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Research Article

MUSTARD OIL MICROEMULSION FORMULATION AND EVALUATION OF BACTERICIDAL ACTIVITY

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ABSTRACT

Microemulsion was formulated using mustard oil, non-ionic surfactant Tween 20 and water. MF5 formulation showed higher stability with droplet size in the range of 118-430nm with a polydispersity index of 0.236. Low polydispersity index confirms the homogeneity of emulsion formulation. Mustard oil based microemulsion formulation demonstrated effective antibacterial activity against *E. coli.* Kinetics of killing of bacterial polutaion showed that 10-fold diluted MF5 formulation was effective in 5 log reductions of *E. coli* population in an interaction period of 60 min. Alteration in bacterial surface functional groups were observed in microemulsion-treated cells. These results pertaining to FTIR of untreated and treated *E. coli* substantiated the kinetics of killing of bacteria upon microemulsion treatment.

Keywords: Microemulsion, Low energy, Mustard Oil, Tween 20, Antibacterial activity

INTRODUCTION

Microemulsions are homogeneous thermodynamically stable dispersions of oil, water and a surfactant, usually in combination with a cosurfactant. The term microemulsion was coined by Schulman and coworkers (1959)[1]. Oil and water are two immiscible liquids. Hence, the emulsion formed is unstable and tends to separate into constituent phases. Emulsifier decreases the interfacial tension at the oil/water interface and stabilizes emulsion. Surfactants are a group of amphiphilic surface active agents that acts as emulsifiers and helps in making the emulsion stable.

Potential advantage of microemulsion is that they are thermodynamically stable and require very less energy to formulate emulsion. Microemulsions are potent antimicrobial agents[2,3,4]. Microemulsions have proven to be one of the potent delivery agents of lipophilic drugs and its controlled release[5,6,7].

Essential oils are illustrious antibacterial[8,9,10], antifungal[11], antioxidant[12], antigiardial[13] and antidiabetic[14] agents. Different essential oils and oil based formulations are reported to be efficient antimicrobial agents that can be used to prevent food spoilage[15,16,17]. The antibacterial activity may be due to the ability of oil components to damage the bacterial membranes and hence resulting in lysis of the cell.

The aim of our present study is to formulate microemulsion using mustard oil, Tween 20 and water. Mustard oil is food grade oil that is reported to have antibacterial property[18]. Tween 20 is a non-ionic, non-toxic surfactant that is not affected by change in pH. The antibacterial efficacy of the formulated microemulsion is also investigated.

MATERIALS AND METHODS

Chemical Reagents

Mustard oil (refined & food grade) was purchased from local super market. Tween 20 (Polyethylene glycol sorbitan monolaurate) was procured from Sigma Aldrich, India. Deionised milli-Q (Millipore Corporation) water was used for all the experiments.

Microemulsion Preparation

Microemulsions were formulated using mustard oil, Tween 20 and water. Mustrad oil used for this formulation was food-grade oil. Nonionic surfactant Tween 20 was used as surfactant for formulating microemulsion with an average HLB (Hydrophilic-lipophilic balance) value of 16.7 that is favourable for formulation oil-in-water emulsion. Also being a small molecule surfactant molecule Tween 20 gets adsorbed easily to the oil/water interface and helps in formation of oil-in-water emulsion when compared to high molecular weight polymers. 6 % v/v of mustard oil was fixed for all the formulations. Microemulsions were prepared by to adding water organic phase (oil & surfactant). Different formulations were prepared by mixing oil and surfactant in various ratios such as, 1:1 (MF1), 1:2 (MF2), 1:3 (MF3), 1:4 (MF4) and 1:5 (MF5) respectively. Water was added dropwise to the organic phase (mixture of oil & surfactant) under stirring condition using a magnetic stirrer at 400 rpm. All the formulated emulsions were then characterized and their stability was assessed.

Characterization of Microemulsion

Droplet size distribution

Droplet size measurement formulated microemulsion was performed using 90 plus particle size analyser (Brookhaven Instruments Corporation, USA). This instrument utilizes dynamic light scattering method to determine droplet size. Microemulsion formulation was diluted with double distilled milli-Q (Millipore corporation) water prior to experiment to do away with the effect of viscosity caused due to ingredients.

Stability Studies

Resistance of formulated micremulsions to centrifugation was studied by centrifuging the emulsions at 10,000 rpm for 30 min. Emulsions were checked for phase separation (if any) after being subjecting to centrifugation.

Intrinsic stability was examined by storing all the microemulsion formulations at room temperature. Phase separation or creaming (if any) of the formulations was then observed. All the experiments were performed in triplicates.

Antibacterial Studies

Kinetics of killing

Mustard oil microemulsion formulation MF5 was chosen for studying antibacterial efficiency due to its lowest droplet diameter and comparatively high stability. Overnight culture of E. coli (NCIMB 2809) was centrifuged (15 min, 5000 g) and washed twice in phosphate buffered saline (PBS, pH 7.4). Test culture was then prepared with a known inoculum size (1 x 10^5 CFU/ml), and challenged with MF5 microemulsion formulation. For viable count enumeration, 0.1 ml of the interacted sample was taken from each tube, serially diluted and spreaded onto nutrient agar plates[2]. Viable colonies were counted after being incubated for 24 hrs at 37°C. All the experiments were performed in duplicates.

FTIR

Structural modifications of functional groups on bacterial surface upon treatment with mustard oil microemulsion were analyzed by FTIR spectroscopy. A known concentration of *E. coli* inoculum

(1x10^5 CFU/ml) was interacted with 10-fold diluted MF5 mustard oil microemulsion. Interaction was carried out for 60 min. Samples were prepared by adding required amount of pellet with potassium bromide crystals. FTIR spectroscopic analysis was performed using Perkin Elmer Spectrum1 FT-IR instrument. Scanning was done in the range of 450-4000 cm^{-1} with a resolution of 1.0 cm^{-1}.

RESULTS AND DISCUSSION

Selection of Microemulsion formulation

Different microemulsion formulations (MF1, MF2, MF3, MF4 and MF5) were prepared using mustard oil, Tween 20 and water. Results pertaining to MF5 formulation showed highest stability. Phase separation was observed in MF1, MF2 and MF3 formulations after centrifugation. Upon storage for one week duration, phase separation was observed in MF4 but not in MF5. It showed higher stability when compared to other formulations. Hence, MF5 formulation was used for further characterization and application studies.

Droplet size distribution

Microemulsion was formulated using mustard oil, Tween 20 and water. Based on intrinsic stability assessment, MF5 formulation was chosen for droplet size characterization. Figure 1 shows the droplet size distribution of MF5 formulation. Emulsion droplets were found to be in the range of 118-430 nm. Similar results were reported for formulation of fluconazole based topical microemulsion[7]. Polydispersity index of the formulation was 0.236, which demonstrates the homogeneity of the emulsion droplets.

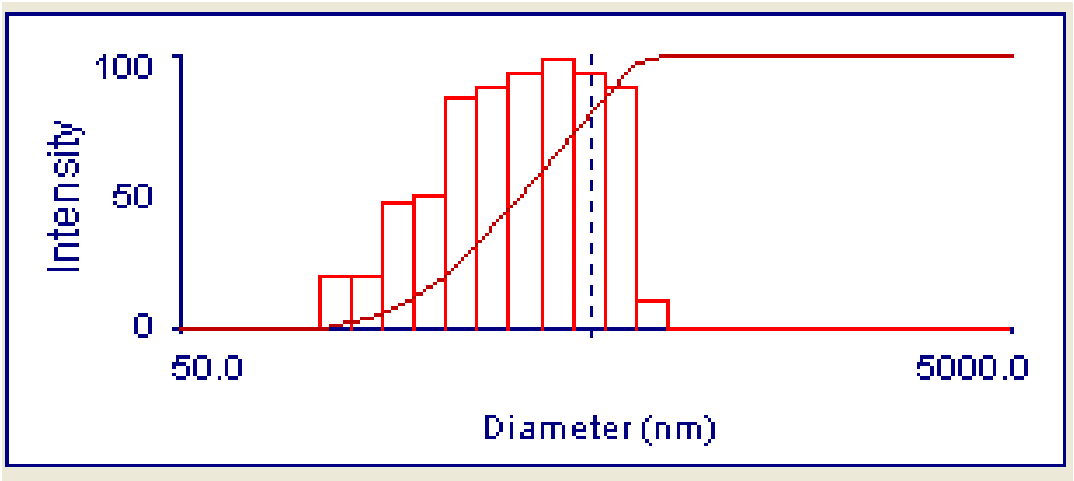

Fig. 1: Droplet size distribution of MF5 formulation

Antibacterial Studies

Kinetics of killing

Mustard oil microemulsion (MF5) was opted to study antibacterial efficiency due to its stability and low droplet diameter. Figure 2A shows the agar plate with untreated (control) bacterial cells. A lawn of *E. coli* was formed. Upon treatment with mustard oil microemulsion there was a decrease in bacterial colony number that is directly related to interaction time (Fig 2B-2F). A gradual decrease in number of bacterial colony was observed with the increase in interaction time. No colonies were found in the agar plate used for growing viable colonies after treting *E. coli* with MF5 formulation for 60 min. This suggests that all the cells were killed resulting in complete loss of viability.

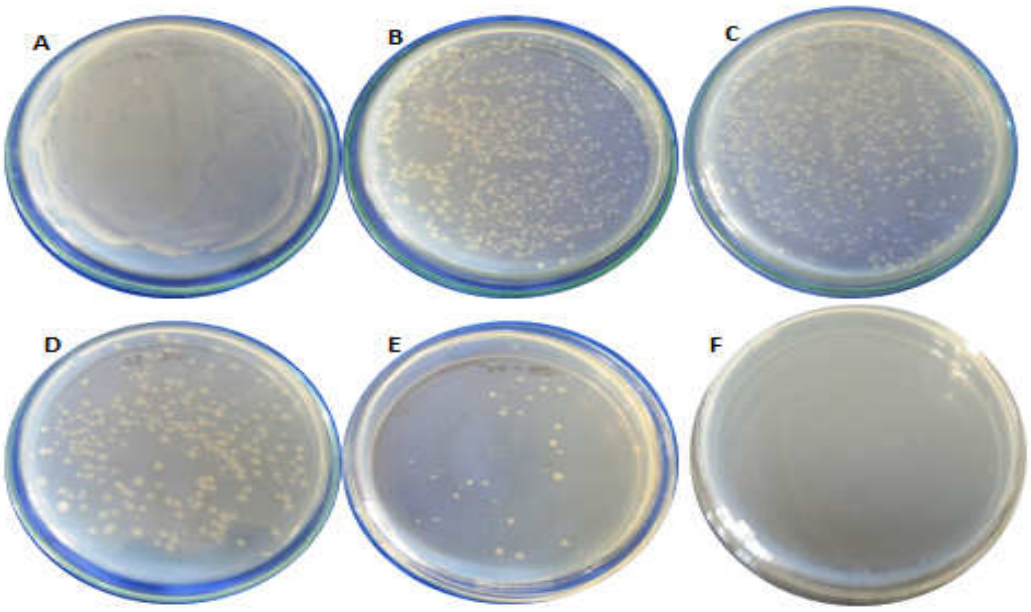

Fig. 2: Antibacterial activity of mustard oil microemulsion at different interaction time. Fig 2A shows untreated (control) bacteria cells, Fig 2B (5 min), Fig 2C (15 min), Fig 2D (30 min), Fig 2E (45 min) and Fig 2F (60 min) respectively.

Kinetics of killing experiment showed the gradual reduction in bacterial population by increasing the interaction time (Fig 3). Ten-fold diluted mustard oil microemulsion treatment was done for *E. coli*. Ten-fold diluted MF5 formulation showed 2 log reductions in bacterial population after treating for 15 min. Approximately 50 % reduction in bacterial population was observed after an interaction period of 30 min. Four log reductions in *E. coli* were seen after treatment for 45 min and a complete loss of viability was observed after interaction period of 60 min. These results go along with the earlier reports that microemulsions are potent antibacterial agents[3].

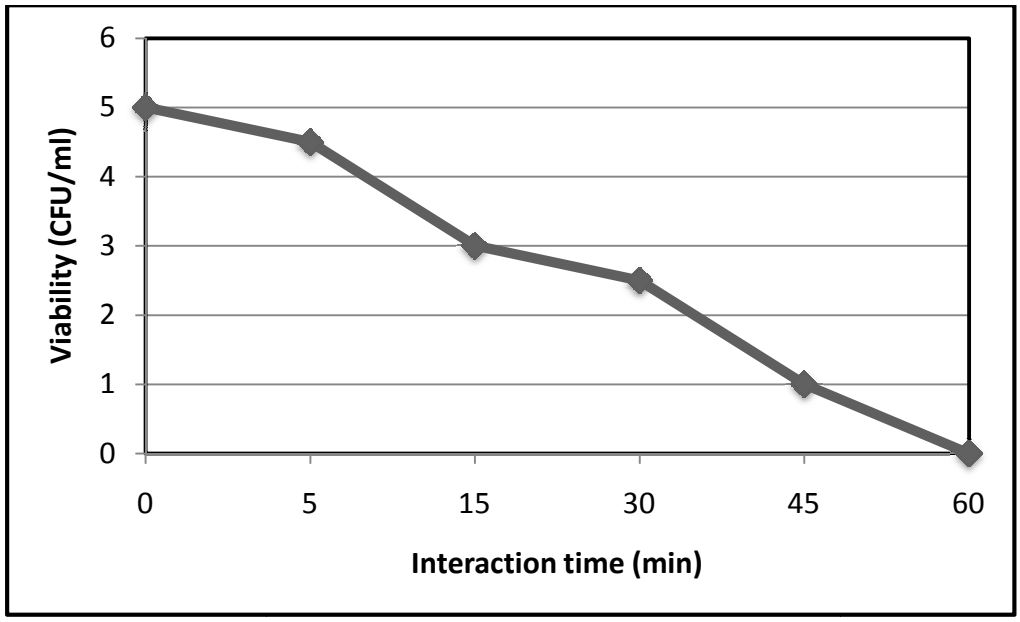

Fig. 3: Time dependent decrease in *E. coli* upon treatment with MF5 mustard oil microemulsion.

FTIR

Structural modification of bacteria at molecular level upon microemulsion treatment was studied by fourier transform infrared spectroscopy. Figure 4 shows the spectral changes observed in untreated (control) and microemulsion treated *E. coli* cells in the frequency range of 4000-450 cm-1.

All the bands were assigned based on previous reports. The bactericidal effect of mustard oil microemulsion on *E. coli* caused changes in spectral features in the different regions such as, 1700-1500 cm-1 corresponding to protein region, 1500-1200 cm-1 corresponding to mixed region, 1200-900 cm-1 corresponding to carbohydrate region and 900-720 cm-1 corresponding to fingerprint region respectively. Intensity of band at 1237 cm^{-1} was changed which is endorsed to deformation in bacterial membrane phospholipids[19]. These results suggest that upon treatment with mustard oil microemulsion, alteration in functional groups on the *E. coli* surface. This change in membrane functional groups and membrane damage would have caused leakage of intracellular materials leading to cell lysis and death.

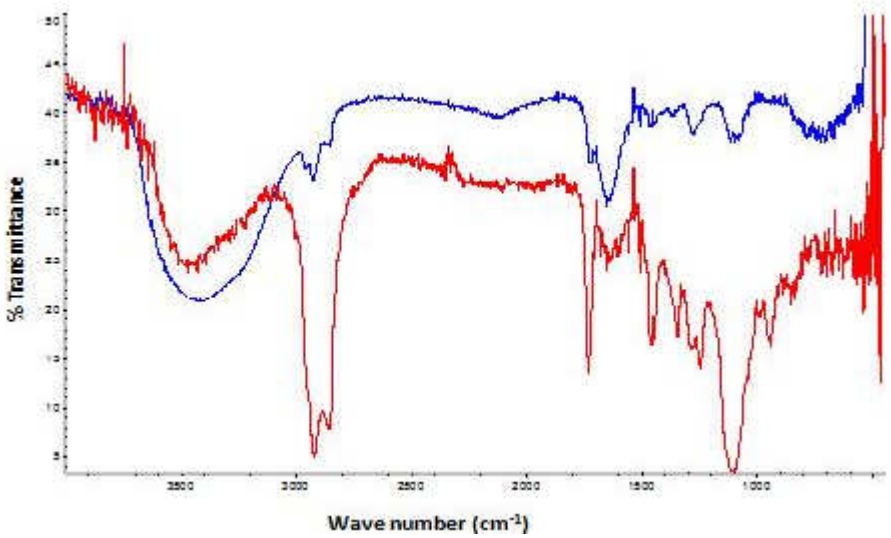

Fig. 4: FTIR spectra of untreated ( ) and mustard oil microemulsion treated ( ) bacteria cells

CONCLUSION

A dilutable food-grade microemulsion was formulated using mustard oil and non-ionic surfactant Tween 20 by spontaneous emulsification which exhibited bactericidal activity against *E. coli*. In the present study we observed that surfactant concentration and mixing ratio of oil and surfactant had significant effect on droplet diameter and stability of microemulsion. The formulated microemulsion also exhibited significant antibacterial activity against *E. coli* after being diluted. FTIR studies suggested that mustard oil microemulsion demonstrated bactericidal activity by altering the functional groups present on bacterial surface. Further, this mustard oil based microemulsion can be used against microbial spoilage for food preservation.

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