ORIGINAL ARTICLE

Nanoemulsion formation and characterization by spontaneous emulsification: Investigation of its antibacterial effects on *Listeria monocytogenes*

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A transparent oil-in-water nanoemulsion system consisting of eucalyptus oil, Tween-20 as organic phase and water as an aqueous phase was developed using a low energy emulsification method. Physicochemical properties such as droplet size, optical transparency, and long-term stability was studied. The stable eucalyptus oil nanoemulsion (1:2) having mean droplet size in the range of 50-100 nm with a polydispersity index <0.2. The optimized nanoemulsion formulation exhibited significantly higher antibacterial activity by the well diffusion method against *Listeria monocytogenes*. Further, alteration in the membrane integrity was assessed, and it is found higher for nanoemulsion treated cells than control cells. Atomic force microscopic observations showed distorted morphology of treated bacterial cells. These results propose the possible use of eucalyptus oil nanoemulsion in the food industries.

Key words: *Antibacterial, eucalyptus oil, Listeria monocytogenes, nanoemulsion, spontaneous emulsification*

INTRODUCTION

Eucalyptus oil (*Eucalyptus globulus*), the genus comes under the myrtaceae family include about 900 species and subspecies.[1] Eucalyptus oil, especially *E. globulus*, is the most representative species in the international pharmacopeia.[2] It is a well-known medicinal plant due to the bioactive components present in it. The main compound present in eucalyptus oil is "eucalyptol or (1,8 cineol)," which is a prospective source of biological and pharmacological properties.[3,4] Since, eucalyptus oil possesses a broad spectrum of antimicrobial activity; it had been long used as flavouring agents and preservatives in food industries.[5] Several studies have been focused on antifungal properties by using eucalyptus oil[6] and only few studies reported on antibacterial properties.[7,8]

Listeria monocytogenes are nonspore forming Gram-positive with a short rod shaped bacterium. It is a psychrotolerant bacterium commonly found in infected cow's milk and is known to cause listeriosis in humans. Furthermore, several different kinds of food products have been associated with listeriosis outbreaks.[9,10] Therefore, the food industry is constantly exploring efficient and cost-effective technology to control the growth of pathogenic microorganisms to ensure food quality and safety of consumer foods.[11] In previous studies, antilisterial properties of plant based essential oils have been reported.[12-14]

There is a growing interest in food and beverage industries for the utilization of nanoemulsion as a colloidal system for delivery of bioactive components such as antimicrobials, colors, flavours, micronutrients and nutraceuticals.[15-19] The applications of nanoemulsions as attractive systems has gained interest nowadays due to their unique properties such as extremely small droplet diameter in the range of 20-200 nm,[20] high physical stability, high bioavailability and optical transparency compared with other conventional emulsions.[21]

Previous studies showed encapsulation of essential oils into emulsion due to the hydrophobic nature of oil this leads to higher solubility and dispersibility in aqueous media and also to reduce organoleptic

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properties in the food system.[22] Recently, there is growing interest in designing structured delivery systems to improve the dispersion stability and antimicrobial activity with examples being nanoemulsions, microemulsions and liposomes.[22-25] However, each of these examples has certain limitations, e.g., high energy emulsification used to prepare nanoemulsions and large quantity of surfactant required for formulating microemulsions. Until date, no reports are available for the formation of eucalyptus oil nanoemulsion with low energy emulsification. Therefore, the objective of the present study focuses on the antibacterial activity of eucalyptus oil nanoemulsion toward *Listeria* sp. was studied, which is cost-effective method compared to the high energy emulsification method.

MATERIALS AND METHODS

Materials

Eucalyptus oil (*E. globulus*) was procured from Himedia chemicals, India and Tween-20, BIOXTRA from Sigma Aldrich, India. All other reagents used were of analytical reagent grade. Anti-bacterial activity was carried out against Gram-negative bacteria, *L. monocytogenes* (MTCC 1143). For all experiments, double distilled water (Cascada Bio Water, Pall Corporation, USA) was used.

Methods

Nanoemulsion formation by spontaneous emulsification

Nanoemulsions were formulated spontaneously using eucalyptus oil, Tween-20 and water by the low energy emulsification method. The bio-based and nonionic surfactant Tween-20 was used as surfactant for formulating nanoemulsion that contains hydrophilic-lipophilic balance value of 16.7, which is favorable for the formation of oil-in-water emulsion. A, 6% v/v of eucalyptus oil was fixed for all the formulations, and different ratios were prepared by mixing oil and surfactant in various ratios such as 1:1, 1:2, 1:3 and 1:4, respectively. The nanoemulsions were prepared by simply mixing and heat treatment method. In simple mixing method, water was added drop-wise to the organic phase contains oil and surfactant under stirring condition at 400 rpm using a magnetic stirrer. In the heat treatment method, the organic phase was heated at 70°C and water was added to the system at 25°C. All the formulated emulsions were subjected to stability study, and further characterization was carried out.

Physico-chemical characterization

Turbidity of emulsion

Turbidity of all the formulated nanoemulsions was analyzed by measuring the transmittance of undiluted emulsions at a wavelength of 600 nm ultraviolet (UV-Vis Spectrophotometer 2201, Systronics, India).

pH measurement

The pH value of the nanoemulsions (simple mixing and heat treatment) was measured by immersing the electrode directly into the emulsion using a calibrated pH meter (model HI 8417, Hanna Instruments Inc., Woonsocket, USA), at 25°C ± 1°C. The measurement was carried out in triplicates.

Viscosity determination

The viscosity of the nanoemulsion formulations (simple mixing and heat treatment) were measured as such without dilution using a Brookfield Viscometer, USA (model LVF 69726). Viscosity measurements were carried out in triplicates.

Thermodynamic stability study

The formulated emulsion was centrifuged to prove the stability at 3500 rpm for 30 min and was observed for phase separation if any. Followed by heating-cooling cycle, this was carried out by keeping the formulated emulsion at 40°C and 4°C alternatively each for 2 days. The cycle was repeated for 3 times. Further, freeze-thaw cycle was performed by keeping the emulsion alternatively at −21°C and 25°C for 2 days at each temperature. The cycle was repeated for 2 days. This was done to check the stability of emulsions at varying the temperature. Formulations that passed the thermodynamic stress tests were taken for further optimization studies.

Droplet size measurement

The measurement of droplet size and polydispersity index of nanoemulsion formulations was determined using 90 plus particle size analyzer (Brookhaven Instruments Corporation, USA). The droplet radius (*R*) can be calculated from Stokes-Einstein equation, Equation 1.

$$D = kT / 6 \eta R \quad (1)$$

Where, *D* is the diffusion coefficient, *k* is the Boltzmann's constant, *T* is the absolute temperature and η is the viscosity of the medium. All the formulations were diluted with double distilled water prior to each experiment to minimize multiple scattering effects caused due to the viscosity of samples due to the ingredients used in the experiment.

Antibacterial activity

Well diffusion method

The eucalyptus oil nanoemulsion was prepared by simply mixing, and heat treatment method was studied for well diffusion assay.[26] A single isolated bacterial colony was streaked onto the nutrient agar medium for 28 h prior to testing. A few colonies were inoculated into the nutrient broth and were adjusted to 10^7-10^8 CFU/ml using phosphate buffered saline (PBS). The wells were made using sterile cork borer on the surface of the agar plates that were previously seeded by spreading 100 µl of bacterial culture. A 100 µl of the sample was added to the wells, and the plates were incubated for 24 h at 37°C. The resulting zone of inhibition was measured in millimeters.

Leakage of ultraviolet-absorbing substances

The release of UV absorbing substances was measured using UV-Vis Spectrophotometer. In brief, an overnight bacterial culture grown at 37°C was harvested, washed, resuspended and adjusted to 1×10^8 CFU/ml with PBS. Different dilutions of nanoemulsion (10-fold, 100-fold and 1000-fold) were made and added to the cell suspension. Sodium benzoate (500 mg/L) was used as a positive control. The cells without nanoemulsion treatment were used as a control. All the samples were incubated at 37°C for 60 min. After treatment, the samples were centrifuged at 6000 rpm for 10 min to check the release of cytoplasmic cell contents from the bacterial cell. The supernatant was used for measuring the absorbance at 260 nm, and it was taken as a percentage using UV-Vis Spectrophotometer (Systronics 2201, India).

Atomic force microscopy

The morphology of untreated and treated bacterial cells was examined by atomic force microscopy (AFM).[27] The nanoemulsion treated, and untreated cells were washed twice with sterile distilled water to eliminate the salt interference during the analysis. This was performed in contact mode using Nanosurf Easy Scan 2, Switzerland. A drop (5-10 µl) of each bacterial suspension was deposited onto a clean glass slide, and then air dried for 15 min before imaging.

RESULTS AND DISCUSSION

Nanoemulsion formation

Formulation by simple mixing and heat treatment method: Effects on turbidity

The nanoemulsion was formulated using eucalyptus oil (6%), Tween-20 and water. In simple mixing and heat treatment method, the turbidity measurements were made immediately after samples had been mixed using a stirrer to make them homogeneous, whereas, visual observations were made after the samples had been allowed to place at ambient temperature overnight [Figure 1a and b]. In the heat treatment method, there was a distinct change in appearance, and the emulsions were optically transparent compared to simply mixing method. Optical properties of emulsions prepared by simply mixing and heat treatment methods are shown in Table 1.

From 6, 12, 18 and 24% v/v Tween-20, the heating systems remained optically transparent, and the turbidity was maintained constant. In simple mixing method, the turbidity decreased progressively when surfactant concentration is low. These results indicated that, the surfactant-to-oil ratio (SOR) and heating of the organic phase had a significant impact on the nature of the colloidal dispersions formed from eucalyptus oil and surfactant. In the heat treatment method, the formation of transparent systems either at high or low surfactant concentration is due to the changes in molecular characteristics of the surfactant with temperature. Tween-20 is a nonionic surfactant with a hydrophilic head group that will become progressively dehydrated during heating. As a result, packing, interfacial tension, and oil water solubility of the surfactant molecules will change during heating (Israelachvili, 1992).[28] Apparently, between two different phases there was a kinetic barrier at ambient temperature that prevents it from moving from an opaque to the transparent system of an emulsion.

Physico-chemical characterization

pH measurement

In simple mixing method, a continuous decrease in pH was observed, when water concentration increased from 1:4 to 1:1 ratio by maintaining the oil concentration constant. A similar trend of decrease was also observed in heat treatment method. pH measurement of both the methods is shown in Figure 2.

Viscosity measurement

The viscosity of the simple mixing and heat treatment formulations were shown in Figure 3. The viscosity of the emulsion increased with increasing concentration of the surfactant, Tween-20. In the heat treatment method, there was a slight decrease in viscosity than a simple mixing

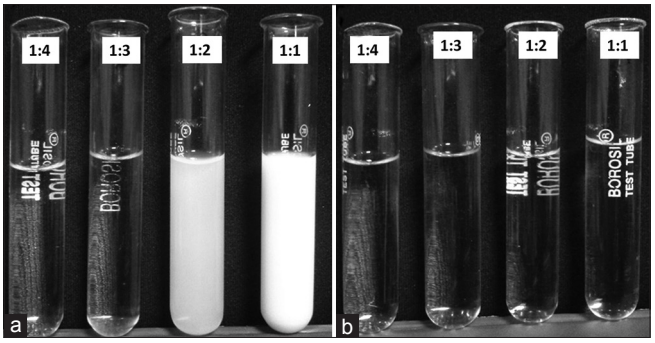

Figure 1: Effect of surfactant concentration on visual appearance of nanoemulsion by low energy emulsification (a) simple mixing method; (b) heat treatment method

Table 1: Optical properties of simple mixing and heat treatment methods

Oil:surfactant (v/v %)	Simple mixing method		Heat treatment method	
	Optical properties	Percentage of transmittance (600 nm)	Optical properties	Percentage of transmittance (600 nm)
1:4	Transparent	96.8±0.213	Transparent	97.6±1.289
1:3	Transparent	96.4±0.132	Transparent	97.35±0.912
1:2	Translucent	81.5±1.231	Transparent	96.88±1.232
1:1	Milky white	0.53±2.001	Transparent	96.57±1.332

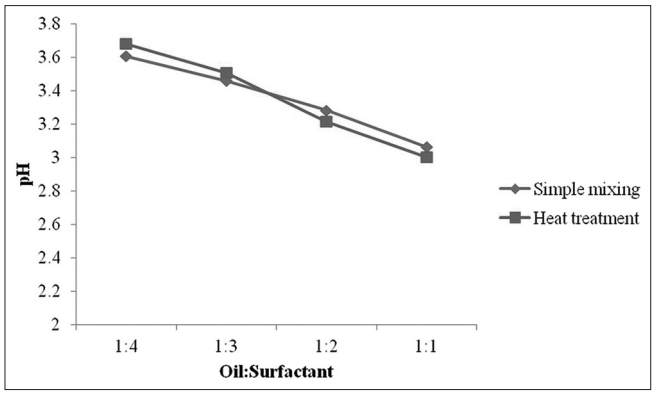

Figure 2: Effect of simple mixing and heat treatment methods on pH

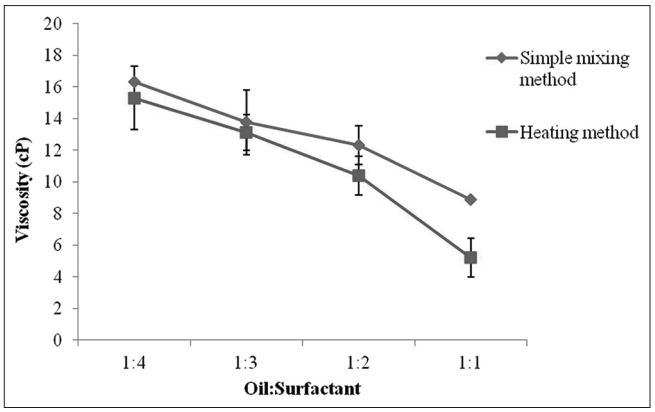

Figure 3: Effect of surfactant concentration on viscosity of nanoemulsion by simple mixing and heat treatment methods

Table 2: Thermodynamic stability of nanoemulsions prepared by low energy emulsification methods

Oil:surfactant (v/v %)	Thermodynamic stability study	
	Simple mixing method	Heat treatment method
1:4	Stable	Stable
1:3	Stable	Stable
1:2	Stable	Stable
1:1	Unstable	Stable

method, which is due the system heating at 70°C, which was an indication of a gel to liquid transition state of a surfactant in the emulsion. When heating the surfactant molecules leads to less availability to participate in the formation of gel phase.[15] The increase in viscosity is attributed due to the water molecules that are trapped in the cross-linking portions of surfactant. Therefore, there may be an increase in the hydration of water molecules around the hydrophilic portion of surfactants as explained by previous researchers.[29,30]

Thermodynamic stability study

All formulations prepared by simply mixing and heat treatment method was subjected to different stress tests such as centrifugation, heating cooling and freeze thaw cycle [Table 2]. In simple mixing method, the formulations from 1:4 to 1:2 were found to be stable and to heat treatment method, all the formulations were passed due to clear and transparent nature of the emulsion system. Those formulations that survived thermodynamic stability tests were taken for particle size analysis.

Droplet size distribution and effect of surfactant concentration

The surfactant concentration had a direct correlation with droplet diameter and the stability of emulsions. The droplet size was analyzed for samples prepared by simply mixing and heat treatment method. In simple mixing method, the freshly prepared samples after 1 day storage at room temperature showed the mean diameter of 47.7 nm, 59.72 nm, 47.8 nm and 280.9 nm for 1:4, 1:3, 1:2 and 1:1 respectively. After 1 month storage at room temperature, the samples were analyzed for size. The mean diameter of 1:2 and 1:1 showed a similar droplet size distribution; whereas, other ratios (1:4 and 1:3) showed micron size range due to Ostwald ripening.[31,32] This is due to the usage of the large amount of surfactants than the oil amount used in the formulation. Nanoemulsions can be formed by a simple mixing method with the use of less surfactant, and it is highly stable (1:2). The nonionic surfactants stabilize emulsions by generating a steric barrier via the bulky molecular groups that are directed towards the continuous medium.[33] The dispersions containing (1:2) 6% and 12% Tween-20 are considered to be nanoemulsions, since, the droplet radii <100 nm ($d<200$) was prepared by simple mixing method and the size distribution is shown in Figure 4. This observation can be substantiated with the fact that nonpolar tail in Tween-20 is saturated and linear. Dai *et al*. have reported the fabrication of nanoemulsion with reduced droplet size in the presence of double bonds in the nonpolar chain of nonionic surfactant.[34]

In the case of heat treatment method, all the formulations were in the micron size range except 1:2 ratios, which showed 278 nm, and other ratios were in micron size (data not shown). This is due to the molecular geometry and solubility characteristics of nonionic surfactants (Israelachvili, 1992).[28] Other formulations such as 1:4 and 1:3 ratios contain more amount of surfactant that may lead to structural modifications in the droplets due to Ostwald ripening process. Though, it is transparent and highly stable the size appeared in the micron range that is not applicable for further studies such as antibacterial activity.

Antibacterial activity

Well diffusion method

All the formulations prepared by simply mixing method were screened for antibacterial activity against *L. monocytogenes*. Oil alone (6% in dimethyl sulfoxide [DMSO]) showed zone of inhibition of around 15 mm against the tested pathogen. Furthermore, Tween-20 was tested and didn't exhibit any antibacterial effect on the concentration used in the present study. For nanoemulsion, it showed zone of inhibition of

30 mm for all formulations tested by a simple mixing method (data not shown). This was a comparatively higher antibacterial activity than the oil (6% in DMSO) alone tested. Hence, we conclude that the anti-bacterial activity of the formulations was due to the reduced droplet size. The encapsulation of essential oil with the help of food grade emulsifier at the nanoscale range with spontaneous emulsification technique represents a feasible and efficient approach in increasing the physical and chemical stability of the bioactive compounds and thereby, protecting them from interactions with the food components. Also, the nano sized droplets possess to increase their bioactivity through the activation of passive mechanisms of cell absorption.[22] Recently, there has been growing interest in utilizing nanoemulsions to encapsulate bioactive components for applications in food and beverage products (McClements, 2011).[35]

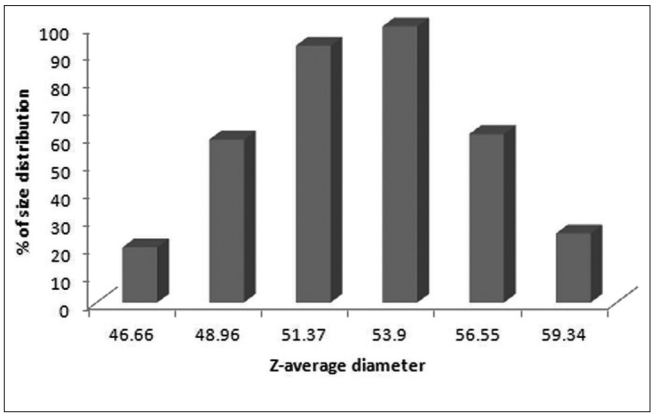

Figure 4: Droplet size distribution of eucalyptus oil nanoemulsion with 1:2 (v/v %) ratio of oil and surfactant by simple mixing method

Leakage of ultraviolet-absorbing substances

The cytoplasmic contents release from the cells of *L. monocytogenes*, when interacted with diluted nanoemulsion (10-fold, 100-fold and 1000-fold) formulation can be quantified by measuring the absorbance at 260 nm. In Figure 5, there is an increased release of cytoplasmic contents from 10-fold as compared to the positive control (sodium benzoate); and 100 and 1000-fold dilution showed decreased levels of cell content release by interacting up to a period of 60 min. The nanoemulsion droplet is capable of fusing with lipid bilayer present in the cell membrane of the pathogen. These results in membrane compromised, and thereby destabilization occurs in the membrane integrity. This confirms lysis and death of the treated cell with diluted nanoemulsion.[36] Thus, the changes in the membrane structure would have altered their permeability and caused the subsequent release of intracellular components.

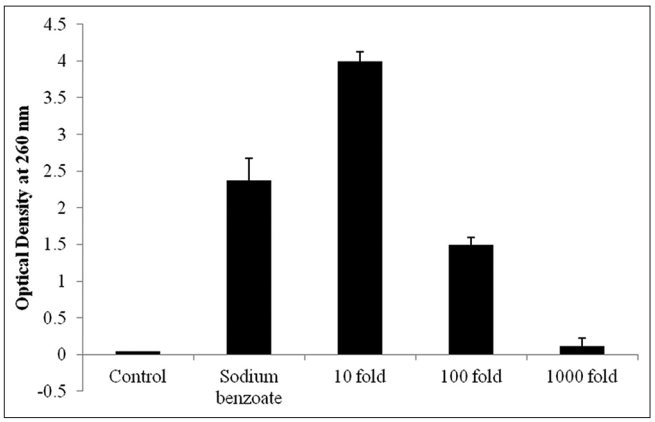

Figure 5: Leakage of ultraviolet absorbing substances in the supernatants of nanoemulsion (10-fold, 100-fold, 1000-fold) treated *Listeria monocytogenes* cells

Effect of nanoemulsion on *Listeria monocytogenes* cell surface by atomic force microscopy

The morphology of control cells of *L. monocytogenes* were smooth and intact cell membrane [Figure 6a]. Exposure to 10-fold diluted nanoemulsion for 30 min alters the cell morphology to rough appearance, and the cells appeared to be shrunken [Figure 6b]. In the previous literatures, the AFM technique was utilized in the study of morphological damage that occurs in bacteria.[37,38] In recent reports using essential oils like carvacol, eugenol was studied through AFM examination.[39] Our results suggested that rough surface morphology and shrinkage of cells was evident in the cells treated with nanoemulsion as compared to the control ones. Loss of membrane permeability and damaged cell surface further supports the evidence that the mode of bactericidal action of the nanoemulsion against *L. monocytogenes* is through membrane disruption and further cell death occurs.[40]

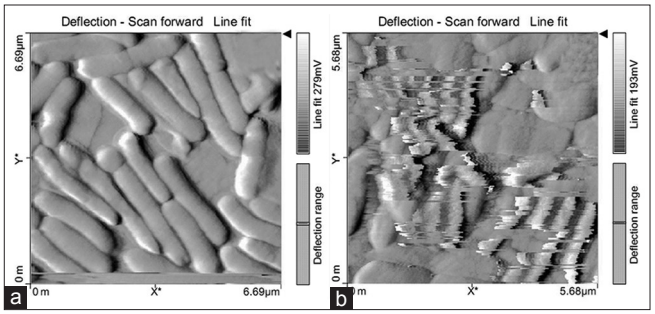

Figure 6: Atomic force microscopy images of *Listeria monocytogenes* (a) untreated bacteria, showed intact cell morphology (b) bacteria treated with 10-fold nanoemulsion for 30 min showed membrane damage

Eucalyptus oil based stable food-grade nanoemulsion using a low energy emulsification method with a droplet diameter of 47 nm demonstrated bactericidal activity against food borne pathogen *L. monocytogenes*. The present study illustrates that simple mixing and heat treatment method had significant effect on the nanoemulsion droplet diameter and its stability. The SOR had direct relation to its droplet size and stability. Simple mixing method exhibited significant antibacterial activity against *L. monocytogenes* with a zone size of 30 mm than oil alone tested that showed 15 mm zone size. To the best of our knowledge, this is the first report showing eucalyptus oil nanoemulsion with a reduced droplet diameter

by low energy emulsification method. The formulated eucalyptus oil nanoemulsion has the potential as an effective antibacterial agent in food industries.

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