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Research Article

ANTIBACTERIAL ACTIVITY OF EUCALYPTUS OIL NANOEMULSION AGAINST *PROTEUS MIRABILIS*

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ABSTRACT

The present study deals with the formulation and characterization of bio-based oil in water nanoemulsion and its potential antibacterial activity against *P.mirabilis*. The nanoemulsion was formulated using eucalyptus oil, Tween 20 and ethanol by high energy method, Ultrasonication and the mean droplet size was found to be 20.17 nm as confirmed by dynamic light scattering. The nanoemulsion was highly stable, transparent and found to be effective bactericidal activity against tested pathogen. Growth inhibition was found to be 100% when treated with nanoemulsion as confirmed by dilution plate count and antibacterial susceptibility method.

Keywords: Eucalyptus oil, Tween 20, Nanoemulsion, Ultrasonication, Antibacterial activity

INTRODUCTION

Currently there is a growing interest in the study of nanoemulsions with plant based oils due to its bioavailability and biocompatibility. Nanoemulsions or sub-micron emulsion or mini-emulsion can be defined as oil-in-water (O/W) emulsions with mean droplet diameters ranging from 50 to 1000 nm, and their average droplet size is between 100 and 500 nm[1]. This can be achieved by high-energy and low-energy emulsification methods. In high energy emulsification[2] system make use of mechanical energy using Ultrasonicator or High pressure homogenizer and low-energy emulsification methods, which use the chemical energy stored in the components of the system to be emulsified using PIT[3] or PIC methods[4]. Emulsifiers are surfactants play a major role in the formation of stable nanoemulsions in aqueous solutions. The interfacial tension between the oil and water phases was reduced. Low amount of surfactant required to form nanoemulsion using high energy method to disrupt the droplets into nanosize droplets. Moreover, they form a strong barrier between the droplets thus prevents coalescence in newly formed droplets[5]. Essential oils has a rich source of bioactive compounds and have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties[6,7,8]. Tween 20 is a non-ionic surfactant that readily miscible at the oil-water interface, and moreover less sensitive to pH and ionic strength, it is used for various applications[9] including nanoemulsions[10,11].

In the present study, eucalyptus oil was selected as an oil phase, Tween 20, Bioxtra (Polyoxyethylene sorbitan monolaurate) as the surfactant, ethanol as the cosurfactant and water as the aqueous phase. There has been very few reports in the formulation and characterization of nanoemulsion using plant based oil with biologically based surfactant for antibacterial studies.

MATERIALS AND METHODS

Materials

Eucalyptus oil (Himedia, India), Tween 20 (Polyoxyethylene (20) sorbitan monolaurate) (Sigma aldrich, India), as surfactant and Ethanol as cosurfactant. Water was taken from Milli-Q-water purification system (Millipore, MA, USA).

Microorganism

The bacterial strain, *Proteus mirabilis* (NCIM 2388) was purchased from National Chemical Laboratory (Pune, India). The strain was cultured in a 50 ml nutrient broth at 37 ºC overnight. 1 ml of this grown culture was reinoculated again into a 50ml nutrient broth and, the growth was adjusted to 1×10^8 colony-forming units (cfu)/ml] at 600 nm using phosphate buffered saline (PBS).

Table 1: Selected Nanoemulsion Formulations (%(V/V))

Components	F1	F2	F3	F4	F5	F6
Eucalyptus oil	10	10	10	10	33.33	25
Tween 20	80	40	10	5	33.33	25
Ethanol	-	40	-	5	-	25
Water	10	10	80	80	33.33	25

Preparation of Nanoemulsions

Nanoemulsion formulations were prepared according to the composition presented in Table 1. The variables were the presence or absence of cosurfactant. The nanoemulsions were prepared by mixing the oil with the surfactant or surfactant/cosurfactant mixture before adding the required amount of water and then the mixture was equilibrated using an Ultrasonicator for 10 min (Sonics, USA).

Characterization of the nanoemulsion

Droplet size and size distribution

The droplet size and size distribution of the eucalyptus oil nanoemulsion were determined using a Brookhaven particle size analyzer (90S). Nanoemulsions were diluted to 1:30 dilution with water to reduce multiple scattering effects prior to each experiment. Droplet size was described as the size in nm, and the polydispersity index (PDI). Each measurement was carried out in triplicate.

Thermodynamic stability studies

The physical stability of a nanoemulsion formulation depends upon its preparation and mixing ratios of oil phase and aqueous phase. In addition, poor formulation can lead to phase separation, affecting not only formulation performance, but also visual appearance. Thermodynamic stability tests were perform to overcome metastable formulation[12].

1. Centrifugation

Selected formulations were centrifuged at 3500rpm for 30 minutes. The formulations that did not show any phase separations were taken for heating and cooling cycle.

2. Heating and cooling cycle

Six cycles between refrigerator temperature (4°C) and 45°C with storage at each temperature of not less than 48 hours were done. The formulations, which were stable at these temperatures, were subjected to a freeze-thaw cycle test.

3. Freeze-thaw cycle

Three freeze-thaw cycles were done for the formulation between –21°C and 25°C. The formulations that survived thermodynamic stability tests were selected for further study.

Microscopic studies

Phase contrast microscopy

The morphology of the nanoemulsion formulation before and after sonication was studied by phase contrast microscopy (Carl Zeiss, USA).

Antibacterial activity of the selected nanoemulsion

Bacterial susceptibility test

A single isolated bacterial colony was streaked onto the nutrient agar medium overnight prior to testing. A few colonies were inoculated into nutrient broth, and it was adjusted to 1×10^8 cfu/ml approximately using PBS. Ten fold dilution of the concentrated nanoemulsion were made with 1×10^8 cfu/ml and incubated at 37 ºC in an orbital rotary shaker for 4 h. Duplicate aliquots from the interacted cells (100µl) were plated onto nutrient agar plates. After overnight incubation, colonies were counted using colony counter. All the bactericidal assays were done in triplicates\, and the average results with standard error were plotted.

Dilution plate count method

Bacteria were grown on the appropriate solid medium overnight prior to testing. A few colonies were suspended into nutrient broth and it adjusted to approximately 1×10^8 CFU/ml using phosphate buffer saline. Undiluted nanoemulsion formulation was taken and incubated at 37^0C in orbital rotary shaker for 4 h. Ten fold serial dilutions were made in each interval and duplicate aliquots were plated on nutrient agar plates. After overnight incubation, colonies were counted. All bactericidal assays were repeated at least three times, and average results were reported.

RESULTS AND DISCUSSION

Selection of formulated nanoemulsion

Nanoemulsion formulation from F1 to F6 was formulated. After sonication, the samples were selected based on thermodynamic stability study. F6 formulation was found to be highly stable and less viscous\, and this was selected for further characterization studies.

Characterization of nanoemulsion formulation

Droplet size distribution

Eucalyptus oil nanoemulsion was formulated with Tween 20 and Ethanol. The Z-average particle size was found to be 20 nm and the typical size distributions of such dispersion are shown in Fig. 1. and the polydispersity index remained below 0.2, which reflects their relative homogeneity.

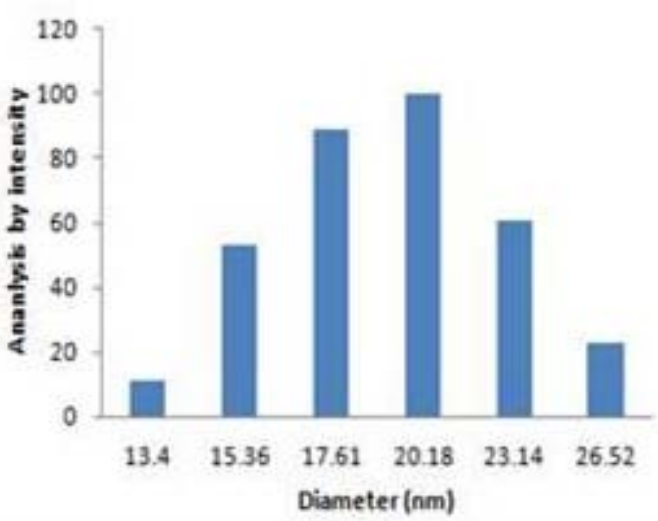

Fig. 1: Particle size distribution of formulated nanoemulsion by DLS

Thermodynamic stability studies

Stress test including centrifugation, heating cooling cycle and freeze thaw cycles showed that F6 formulation had a good physical stability without any phase separation, creaming and flocculation. Thus, it can be concluded that the nanoemulsion formulations were not only physically stable but also chemically stable.

Microscopic studies

Phase contrast microscope

One drop of nanoemulsion was kept in glass slide, and it was covered with a cover slip then the slide was observed under the microscope. Before sonication, the sample showed oil droplets. After sonication, the sample doesn't show droplets. It reveals that the droplet size was reduced to nanosize because of sonication, and it was well correlated with particle size measurement. Light microscopy is more sensitive than laser diffraction to detect a few larger particles present in formulation[13] and also recommended getting a fast indication of the presence of microparticles or aggregation in sample.

Antibacterial activity

Using nanoemulsion as an antimicrobial agent is a promising and new innovation[14]. The formulated nanoemulsion was tested on *P.mirabilis* using bacterial susceptibility method and dilution plate count method. In bacterial susceptibility method, it was observed that the bacterial strain was susceptible to the formulated nanoemulsions and showed no growth compared to Tween 20, ethanol and oil alone (Fig. 3, A, B, C, D, E). These results are well correlated with previous report using sunflower oil microemulsion[15].

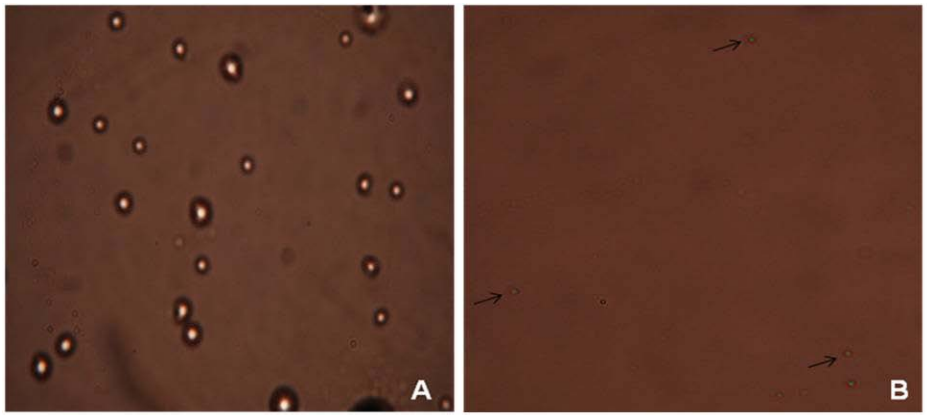

Fig. 2: Microscopic image: Before sonication (A) and after sonication (B) of the formulated nanoemulsion

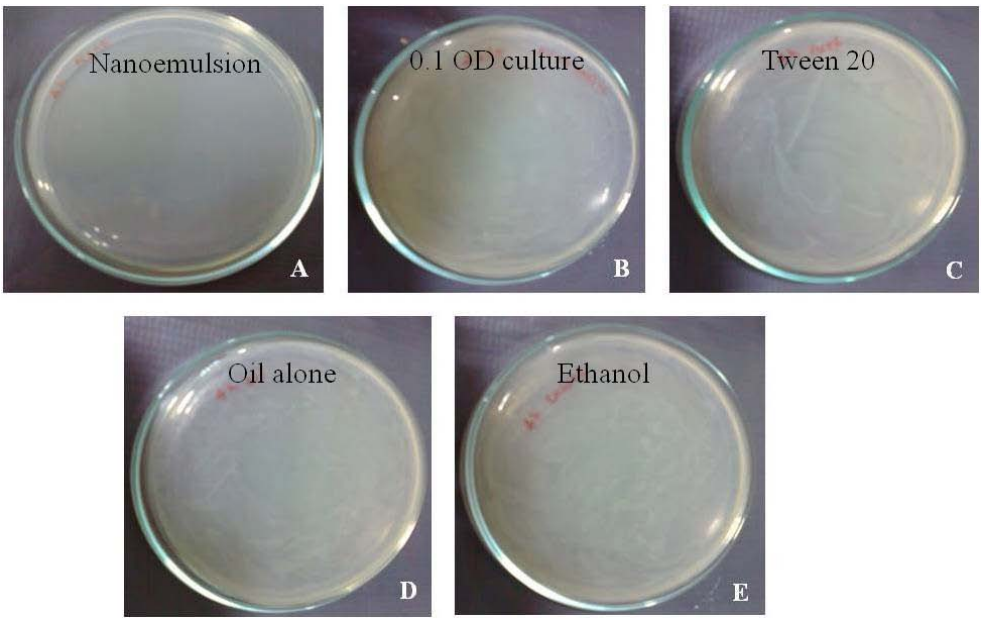

Fig. 3: Antibacterial susceptibility (*P. mirabilis*) of the formulated nanoemulsion by spread plate method

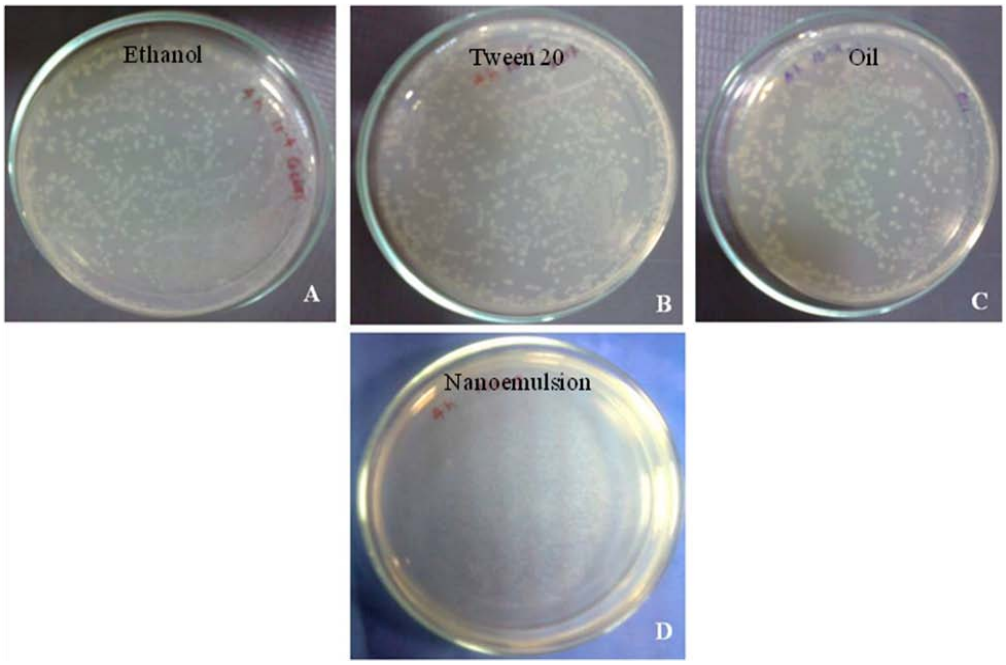

Fig. 3: Antibacterial activity (*P.mirabilis*) of the formulated nanoemulsion by dilution plate method

In dilution plate count method, the bacterial cell was adjusted to 0.1 OD (1×10^8 cfu/ml). This was serially diluted to ten-fold with phosphate buffer saline, to count visible colonies. The growth inhibition (>100 % reduction) of the bacteria by dilution plate count method was found to be within 4h itself. The results are in agreement with those previous reports, using less volume of kaffir lime oil emulsion showed effective antibacterial activity. In a fig. 3 A, B, C, D shows the absence of growth when treated with nanoemulsion within 4h compared to oil, surfactant and cosurfactant alone. It reveals that nanodroplets found to be effective in killing the bacterial cells.

CONCLUSION

The results of the current work show that it is possible to obtain plant based oil-in-water nanoemulsion stabilized by Tween 20 with cosurfactant ethanol. It was concluded that eucalyptus oil nanoemulsion using high energy method forming nanosize droplets would have higher antibacterial activity than eucalyptus oil alone. These results clearly indicate that the nanoemulsion is stable, transparent formulation with effective killing rate against bacterial growth. Our future studies would be on the formation of topical product of this nanoemulsion formulation against uropathogens.

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