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Research Article

ANTI BACTERIAL ACTIVITY OF SUNFLOWER OIL MICROEMULSION

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ABSTRACT

The aim of the study was to investigate the antibacterial activity of refined Sunflower oil, Tween 20 (polyoxyethylene (20) sorbitan monolaurate), water microemulsion system. Pseudo-ternary phase diagram was constructed to obtain the concentration range of oil, surfactant and water. Three microemulsion formulations were prepared, of which oil, tween 20 and water were made to 100 %. Conductivity and pH were used to characterize microemulsion. The concentration of refined sunflower oil varied from 5 % to 15 %, the surfactant concentration varied from 10 % to 30 % and water concentration varied from 55 % to 85 %. When water concentration increases, conductivity of the microemulsion system increases upto 50 % of water concentration and after that become stable. When oil and surfactant concentration was increased, pH of the microemulsion system decreases. Kinetic studies showed inhibition of bacterial growth in all formulated microemulsions. Bacterial growth was enhanced in the case of oil and surfactant alone.

Keywords*:* Refined sunflower oil; Microemulsions; Pseudo-ternary phase diagram; Anti-bacterial activity.

INTRODUCTION

Emulsions are pharmaceutical preparations consisting of at least two immiscible liquids; typically oil and water. A microemulsion is defined as a dispersion consisting of oil, surfactant, co surfactant and an aqueous phase. It is a single optically isotropic and thermodynamically stable liquid solution with a droplet diameter usually within the range of 10–100 nm[1]. Microemulsions have a number of special properties such as enhanced drug solubility, good thermodynamic stability, ease of manufacturing and permeation enhancement ability over conventional formulations, which have been exploited in drug delivery systems[2,3], pharmaceutics[4], and food industries[5].

In the present study, refined sunflower oil was used as an oil phase, polysorbate 20 (polyoxyethylene sorbitan monolaurate) as the surfactant and water as the aqueous phase. An oil phase plays a very important role in the formation of a microemulsion. Sunflower oil which is non-volatile oil extracted from sunflower (*Helianthus annuus*) seeds was chosen as our oil of interest. Even though many applications of sunflower oil has been reported in the food and cosmetic industry, not many reports are available regarding the use of this oil in the formulation of microemulsion for antibacterial studies.

Tween 20 which was readily miscible with sunflower oil was selected as a surfactant in this study, because of its non-ionic nature and its less sensitivity to pH and ionic changes. Moreover tween 20 is known to be effective in increasing microemulsion region[6].

Pseudo-ternary phase diagram was constructed using different compositions of the oil/surfactant/water mixtures. The formation of microemulsions and its structural changes were monitored by changes in electrical conductivity[4, 7]. pH dependent changes and the stability studies were also performed to optimize the formed microemulsion system.

Though there are some studies on the anti-bacterial[8, 9] and antifungal activity of microemulsion[10], there are not many reports on the antibacterial preparations from natural resources, especially using refined sunflower oil.

MATERIALS AND METHODS

Materials

Refined Sunflower oil, Tween 20(polyoxyethylene (20) sorbitan monolaurate) (sd-fine media chem. Limited, Mumbai) were all food grade additives. The water used was double distilled.

Construction of pseudo-ternary phase diagram

Pseudo-ternary phase diagram was constructed to find out the concentration range of all components (Oil/surfactant/water) in which they form microemulsion. The pseudo-ternary phase diagram was constructed using water titration method at ambient temperature[11]. Mixtures of oil and surfactant were prepared in different percentages keeping 1:2 ratio of oil to surfactant. This mixture was titrated dropwise with water under gentle magnetic stirring and was sonicated for 10 min. The samples were classified as microemulsions when they appeared as

clear liquids. After being equilibrated the systems was visually characterized.

Preparation of Microemulsions

The microemulsions were prepared by mixing the oil with the surfactant mixture before adding the required amount of water under magnetic stirring. Then the mixture was equilibrated using a sonicator for 10 min (Sonics, USA).

Characterization of the selected microemulsions formulations

Centrifugation

In order to check out the stability of the selected formulations, the microemulsions were centrifugated at 10,000 rpm for 30 min.

Conductivity measurements

The solubilization of water phase in the selected oily mixture was monitored quantitatively by measuring the electrical conductivity (σ). The water was added drop by drop to the initial mixture of oil and amphiphiles, and the σ of formulated samples was measured using a conductivity meter.

pH

The pH values of the samples were measured by a pH meter (model HI 8417, Hanna Instruments Inc., Woonsocket, USA), at 20±1 ^{0}C.

Test of antibacterial activity of refined sunflower oil microemulsions

The antibacterial efficacy of refined sunflower oil microemulsions were tested and compared to that of pure sunflower oil using agar well diffusion assay, and spread plate method. Growth kinetic studies were also performed.

Bacterial susceptibility to microemulsions

Susceptibility of *E. coli* (ATCC 25922, 13534), *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 25619) to different concentration of microemulsions (5 %, 10 %, 15 % of oil and 10 %, 20 %, 30 % of surfactant) was examined. Approximately 10^7 – 10^8 colony-forming units of microorganism were inoculated on nutrient agar plates. The plates were then supplemented with 1ml of each microemulsion and the plates were incubated for 24 hours at 37ºC. Microemulsion free plates incubated under the same conditions were used as controls. At least two independent experiments were carried out for each set of conditions.

Agar-well diffusion method

Well diffusion method was performed with each of the formulated microemulsions as well as oil and surfactant alone. Using 6 mm diameter well cutter, wells were made with equal distance, after the medium was set. A drop of the soft agar was dropped into the well to seal the bottom. The bacterial suspension (10^7 – 10^8 CFU/ml) was swabbed on the surface of nutrient agar, (Muller Hinton agar (Himedia Laboratories Ltd., Mumbai) and Luria Bertani (LB) agar) before making wells on the plate. After allowing for 10min setting, 100 µl of each sample was added in to the well. The plates were then incubated without inverting at 37ºC for 24 hours and the average diameter of the inhibition zone surrounding the well was measured using a scale. Experiments were performed in duplicates.

Kinetics of Killing

Sterile Erlenmeyer side armed flasks (250 ml), each containing 50ml nutrient broth were inoculated with one ml of the freshly prepared bacterial culture carrying approximately 10^7–10^8 CFU/ml. Experiments were performed in triplicates for each of the formulated microemulsions both in diluted and undiluted forms and their respective concentration of oil and surfactant. These were then incubated in an orbital shaker at 200 rpm in room temperature (28-30ºC) to minimize the aggregation of the samples. Growth rate was determined by measuring absorbance at 600 nm at regular intervals. A positive control (flask containing microemulsions and nutrient media, devoid of inoculum) and a negative control (flask containing inoculum and nutrient media, devoid of microemulsions) were included in this experiment. The negative controls indicated the microbial growth profile in the absence of microemulsions. From the experimental values (flasks containing nutrient media, inoculum and microemulsions) the absorbance values for positive controls were subtracted [12].

RESULTS AND DISCUSSION

Pseudo-ternary phase diagram

Pseudo-ternary phase diagram of the investigated ternary system water/tween 20/refined sunflower oil is shown in Fig.1. Shaded area in the figure refers to the microemulsion region while the outside area indicates multiphase turbid regions. A continuous single-phase microemulsion region was observed over the oil when water content ranges from 0-100%. From the phase diagram, the concentration of oil, water and surfactant required to form microemulsion were determined (Table-1). The oil-surfactant ratio was maintained as 1:2 in all mixtures of microemulsion. Based on the phase diagram, three microemulsion formulations were selected from the microemulsion region for anti-bacterial studies.

Table 1: It shows the composition of the selected microemulsion formulations (% w/w)

Refined sunflower oil	Tween 20	Water
5	10	85
10	20	70
15	30	55

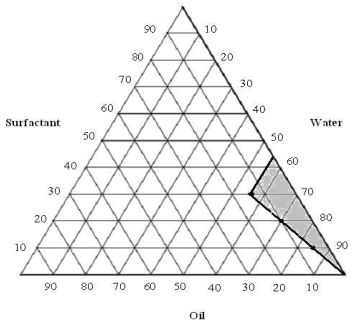

Fig. 1: It shows the pseudo-ternary phase diagram of refined sunflower oil/tween 20/water

Characterization of the selected microemulsions formulations

Conductivity measurement is the most frequently used technique to understand the microstructure and structural changes of selected microemulsions[13]. It is on the basis of the percolation theory, which has been generally accepted to determine the microstructures of microemulsions[14]. The electrical conductivity is expressed as μS/cm and the water concentration is expressed as percentage. The electrical conductivity (σ) of the selected oil and surfactant mixture, as a function of concentration of water (ϕω), is shown in Fig.2. According to obtained conductivity data, the investigated microemulsions can be designed as a type of system where the σ is fairly high and varies with Φw. Our results showed that the electrical conductivity of the selected oil and surfactant mixture was very low as long as Φw was smaller than 15 % (w/w). During the water titration up to ϕω ≈ 50 % (w/w), σ increases fast. At ϕω > 50 % (w/w), the conductivity of the system was not significantly affected by the further addition of water. While the water volume fraction increases, the electrical conductivity of the system slightly increases until critical Φw was reached and a sudden increase in conductivity was observed. This follows percolation theory[14]. Our microemulsion system contains non-ionic amphiphiles, which exhibited electroconductive behavior. In the region of low water content, water in oil microemulsion is formed that has a high resistance to conductivity (oil continuous)[15]. Beyond the percolation threshold (ϕπ ≈ 15 % (w/w)) conductivity increases linearly and sharply up to ϕω ≈ 50 % (w/w). It can be concluded that beyond ϕπ a network of conductive channels exists, which corresponds to the formation of water cylinders or channels in an oil phase due to the attractive interactions between the spherical microdroplets of water phase in the water in oil microemulsions. Volume of water to oil increases, the resistance decreases, and in the turbid region lamellar micelles of hexagonal shape was formed. Above a critical ratio, inversion occurs and the resistance decreases producing an oil in water microemulsions. For the ϕω > 50 % (w/w) the electrical conductivity increases non-linearly up to a maximum at ϕω ≈ 55 % (w/w). The peak occurs at around 50 % aqueous content. This would be a transition point to oil in water emulsion[16]. Thus, the σ vs. Φw curve illustrates the occurrence of the three structural regions: water in oil region [ϕω < 15 % (w/w)], bicontinuous region [15 % (w/w) < ϕω < 50 % (w/w)], and oil in water [ϕω > 50 % (w/w)].

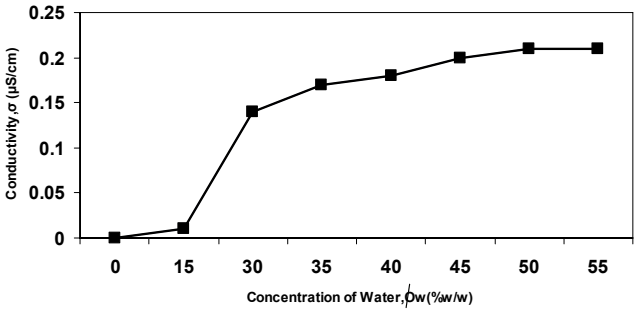

Fig. 2: It shows the electrical conductivity (σ) as a function of water phase volume fraction (ϕω) in the system with oil (15 %) and tween 20 (30 %)

Examination of the physical stability of the microemulsion was performed by centrifugation at 10,000 x g in room temperature for 30 min. No phase separation was seen, indicating that the emulsion is stable. Such observed stability would be a function of both the chemical nature of the microemulsions and their inherent antibacterial nature[8]. Microemulsion pH was in the range of 4.53-4.93. It was observed that when oil and surfactant concentration was increased, the pH of the system decreased from 4.93, 4.55, and 4.53 for oil and surfactant combinations respectively (Fig.3).

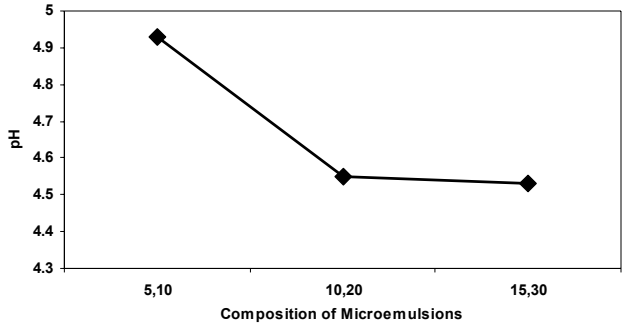

Fig. 3: It shows the variation in ph of selected microemulsions

Antibacterial activity of sunflower oil microemulsions

Three formulated microemulsions were tested on bacteria using well diffusion method and spread plate method. Growth studies were done to find out the effects of microemulsions on the bacterial survival. In well diffusion method, it was observed that the bacterial strain was susceptible to the formulated microemulsions. Bacterial susceptibility to each microemulsion was determined by the zone of inhibition. The inhibition (>90 % reduction) zone diameters of the well diffusion method was clear with a good definition. Antibacterial zone was easy to measure after 24 hours; it could be because of the homogeneous distribution of the strain and culture medium before pouring onto the plate. Bacterial strain treated with microemulsion concentration (15 % of oil and 30 % of surfactant) exhibited high antibacterial zone compared to 10 % of oil and 20 % of surfactant which showed medium antibacterial zone and 5 % of oil and 10 % of surfactant showed low antibacterial zone. Of the three microemulsions, it was observed that microemulsion with concentration 15 % of oil and 30 % of surfactant showed maximum antibacterial effect. Oil and Surfactant alone showed no zone of inhibition. LB agar plates were incorporated with formulated microemulsions and it was inoculated with 10^7-10^8 CFU. Microemulsion concentration of 5% oil and 10% surfactant, showed 60% inhibition of bacterial growth. The extent of inhibition increased upto 90% in plates with microemulsion concentration of 10% oil and 20% surfactant. Microemulsion concentration of 15 % oil and 30 % surfactant showed complete inhibition of bacterial growth (data not shown).

Growth studies were done in four organisms (*E. coli 13534, E. coli 25922, S. aureus, P. aeruginosa*) to find out the effects of microemulsions on the bacterial survival. We studied the rate at which there was inhibition of bacterial growth in different microemulsions. Microemulsion concentration of 15 % oil and 30 % surfactant was found to be more effective in inhibiting bacterial growth, than that of other two microemulsions. In fact, bacterial suspension treated with concentration (15 % oil and 30 % surfactant) showed complete loss of viability within 1 hour. After 2 hours of incubation the 10 and 100-fold dilutions of the microemulsions were equally sensitive to *E. coli, S. aureus, P. aeruginosa* (data not shown).

In contrast, microemulsions with concentration 10 % of oil and 20 % of surfactant was found to be active against bacteria within 3 hours, where as microemulsions with concentration 5 % of oil and 10 % of surfactant was less effective. As these microemulsion structures are quite sensitive to compositional changes, they lost all antibacterial properties when diluted 10-fold and 100-fold (data not shown). Therefore, it is likely that the addition of water leads to significant changes in structure that affect its antibacterial properties[17]. Microemulsions of oil and surfactant alone showed no anti-bacterial effect (Fig. 4, 5, 6, 7).

The high levels of inherent antibacterial activity observed in these microemulsions, demonstrated, for example, by the results of the spread plate method and kinetics of killing experiments, indicate that these microemulsions are indeed self-preserving systems. Such rapid antibacterial activity is indicative of a direct

attack on the structural integrity of the cell rather than a secondary effect through metabolic inhibition[18]. This level of dysfunction of membrane structure could potentially result in the death of the cell and may explain the rapid loss of cell viability observed in the kinetics of killing experiment.

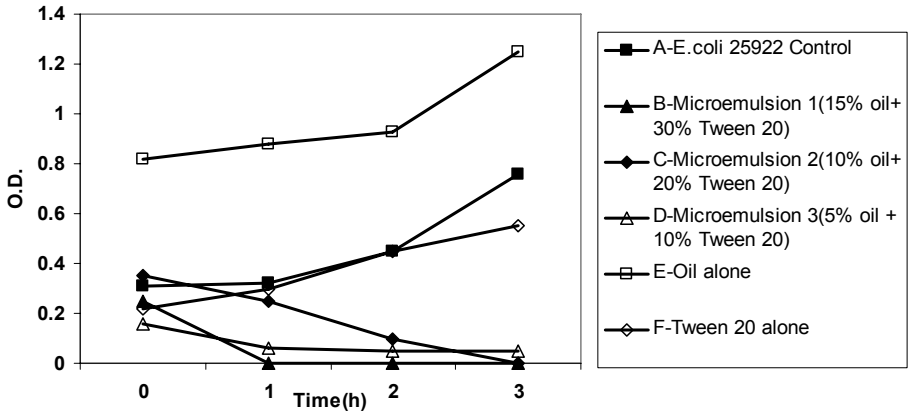

Fig. 4: it shows the bacterial growth curve (e. coli 25922) in lb media of three formulated microemulsions

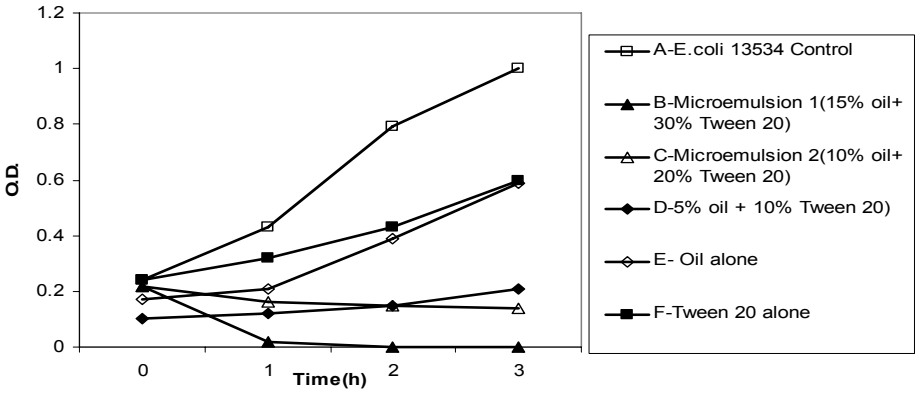

Fig. 5: It shows the bacterial growth curve (e. coli 13534) in lb media of three formulated microemulsions

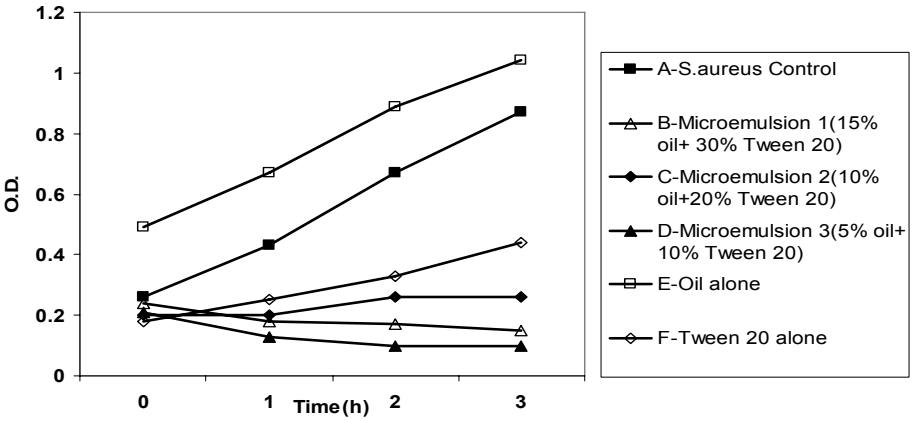

Fig. 6: It shows the bacterial growth curve (*s. Aureus*) in lb media of three formulated microemulsions

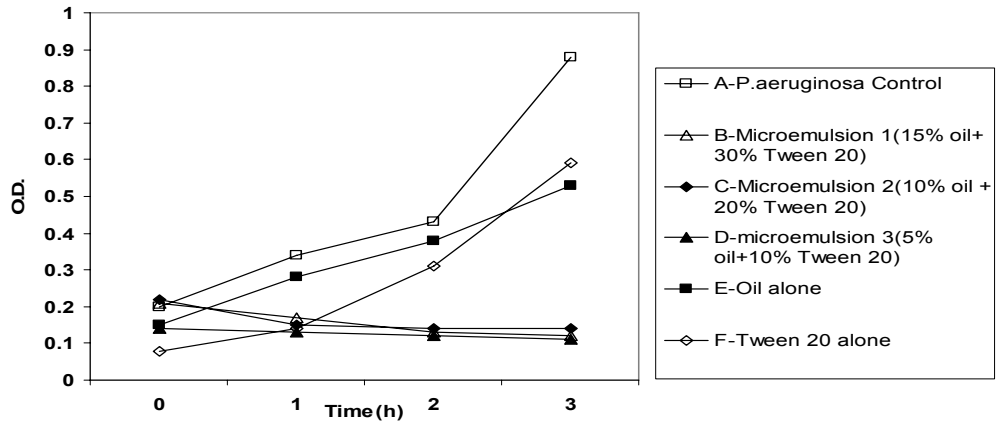

Fig. 7: It shows the bacterial growth curve (*p. Aeruginosa*) in lb media of three formulated microemulsions

CONCLUSION

The results of this work show that it is possible to obtain refined sunflower oil-in-water microemulsions stabilized by Tween 20. From the pseudo-ternary phase diagram it was possible to describe the critical mixtures between the components that provide a system with various microstructures. Conductivity data confirmed the continuous structural transitions during increasing of water phase volume fraction in the selected oil/surfactant mixture. It was concluded that refined sunflower oil microemulsions would have higher antibacterial activity than refined sunflower oil alone. These results clearly indicate that the microemulsions are stable, self-preserving antibacterial agents, with a highly effective killing rate against bacterial growth. Our future studies would be on the formation of nanoemulsion of this system towards biomedical applications.

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