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Ultrasonic emulsification of eucalyptus oil nanoemulsion: Antibacterial activity against *Staphylococcus aureus* and wound healing activity in Wistar rats

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ABSTRACT

The plant derived essential oil nanoemulsion was prepared using a mixture of components containing eucalyptus oil as organic phase, water as continuous phase, and non ionic surfactant, Tween 80, as emulsifier at a particular proportion of 1:1 v/v%. The ultrasonication was applied for varied processing time from 0 to 30 min to study the effect of time on the formation of nanoemulsion and physical stability of formulation by this method. The transparency and stability of emulsion was enhanced when the sonication time was increased compared to hand blender emulsion. The most stable nanoemulsion was obtained in 30 min sonication having the mean droplet diameter of 3.8 nm. The antibacterial studies of nanoemulsion against *Staphylococcus aureus* by time kill analysis showed complete loss of viability within 15 min of interaction. Observations from scanning electron microscopy of treated bacterial cells confirmed the membrane damage compared to control bacteria. Furthermore, the wound healing potential and skin irritation activity of the formulated nanoemulsion in Wistar rats, suggested non-irritant and higher wound contraction rate with respect to control and neomycin treated rats. These results proposed that the formulated system could be favourable for topical application in pharmaceutical industries.

1. Introduction

Wounds are inescapable events of life, which occurs unavoidingly due to injuries. The cuts that are formed in skin epidermis leads to infections caused by certain microorganisms like bacteria, fungi etc. For early healing of wounds, herbal materials have been in use from ancient time onwards and their eco-friendly nature promises output without any side effects.

MRSA, caused by *Staphylococcus aureus* which is a common opportunistic microbe that is found in skin abrasions and open wounds which is acquired due to infections from hospitals. Inadequate control measures to manage the infected wounds can lead to cellulitis and ultimately bacteraemia and septicaemia [6].

The essential oils from plants are considered to be safer compared to synthetic products for application as an antibacterial agent because of the bioactive components present. Among plant derived oils, essential oils have shown to possess insecticidal, antifungal, and antibacterial properties [3]. The plant derived essential oil, Eucalyptus oil obtained from *Eucalyptus globulus* contains 45.4% 1,8-cineole (eucalyptol) approximately. This eucalyptol is found to have strong antimicrobial activity against human and food borne pathogens [19,1]. The intra-dermal administration of essential *E. globulus* oil increased the capillary permeability and favours wound healing [23].

The antimicrobial nanoemulsions (NE) are oil in water type with nanosized droplets having a broad spectrum activity against enveloped virus, fungi and bacteria [7,8,16,18]. NE is defined as mixing of two immiscible liquids with or without emulsifier that contains nanodroplets having the mean droplet radii around $r < 100$ and found to have good kinetic stability that appears transparent or lightly opaque. This can be prepared by using high energy methods such as ultrasonication, shearing and homogenization [26,14,9]. The advantages of nanoemulsion over conventional emulsion is the possibility to dilute them with water without changing the droplet size distribution and moreover with the use of reduced amount of surfactant is an added advantage for preparation by NE methods compared to microemulsions [15,4].

The objective of the present study was to evaluate eucalyptus oil NE formulation for its antibacterial and wound healing activities using time kill analysis and by experiments in excised rats.

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2. Materials and methods

2.1. Materials

Eucalyptus oil (Cineole: 60%), Triton X-100, Nutrient broth and Nutrient agar were purchased from Himedia, India. Tween 80, Bioxtra (Polyoxyethylene (20) sorbitan monolaurate), were purchased from Sigma Aldrich, India. Ultrapure water was obtained from Cascada™ Biowater System, Pall Corporation, USA, with a resistivity not less than, 18.2 MΩ cm, was used for the preparation of all solutions. All other chemicals used were of analytical reagent grade. The bacterial strain, *S. aureus* (MTCC 96), was purchased from IMTECH, Chandigarh. The strain was cultured in a 50 ml nutrient agar at 37 °C for 24 h. Single colony was taken from this grown culture was reinoculated again into a 50 ml nutrient broth, and the growth was adjusted to 1×10^8 colony-forming units (cfu)/ml] at 600 nm using 0.85% saline.

2.2. Preparation of nanoemulsion: Ultrasonic cavitation

Nanoemulsion was prepared by the procedure previously reported [25] and further analysis was done to optimize the NE formation versus sonication time. Initially coarse emulsion (16.66% Oil, 16.66% Tween 80, 66.68% Water in v/v proportion) was made with magnetic stirrer at 250 rpm for 10 min. Coarse emulsion was subjected to 20 kHz ultrasonic processor (Sonics, USA) with a maximum power output of 750 W. This simple bench-top ultrasonic device consisted of an electrical generator, a transducer and a titanium sonotrode (horn). The mechanical ultrasonic vibrations at the sonotrode could be fixed with the amplitude of 40%. Sonication process was carried out for different emulsification time (0, 5 10, 15, 20, 25 and 30 min), in which each cycle consisted of 30 s pulses on and 30 s pulses off. Then the emulsion was removed for every 5 min interval, to study size distribution and stability.

2.3. Characterization of nanoemulsion

2.3.1. Particle size distribution and polydispersity index

The droplet size distribution and polydispersity index of the eucalyptus oil NE was determined using 90 plus particle size analyzer (Brookhaven Instruments Corporation, USA). Droplet size was analysed by dynamic light scattering technique (DLS). Similarly, for the emulsification study, nanoemulsion size distributions were monitored during sonication by samples collected at specified time intervals (0, 5, 10, 15, 20, 25 and 30 min) and characterized using DLS. The NE was diluted in the ratio 1:30 with double distilled water, to minimize the multiple scattering effects prior to each experiment. The droplet size was described in terms of nm.

2.3.2. Turbidity measurement

The turbidity of NE was assessed via absorbance at 600 nm from 0 to 30 min ultrasonication using UV–Visible Spectrophotometer (UV–Vis Spectrophotometer 2201, Systronics, India). Each measurement was carried out in triplicates, and the results were calculated as mean ± SE.

2.3.3. pH measurement

The pH values of the NE were determined at regular intervals for every 5 min sonication at room temperature with a pH meter (model HI 8417, Hanna Instruments Inc., Woonsocket, USA).

2.4. Antimicrobial activity

2.4.1. Kinetics of killing

The kinetics of killing assay was performed by Hamouda et al. [8] with some modification by adding equal amount of adjusted bacterial cultures (1×10^7 cfu/ml) to NE at regular intervals of 0, 15, 30 and 60 min respectively. For viable counts, 1 ml sample from the inoculated emulsions were serially diluted to 10-fold with 0.85% saline and plated as duplicates. These plates were incubated at 37 °C for 24 h.

2.4.2. Membrane integrity of bacteria

The membrane permeability of the bacteria was estimated by Hou et al. [10] with a slight modification. The overnight culture of bacteria at 37 °C was washed and resuspended in a sterile normal saline (0.85% NaCl), reaching a final density of approximately 1.0×10^7 cfu/ml. The bacteria in sterile normal saline (0.5 ml) was added to 9.5 ml of 10-fold dilution of NE for 60 min incubation at 37 °C and sterile normal saline without any treatment was used as control. The bacterial incubation with Triton X-100 (A_0) was used as positive control. The mixture was centrifuged at 6000 rpm for 10 min and the supernatant was read at 260 nm using an ultraviolent spectrometer (A_1). The leakage of UV absorbing materials was calculated as $A_1/A_0 \times 100$.

2.4.3. Scanning electron microscopy

Both interacted and non-interacted bacterial cells were analyzed microscopically. The bacterial cell, after being treated with NE for a minute, was harvested by centrifugation for 10 min at 5000 rpm. The pellet coated onto glass piece (1 cm × 1 cm) was dipped into 2.5% glutaraldehyde and kept for 2 h to fix the cells. Further the slide containing the cells was dehydrated with water/alcohol solutions at various alcohol concentrations (30%, 50% and 70%) for 10 min each. The samples were then coated with gold by sputtering under vacuum in argon atmosphere. The surface morphology of the coated sample was observed by a scanning electron microscope (FEI Quanta FEG 200).

2.5. Animal study

2.5.1. Skin irritation test

The skin irritancy test was carried out on male Wistar albino rats (120–130 g) to determine localized response of the optimized nanoemulsion according to the "Institutional Animal Ethical Committee, VIT University, Vellore, India. The animals were housed in polypropylene cages, four per cage, with free access to standard laboratory diet (Lipton Feed, India) and water ad libitum. The hair on the back was removed 24 h prior to use [17]. The rats were divided into three groups with four animals in each group ($n = 4$). Group I served as control (no treatment), Group II received 0.8% (v/v) aqueous formalin solution as a standard irritant and Group III received NE formulation. A single dose of 100 μL of the NE or formalin was applied on a 5 cm^2 area of the shaved dorsal side of the rats daily for three consecutive days [12]. The development of erythema and edema were monitored daily.

2.5.2. Histopathological examination of skin specimens

The rats were sacrificed after three days and skin samples from treated and untreated (control) areas were taken. Each skin sample was stored in 10% (v/v) formalin saline solution. The skin samples were cut vertically in different sections. Each section of skin sample was dehydrated using ethanol and hematoxylin and eosin stain was used to study the morphological changes using Phase contrast microscope (Carl Zeiss, USA) fitted with a digital camera and compared with the control sample.

2.5.3. Wound healing study

All animals were anaesthetized with ether before wound excision. The hair on the back was removed and the wound was created by cutting off 300 mm^2 of skin from a predetermined area. The study comprised of four animals in four different groups. Treatment was done topically in all the cases; Group 1: Normal rats (Without wound), Group 2: Control animals (Only wound), Group 3: NE treated, and Group 4: Standard neomycin ointment [20].

1 ml of each treatment was applied topically ($n = 4$) and wound contraction was measured for every 2 days interval, until the wound got completely healed. The changes in wound area were monitored with the use of millimeter scale on predetermined days i.e., 0, 5, 10, 15 and 20. The percentage of wound contraction rate was determined according to the equation (Eq. 1)

$$\%\,\text{of wound contraction} = \frac{\text{Initial wound are} - \text{Specific day wound area}}{\text{Initial wound area}} \times 100. \quad (1)$$

The wound size of the initial day of wound excision was considered as 100% while calculating percentage of wound contraction rate.

2.6. Statistical analysis

Each measurement was carried out in triplicates and all the values were expressed as mean ± standard error (SE) using Microsoft Excel. The data was statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The difference was considered significant when p-values <0.05.

3. Results and discussion

3.1. Nanoemulsion characterization

3.1.1. Effect of sonication time on nanoemulsion droplet size

Initially coarse emulsion was prepared with oil as dispersed phase and water as continuous phase with non ionic surfactant Tween 80 (Hydrophilic Lipophilic Balance (HLB) value 15) which acts as emulsifier. With an increase in sonication time from 0 to 30 min, the appearance of emulsion changed from milky white to transparent and also steady decrease in the droplet size had a direct correlation with emulsification time (Figs. 1 and 2). Similar trend of decrease in droplet diameter with increase in emulsification time was observed in the case of sunflower oil nanoemulsion formulation [13]. With respect to time based study, ultrasonic emulsification yields nanoemulsion with minimized droplet diameter with low polydispersity index. The polydispersity is a measure of the homogeny and stability of the droplet size in the emulsion. This small droplet size obtained by the emulsion technique has larger surface area that allows rapid penetration of active components [26]. NE is suitable for delivery of active components through the skin because of smaller droplet size.

From the experimental results, the NE that used the lowest possible surfactant concentration maintained the stability and uniform distribution of small droplets [9]. The presence of large hydrophilic head group prevents coalescence and provides steric repulsion between the droplets [22]. High viscosity resulted in reduced diffusion coefficient and this can be reduced by sonication. According to Stokes–Einstein equation (Eq. 2), this results in reduced frequency of collision and therefore lower coalescence.

$$Rh = \frac{KT}{6\pi\eta D} \quad (2)$$

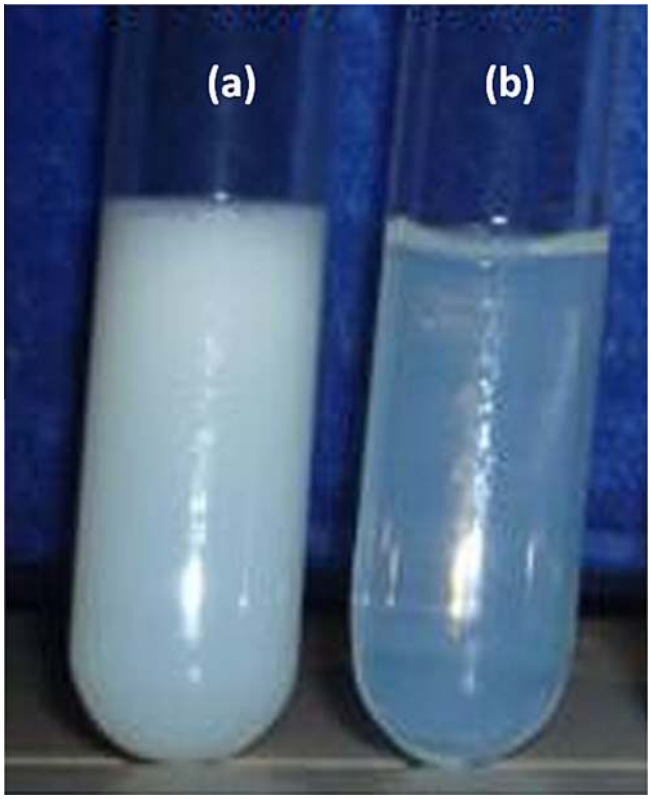

Fig. 1. Visual appearance of eucalyptus oil emulsion (a) before sonication: milky white emulsion (b) after sonication: bluish and transparent nanoemulsion.

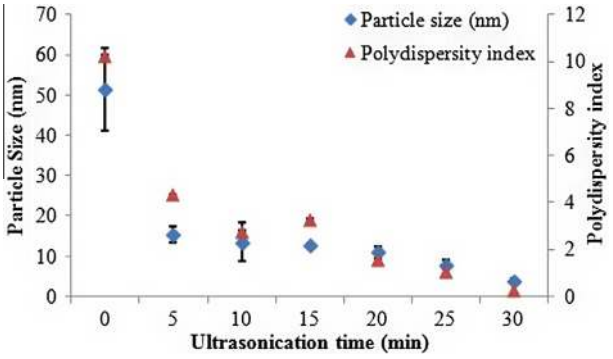

Fig. 2. Effect of sonication time on mean droplet size and polydispersity index.

Where Rh is the hydrodynamic radius of the particle, K is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the medium, D is diffusion coefficient.

Coarse emulsion (without sonication) was milky white in colour and showed immediate phase separation. Also it has shown increased particle size with increased polydispersity index. NE was formulated using low level surfactant that is equivalent to the oil concentration (16.66%, 16.66%, 66.68%). For 10, 15 and 20 min emulsification time, the emulsion got phase separated after one month of storage and found to be unstable. After 25 min emulsifications time, found to be highly stable for three months, there was no change in the droplet diameter and also no phase separation or creaming was observed.

3.1.2. Turbidity measurement

Turbidity of the emulsion was expressed in absorbance at 600 nm for 0 to 30 min sonicated samples. Quantitative

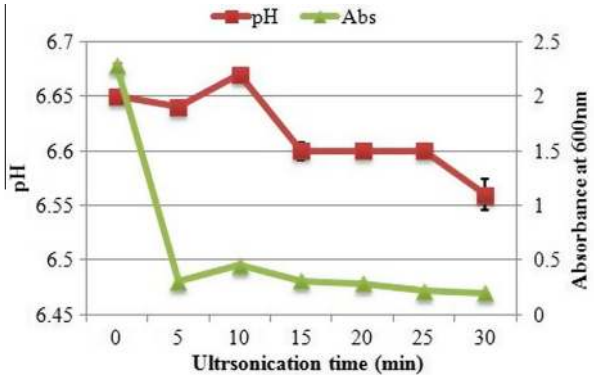

Fig. 3. Correlation between absorbance at 600 nm and pH of nanoemulsion as a function of sonication time.

measurements of the optical transparency of nanoemulsions in the visible and ultraviolet wavelengths are shown through transmission measurements. There was a decrease in absorbance with increased sonication time. The influence of sonication time on the formulated nanoemulsion at different time intervals shows that there is no significant difference in pH. Fig. 3 shows pH and turbidity of the formulated NE versus sonication time. After sonication, the appearance of nanoemulsion was changed to bluish transparent when scatters light, which may be due to Rayleigh scattering effect caused by nanosized droplets [14].

3.2. Antibacterial activity

3.2.1. Kinetics of killing

The optimized NE formulation was tested for its bactericidal activity against the clinical pathogen *S. aureus*. The kinetics of killing experiment demonstrated the loss of viability when interacted with formulated NE by adjusting number of cells (1×10^7 cfu/ml) over time (Fig. 4). There was immediate log reduction (0.324 log cfu/ml) within a min when compare to control cells. The result shows complete loss of viability within 15 min of interaction. All the cells were viable and no cells were killed when they are grown in PBS (control) and Tween 80. The kinetics of killing experiment indicates that the both undiluted and diluted NE (10-fold, 100 fold and 1000 fold) possess significant antibacterial activity.

Recently, there are several reports on NE being used as an antimicrobial agent against food borne pathogens, cariogenic pathogens, fungi and yeast. The particle size of the emulsion droplets decreases from micro to nanodroplets by sonication. This reduction in particle size increases the surface area which may result in greater interaction of NE with bacterial membrane, thereby, resulting in the enhancement of antibacterial activity. Our investigation

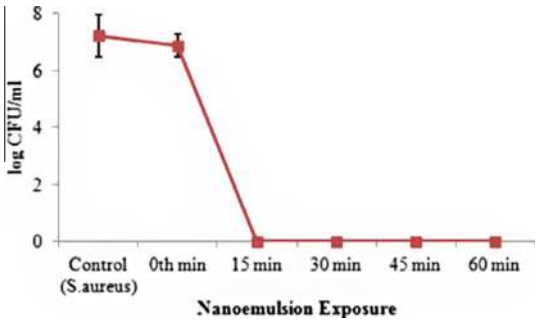

Fig. 4. Time kill study of nanoemulsion (undiluted) activity against *S. aureus* and viable bacterial counts were determined at the times indicated versus log (cfu/ml).

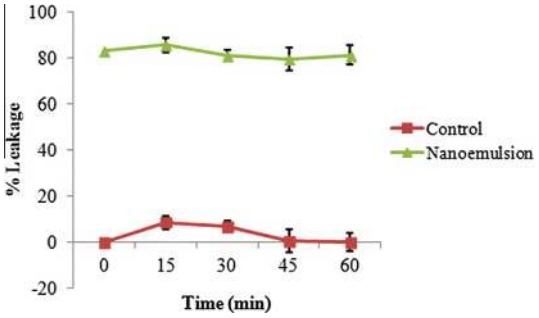

Fig. 5. Leakage of UV absorbing components of *S. aureus* for 0, 15, 30, 45 and 60 min treated and untreated cells by 10-fold dilution of nanoemulsion.

based on the influence of the nanodroplets size on antimicrobial activity agrees well with the previous reports [16,11,25].

3.2.2. Membrane integrity of bacteria

The influence of nanoemulsion on the leakage of cytoplasmic contents from the bacterial strain was expressed in terms of percentage as shown in Fig. 5. The cytoplasmic leakage was studied between the range of 0 to 60 min for both control and NE (10-fold) treated bacteria. It was found that leakage of cell contents was observed immediately in 0 min (83.32%) compared to control bacteria (0.03%). From the membrane permeability studies, it is seen that there is loss of cell contents by Zhang et al. [27] within a min of interaction.

3.2.3. Cell damage study by SEM

A comparison of untreated and NE treated *S. aureus* was studied by scanning electron microscopy. The treated cells of *S. aureus* with NE showed morphological changes when compared to intact cells. The control cells were spherical and remained intact as shown in Fig. 6a. After NE treatment, the cells were remarkably disintegrated (Fig. 6b). Irregular shape and integrity of the cells were distorted after being treated with 10-fold diluted NE within a minute. These results were similar to other nanoemulsion treated *Streptococcus mutans* and *Candida albicans* as obtained from previous literatures [16,21].

3.3. Animal study

3.3.1. Skin irritancy test and Histological study

The skin irritancy test was performed for the optimized NE formulation. There was no skin irritation (erythema & edema) observed through visual observation when rat skin was treated with NE. In formalin (0.8%) treated rats, reddening of the skin appeared. These results indicated that NE is non irritant to the skin.

Histopathological examination of skin sample stained with haematoxylin and eosin of the control animal and NE treated animal was shown in Fig. 7a and b. Pathological changes were not observed in animals treated with NE and it is similar to control animal with well defined epidermal (E) and dermal (D) layers. Due to the low amount of surfactant usage, the NE is non irritant to the skin and found selectively toxic to *S. aureus*. There is an ongoing need for the development of safe antimicrobial formulations with broad range and rapid biocidal activity.

3.3.2. Wound healing activity

Wound contraction activity was studied to check the efficiency of the formulated NE compared to the commercially available drug, neomycin. The wounds treated with the NE showed considerable contraction starting from day 10 onwards. On day 16, a 100% wound healing activity was observed in NE treated rats, whereas,

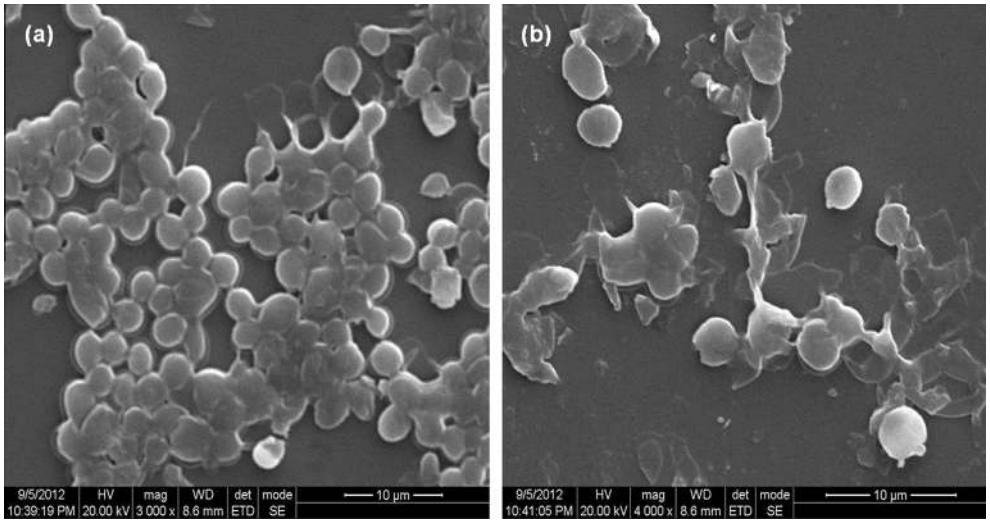

Fig. 6. SEM images (a) untreated (Control) and (b) treated (undiluted nanoemulsion) of *S. aureus.*

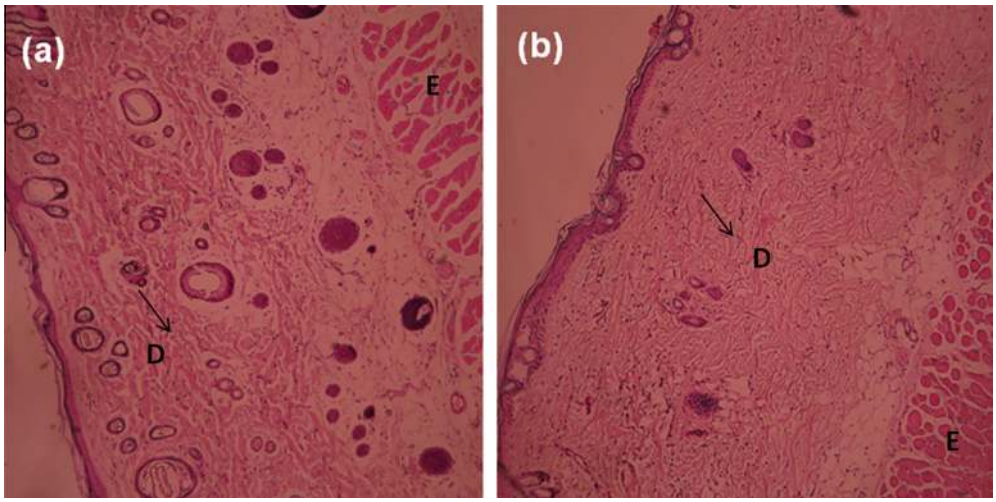

Fig. 7. Photomicrograph of skin irritation sample stained with haematoxylin and eosin (a) control group animal and (b) nanoemulsion treated animal at low power (40 X). E: epidermis; D: dermis (dermis layer is not disturbed by NE treatment (b) as shown in arrow mark and it is similar to control group (a)).

94.2% healing was seen in both untreated (control) and neomycin treated rats. This is in contrast to neomycin, which showed decreased rates of wound contraction leading to delay in total wound closure. Delayed wound healing was observed in control group. Nanoemulsion treated rats showed significant increase ($p < 0.05$) compared to control and neomycin treated groups as shown in Fig. 8. Oil in water based NE system is attractive for topical application studies due to their biocompatibility between water and tissue. As the water evaporates, the emulsion droplets form a continuous film, thereby, promoting healing activity. Animals treated with nanoemulsion showed better wound healing activity compared with neomycin treated and control rats.

Hence we have designed a eucalyptus oil NE that is non toxic, and proved to be effective antibacterial agent due to its property of wound healing activity that was found significantly higher. This may be due to the presence of cineole (eucalyptol), a well known penetration enhancer in transdermal drug delivery system and topical application studies [2,24,5]. Results suggested from this study confirm the rapid bactericidal against *S. aureus* and wound healing activity in Wistar rats of eucalyptus oil nanoemulsion.

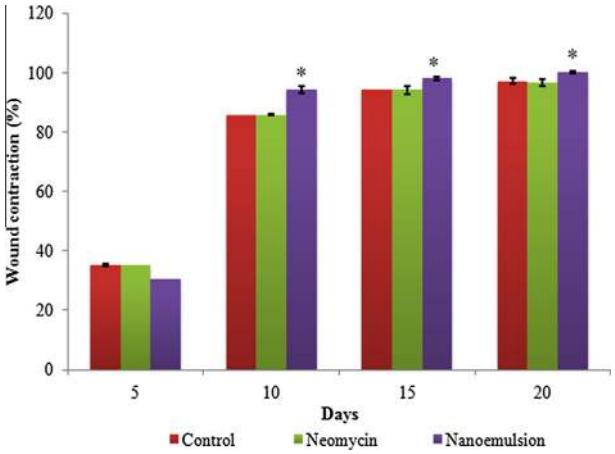

Fig. 8. Effect of nanoemulsion treatment on wound contraction rate in rats (*$p < 0.05$ represents significantly higher for nanoemulsion than control and neomycin treated groups).

4. Conclusion

In conclusion, the topical eucalyptus oil NE was formulated using biobased approach using eucalyptus oil. By virtue of its anti-inflammatory, antimicrobial property in eucalyptus oil and the formation of nano droplet size may be reason for faster wound healing activity and non irritant to the skin. Also, found to be effective bactericidal activity against *S. aureus* and may use as a desirable topical agent in pharmaceutical industries.

Conflict of interest

Authors declare that no conflict of interest.

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