond a certain increase in surfactant concentration and the break-up of the oil/water interface becomes more complicated (Wang et al 2009).

Dai et al (1997) and Wang et al (2009) reported that presence of double bonds in the non-polar chains of non-ionic surfactants favors the formation of nanoemulsion with smaller droplet sizes. The non-polar tails in Tween20 are saturated and are fairly linear but those in Tween80 are unsaturated and are more kinked. Hence, Tween80 was more effective in reducing droplet size of mustard oil nanoemulsion than Tween20. Saberi et al (2013) also reported vitamin E-enriched nanoemulsions with reduced droplet size when Tween80 was used as surfactant than that of Tween20 or Tween40.

4.2.2.2 EFFECT OF SONICATION TIME ON EMULSION DROPLET SIZE

Emulsification time had a direct correlation with droplet diameter of nanoemulsion. Using 6% of basil oil and 18% Tween80, when emulsification time increased from 5 min to 15 min, then droplet size reduced from 45 nm to 29.6 nm. Similar results were obtained using cinnamon oil, mustard oil and sesame oil. Using 6% of oil and 18% Tween80, when emulsification time increased from 10 min to 30 min,
droplet size reduced from 199 nm to 65 nm (using cinnamon oil), from 191 nm to 67.5 nm (using mustard oil) and from 124 nm to 20 nm (using sesame oil) respectively.

Leong et al (2009) and Landfester et al (2004) observed similar decreasing trend of droplet diameter with the increase in emulsification time while formulating sunflower oil and styrene nanoemulsion respectively.

Kamogawa et al (2004) and Jafari et al (2008) reported stable surfactant-free transparent nanoemulsions using megasonic irradiation (frequency in the range of mega Hz). This result suggests that sonication time, sonicator frequency and sonicator power plays an important role in determining nanoemulsion droplet size

4.2.2.3 EFFECT OF SURFACTANT CONCENTRATION ON VISUAL APPEARANCE AND TURBIDITY OF NANOEMULSION

Basil oil based coarse emulsion (before subjecting to ultrasonic emulsification) was turbid in color. This turbidity can be attributed to the micron range droplet size of the emulsion. After subjecting to ultrasonic emulsification, the emulsion became optically transparent. The optically transparency of the sonicated emulsion can be explained by droplet size minimization upon ultrasonic cavitation. Among the sonicated samples, turbidity reduced from 0.568 to 0.003 as the surfactant concentration increased from 6 % (vol/vol) to 24 % (vol/vol). This result suggests that both surfactant concentration and sonication contributes to droplet size minimization and optical transparency of nanoemulsion.

Similar trend of decreasing turbidity with increasing surfactant concentration was observed while formulating mustard oil nanoemulsion. When the Tween80 concentration was increased from 6 % to 24 %, absorbance (600 nm) value was reduced. This decrease in turbidity is due to reduced droplet size. But when Tween80 concentration was further increased to 30 %, a slight increase in turbidity was observed. This increase in turbidity can be explained by increased droplet size at 30 % Tween80 concentration. This result goes along with earlier reports that further increase in surfactant concentration above a
certain level results in increasing particle diameter of vitamin E-enriched nanoemulsion and consequent increase in turbidity Saberi et al (2013).

McClements (2002a; 2002b) explained the theoretical basis of emulsion color and theoretical calculations of the droplet size dependence of the turbidity of nanoemulsions i.e. when the droplet size is lower than the wavelength of light, the emulsion droplets, in Brownian motion, scatter light waves very weakly and hence make the emulsion system optically transparent.

4.2.2.4 EFFECT OF SURFACTANT CONCENTRATION ON VISCOSITY OF NANOEMULSION

As the surfactant concentration increased from 6 % vol/vol to 24 % vol/vol, an viscosity increased from 1.78 cP to 19.75 cP. Eini et al (1976) observed that at elevated surfactant concentration water molecules gets trapped in cross-linking chains of surfactants and increase the viscosity of the emulsion system.

4.2.2.5 DROPLET MORPHOLOGY OF NANOEMULSION FORMULATION

Droplets of the formulated nanoemulsions i.e. basil oil nanoemulsion, cinnamon oil nanoemulsion, mustard oil nanoemulsion and sesame oil nanoemulsion were spherical in morphology.

Li and Chiang (2012) and Anjali et al (2012) reported nanoemulsion droplets of D-limonene and neem oil with spherical morphology respectively.

McClements (2011) reported that droplets of nanoemulsion formulation always tend to have spherical morphology because of their comparatively high interfacial tension and small droplet size, which leads to a high Laplace pressure and favors the reduction of the oil/water interfacial area. But in case of microemulsion system, the droplets can have either spherical or ellipsoid or worm-like morphology depending on the molecular
geometry of the surfactants used. This is attributed to the fact that the interfacial tension is typically much lower in case of microemulsion than nanoemulsion.

AFM provided additional information for size and shape determination (Preetza et al 2010). The morphology of droplets was approximately spherical in shape and smooth surface. Authors have reported AFM image of neem oil nanoemulsion and eucalyptus oil nanoemulsion with spherical shape (Anjali et al 2012; Sugumar et al 2013).

4.3 NANOEMULSION FOR DELIVERY OF LIPOPHILIC BIOACTIVE COMPONENTS

Sesame oil with oil-surfactant mixing ratio of 1:3 v/v and Tween80 as surfactant was used for delivery of eugenol. Eugenol-loaded nanoemulsion was formulated with droplet diameter of 13 nm and was stable for more than 1 month. Sesame oil blended eugenol-loaded nanoemulsion demonstrated lower droplet size and higher stability than only-eugenol (without sesame oil) nanoemulsion.

Donsi et al (2011) and Donsi et al (2012) reported sunflower oil based nanoemulsion for delivery for D-limonene, carvacrol and cinnamaldehyde prepared by high pressure homogenization (HPH) and stabilized by different emulsifiers such as lecithin, pea proteins, sugar ester, and a combination of Tween20 and glycerol mono-oleate.

Donsi et al (2011) also observed that production of stable lecithin-based nanoemulsions required the blending of D-limonene with palm oil. This corroborates with the results of our study that eugenol-loaded nanoemulsion blended with sesame oil demonstrated lower droplet size and better stability.


Gao et al (2011) developed nanoemulsion loaded with Candesartan cilexetil (CC), an inactive prodrug of candesartan, to improve its intestinal absorption. Candesartan cilexetil is poorly insoluble in water; hence it exhibits incomplete intestinal absorption and low oral bioavailability.

Kong et al (2011) developed hyaluronic acid nanoemulsion for transdermal carrier for α-tocopherol as model active lipophilic ingredient. In vitro hemolysis, skin penetration and histological examinations were carried out using as model ingredient to assess skin permeability and bioavailability. Nanoemulsion, being able to penetrate across stratum corneum, diffused deeper into dermis and performed desirable skin permeable capacity.

Droplet size of emulsion is reported to have significant the effect on the the transdermal application and bio-availability of lipophilic drugs. Kotyla et al (2008) formulated δ-tocopherol loaded canola oil nanoemulsion stabilized by Tween80. The droplet size of coarse emulsion (before subjecting to microfluidization) was 2788 nm and the droplet size reduced to 65 nm after microfluidization. Nano-range emulsion enhanced the bioavailability of δ-tocopherol applied transdermally when compared to the micron-sized coarse emulsion.

4.4 STABILITY STUDIES

Thermodynamic stability of the formulated emulsions was investigated by subjecting to centrifugation, free-thaw cycle and heating-cooling cycle; and kinetic stability of the emulsion formulation was studied by checking droplet size in different interval of time. Cinnamon oil microemulsion CMF4 was stable to all the stability studies and demonstrated marvelous kinetic stability for 240 days. This can be attributed to low droplet size of 5.7 nm.
The stability of emulsions to coalescence and flocculation improves as the droplets get smaller because the strength of the attractive forces diminishes more rapidly than the strength of the repulsive forces (Kabalnov and Shchukin 1992; McClements 1994; McClements and Dungan 1993; McClements and Rao 2011).

4.5 ANTIBACTERIAL ACTIVITY

4.5.1 TIME AND CONCENTRATION DEPENDENT INACTIVATION KINETICS

Antibacterial activity of cinnamon oil microemulsion CMF4 is due to the active ingredients present in essential oil, and also due to the reduced droplet size and increased droplet surface area available to interact with bacteria. Complete loss of bacteria viability was observed within 1 min of interaction with undiluted microemulsion. This result goes along with the earlier reports that, microemulsion are capable of causing a 6 log reduction in bacteria population in 1 min (Zhang et al 2009), when they performed kinetics of killing experiments of microemulsion against *E. coli* or *S. aureus* cells.

Sugumar et al (2013a) studied antibacterial activity of eucalyptus oil nanoemulsion against *B. cereus, E. coli* and *S. aureus*. Teixeira et al (2007) examined the antibacterial activity of O/W soybean oil, tri-n-butyl phosphate and ethyl oleate based emulsions stabilized with Triton X-100, Tween80 and n-pentanol, against *Salmonella* spp., *Escherichia coli* 0157:H7, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes*. The reported emulsions were also studied for anti-biofilm action.

Teixeira et al (2007) also reported that microemulsions, unlike nanoemulsions, loose antibacterial efficiency when diluted with water because of considerable structural changes of droplets in microemulsion. In the present work, cinnamon oil microemulsion exhibited significant bactericidal activity even after dilution up to 1000-fold with water.

Al-Adham et al (2003) found that the microemulsion formulations are highly effective against of *P. aeruginosa* biofilms. Teixeira et al (2007) also reported that the
microemulsion was active against biofilms of *E. coli*, *P. aeruginosa*, *S. aureus* and *S. typhimurium*.

4.5.2 ALTERATION IN MEMBRANE PERMEABILITY BY AO/ETBR STAINING

Upon treatment with microemulsion, bacteria membrane gets damaged and membrane permeability gets altered. This alteration in membrane permeability can be analyzed by differential staining with acridine orange and ethidium bromide. Acridine orange is a metachromatic intercalator that is permeable to both live and dead cells. But ethidium bromide is permeable to stains only those bacteria cells that have lost their membrane integrity. Hence the control/untreated live cells appeared green due to staining with acridine orange, whereas cinnamon oil microemulsion treated bacteria cells appeared red.

Jakopec et al (2006) studied nuclear alterations and morphological changes in HeLa cells upon treatment with Diazene-N-phenyl-2-(2-pyridinyl)-diazencarboxamide (JK-279) by staining with acridine orange and ethidium bromide. Sugumar et al (2013) reported alteration in membrane permeability of *B. cereus*, *E. coli* and *S. aureus* upon treatment with eucalyptus oil nanoemulsion by staining with the same combination of dyes as mentioned above.

Dalai et al (2012) demonstrated comparative toxicity of TiO$_2$ nanoparticles against freshwater bacterial isolate, *Bacillus licheniformis*, under light and dark conditions by staining with acridine orange and ethidium bromide. This fluorescence is endorsed with the property of acridine orange and ethidium bromide to intercalate with nucleic acids due to the lofty negative charge density of phosphate groups, sugars and hydrogen bonding opening in DNA and RNA (Lyles and Cameron 2002; Lauretti et al 2003; Nafisi et al 2007).

4.5.3 LEAKAGE OF 260 NM ABSORBING SUBSTANCES

Upon treatment with microemulsion, bacteria membrane gets damaged and intracellular components leak out. This leakage can be quantified by checking the
absorbance at 260 nm. A gradual increase in leakage of 260 nm absorbing was observed when *E. coli* and *S. aureus* cells were treated with 10-fold diluted CMF4 cinnamon oil microemulsion formulation.

Results pertaining to membrane permeability go along with earlier findings that there is a gradual increase in leakage of UV absorbance material upon treatment with microemulsion (Zhang et al 2009). The release of 260 nm absorbing substances was in agreement with the killing kinetics of bacteria by microemulsions. The cell membrane upon interactions with antimicrobials frequently causes fundamental damage to both structure and function of bacteria membrane.

Baker et al (2003) reported that microemulsions fuse with lipid bilayer cell membrane of bacteria and this fusion destabilizes the membrane integrity and function, resulting in lysis and death of pathogen. Changes in membrane structure alter its permeability and cause an increase in leakage of UV absorbing substances.

4.5.4 MODIFICATION OF SURFACE FUNCTIONAL GROUPS BY FTIR

FT-IR is one of the imperative methods to study the modification of bacterial surface functional groups upon treatment with emulsion (Erukhimovitch et al 2005). FTIR spectra demonstrated band shift and modification of functional groups present on *E. coli* and *S. aureus* after incubation with cinnamon oil microemulsion CMF4. Band at 1238 cm\(^{-1}\) and 1078 cm\(^{-1}\) can be attributed to asymmetric and symmetric stretching vibrations of PO\(^{-2}\) and phospholipids (Dukor 2002). Eugenol, the major component of cinnamon oil used in our present study, is reported to inactivate bacteria by deforming phospholipids (Devi et al 2010). In case of *E. coli*, Band shift from 1238 cm\(^{-1}\) to 1249 cm\(^{-1}\) indicates deformation in bacterial membrane phospholipids (Dukor et al 1998).

These results suggest the modification of functional groups on bacterial surface upon cinnamon oil microemulsion CMF4 treatment. FTIR findings corroborates with the results pertaining to alteration in membrane permeability. So the possible mechanism of bactericidal activity suggests that upon incubating with microemulsion bacterial
membrane gets compromised and membrane permeability alters which leads to leakage of intracellular constituents and cell lysis.

4.5.5 MORPHOLOGICAL CHANGES BY SEM

Microscopy is a vital tool to assess the morphological changes of bacteria (Stokes 2003). Scanning electron microscopy demonstrated significant morphological changes in *E. coli* and *B. cereus* cells exposed to cinnamon oil microemulsion CMF4.

Al-adham et al (2000) visualized signs of membrane disfunction in the microemulsion-exposed *P. aeruginosa* cells using transmission electron microscopy. Karthikeyan et al (2011) observed morphological changes in *S. mutans* upon treatment with nanoemulsion. Cell wall margins were unclear in treated bacteria cells and also cell boundaries were irregular.

4.6 ANTIBACTERIAL ACTIVITY IN SITU IN REAL FOOD SYSTEM

Cinnamon oil microemulsion CMF4 treated samples showed a time, concentration and temperature dependent bacterial activity *in situ* in orange juice. Bacterial population reduced up to a time period of 6 hr, followed by a gradual increase in the bacteria population. Antibacterial activity of both cinnamon oil microemulsion and positive control (sodium benzoate) treated groups were better at 4 °C than that at 25 °C. Bactericidal effect of cinnamon oil microemulsion was better than the sodium benzoate.

Devi et al (2011) evaluated in situ antimicrobial activity of sunflower oil based nanoemulsion in food products such as apple juice, milk, raw chicken and mixed vegetable. They observed a considerable reduction in the native cultivable bacterial population and fungal population of all these food products.
4.7 WOUND HEALING ACTIVITY

Cinnamon oil microemulsion was tested for skin irritancy before studying its wound healing application. Neither cinnamon oil alone, nor cinnamon oil microemulsion formulation (CMF4) exhibited any irritation, whereas standard irritant (0.8 % formalin) demonstrated erythema and edema. The skin tissue collected from wistar rats treated with cinnamon oil only and cinnamon oil microemulsion formulation CMF4 didn’t show any discrepancy or irregularities but standard irritant (0.8 % (v/v) aqueous formalin) treated wistar rat demonstrated damage in basal epithelium cells. This result further confirmed that cinnamon oil microemulsion is non-irritant to skin in the used concentration.

Complete healing of wound occurred in 14 days in cinnamon oil microemulsion CMF4 treated group, and 16 days each for cinnamon oil only and standard ointment treated group, and 20 days in case of untreated (control) wistar rats. Further, bacteria were isolated from excised wound and antibacterial activity was studied. Cinnamon oil microemuksion CMF4 demonstrated considerable bactericidal activity against wound isolate *Macrococcus caseolyticus*,. These above results suggest that cinnamon oil microemulsion prevents sepsis of wound and triggers wound healing process.

Hemmila et al (2010) observed that topical nanoemulsion (NB-201) reduced bacterial growth in the burn wound model in Male Sprague-Dawley rats. They also observed that NB-201 nanoemulsion attenuated neutrophil sequestration, decreased the levels of pro-inflammatory cytokines (IL-1b and IL-6) and lowered the degree of hair follicle cell apoptosis.

4.8 LARVICIDAL ACTIVITY

Cinnamon oil microemulsion CMF4 demonstrated time and dose dependent killing of mosquito larva. LC\textsubscript{50} value of microemulsion reduced with increase in time. Anjali et al (2012) reported size and concentration dependent larvicidal effect of neem oil nanoemulsion against *Culex quinquefasciatus* larva.
Sakulku et al (2009) and Nuchuchua et al (2009) reported mosquito-repellent activity of nanoemulsion formulation from citronella oil only, and combination of citronella oil, hairy basil oil and vetiver oil, against *Aedes aegypti*.

Damage to mosquito larva was studied by staining by Hematoxylin/Eosin stain (HE stain). This is one of the popular methods of staining in histology. Hemalum is formed from complex of aluminium ion and haematin (the oxidation product of haematoxylin) and is the active ingredient in the Hematoxylin/Eosin staining solution. Hemalum has a blue/purple color and it stains nucleic acids. Eosin is the counter stain which is pink in color and stains proteins. When a tissue is stained with Hematoxylin/Eosin staining solution, then the nuclei are stained blue but the cytoplasm along with the extracellular matrix stains pink (Fischer et al 2008). The Hematoxylin/Eosin staining reveals ample information about the structural difference in control and treated tissue samples.