Wound Healing Study of Eucalyptus Essential Oil Containing Nanoemulsion in Rat Model

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Abstract: The objective of this investigation was to develop nanoemulsion formulations of *Eucalyptus* essential oil (EEO) and to evaluate its wound healing effects in comparison with standard gentamycin in rat model. Various nanoemulsions of EEO were prepared using aqueous phase titration method and the zones of nanoemulsion were identified by the construction of phase diagrams. EEO nanoemulsions were investigated in terms of physical stability, self-nanoemulsification efficiency and physicochemical characterization. Optimized nanoemulsion of EEO was selected for wound healing study, collagen estimation and histopathological evaluation in rats in comparison with pure EEO and standard gentamycin. Optimized nanoemulsion presented significant wound healing activity in rats as compared with pure EEO upon oral administration. The wound healing activity of optimized nanoemulsion was comparable with standard gentamycin. Optimized EEO nanoemulsion also presented significant enhancement in collagen content as compared with pure EEO and negative control. However, the collagen contents of optimized nanoemulsion treated animals were comparable with standard gentamycin-treated animals. Histopathological studies of optimized nanoemulsion treated rats showed no signs of inflammatory cells which suggested the safety and non-toxicity of EEO nanoemulsion. This study suggested the potential of nanoemulsion in enhancing the wound healing activity of EEO upon oral administration.

Key words: collagen estimation, Eucalyptus oil, nanoemulsion, wound healing

1 INTRODUCTION

Essential oils got great acceptance in pharmaceutical and food industries due to their broad range of therapeutic activities and flavoring effects¹⁻⁴. The main chemicals of essential oils are terpenes which are responsible for most of their therapeutic activity⁴. *Eucalyptus* essential oil (EEO) is isolated and extracted from the dried leaves of *Eucalyptus citriodora* and other species of *Eucalyptus*^{5,6}. Various therapeutic activities such as analgesic, anti-inflammatory⁵, antioxidant⁷, antifungal⁸, antibacterial^{6,9} and antiradical activities⁷ have been reported for EEO in literature. Various terpenes such as α -pinene, limonene, 1,8-cineole, p-cymene, tr-pinocarveol, α -terpineol, globulol, α -eudesmol, β -eudesmol and others have been detected in different species of EEO^{6,8,9}. The main component/biomarker responsible for the most of the therapeutic activities of EEO is 1,8-cineole (cineole or eucalyptol)^{6,10}.

Oral nanoemulsions have got great acceptance as drug delivery carriers for the enhancement of therapeutic activities of various therapeutic agents and bioactive compounds in recent years¹¹⁻¹⁴. Nanoemulsions offer several advantages over other colloidal drug carriers due to their ease of preparation, low preparation cost, physical/thermodynamic stability and nanosized droplet diameters¹⁴⁻¹⁶. In the recent years, wound healing power of several essential oils have been proposed in various animal models¹⁷⁻²⁶. However, the wound healing activities of most of the essential oils using nanosized drug delivery systems have rarely been studied in literature. The wound healing activity of clove essential oil (CEO) via nanoemulsion has been reported recently in literature¹². Alam *et al.* got significant wound healing effects of CEO loaded nanoemulsion as compared with pure CEO¹². The wound healing effects of EEO topical nanoemulsion have also been reported in liter-

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ature²⁷⁾. However, the wound healing effects of EEO nanoemulsion upon oral administration have not been studied in literature. Hence, the objective of this research work was to develop suitable nanoemulsion formulations of EEO and to investigate its wound healing effects in rat model as compared with pure EEO and standard antibiotic (gentamycin) after oral administration. Macroemulsions were not prepared in this work because nanoemulsions have lower droplet size in comparison with macroemulsions and these systems are more suitable for enhancing absorption and therapeutic activity of essential oils^{11, 12, 27)}. Various nanoemulsion formulations of EEO were prepared using aqueous phase titration method via construction of pseudo-ternary phase diagrams. The components of nanoemulsions were safe and nontoxic and includes EEO/oil phase, Tween-85/surfactant, Transcutol/cosurfactant and water/aqueous phase. All the formulations were developed properly, characterized physicochemically and optimized nanoemulsion of EEO was taken for biological wound healing activity in rats.

2 EXPERIMENTAL

2.1 Materials

EEO, dimethyl sulfoxide (DMSO) and ethanol were obtained from "Sigma Aldrich (St. Louis, MO)". Diethylene glycol monoethyl ether (Transcutol-HP) was obtained from "Gattefossé (Lyon, France)". Polyoxyethylenesorbitan trioleate was obtained from "E-Merck (Darmstadt, Germany)". Ultra-pure water (deionized water) was obtained from "ELGA water purification system" in the laboratory. All other chemicals and reagents used were of analytical/pharmaceutical grades which were used without any further purification.

2.2 Construction of pseudo-ternary phase diagrams for EEO nanoemulsion

In order to develop various nanoemulsion formulations of EEO, EEO/oil phase, Tween-85/surfactant, Transcutol/cosurfactant and deionized water/aqueous phase were used in this study. Pseudo-ternary phase diagrams were

developed using aqueous phase titration method^{28, 29)}. In brief, Tween-85 and Transcutol were mixed thoroughly in different mass ratios (1:0, 1:2, 1:1, 2:1 and 3:1). EEO/oil phase and a particular mass ratio of surfactant to cosurfactant (S_{mix}) were then mixed at various mass ratios (1:9 to 9:1). Pseudo-ternary phase diagrams constructed developed using aqueous phase titration method²⁸⁾. In this method, the mixture of EEO and specific S_{mix} was titrated with drop-by-drop addition of aqueous phase and recorded for visual observations based on their clarity^{28, 30)}. The clear/transparent and easily flowable zones of nanoemulsion plotted constructed on respective pseudo-ternary phase diagram in which one axis representing the water, second EEO and third one representing the specific S_{mix} .

2.3 Formulation development of EEO

It was observed from pseudo-ternary phase diagrams that the maximum nanoemulsion zones were shown by 1:1 S_{mix} ratio and hence the mass ratio of 1:1 was selected for the development of nanoemulsion formulations of EEO. Various nanoemulsions of EEO (E1-E5) were selected from the phase diagram of 1:1 mass ratio. The entire zones of nanoemulsion were considered in formulation selection. In formulations E1-E5, the concentration of EEO was varied from 12-28% w/w and the concentration of S_{mix} was kept constant at 24% w/w. The formulation composition of EEO loaded nanoemulsions is furnished in Table 1. Typical photographs of various EEO nanoemulsions (E1-E5) are presented in Fig. 1 which indicated clear/transparent nature of prepared nanoemulsions.

2.4 Physical stability tests

Because in aqueous phase titrations, observations were made visually, there is possibility of formation of metastable or unstable formulation. Therefore, physical stability tests on developed EEO loaded nanoemulsions (E1-E5) were conducted in order to eliminate any unstable/metastable nanoemulsion. These tests were carried at various stress conditions including "centrifugation, heating & cooling cycles and freeze-pump-thaw cycles"^{16, 30)}. EEO nanoemulsions (E1-E5) were centrifuged at 5000 rpm for 30 min and observed for any physical change such as phase separation,

Table 1 Composition of nanoemulsion formulations of EEO (E1-E5) prepared using EEO, Tween-85, Transcutol and water.

Code	Formulation composition (% w/w)				S_{mix} ratio
	EEO	Tween-85	Transcutol	Water	
E1	12	12	12	64	1:1
E2	16	12	12	60	1:1
E3	20	12	12	56	1:1
E4	24	12	12	52	1:1
E5	28	12	12	48	1:1

Fig. 1 Typical photographs of various EEO nanoemulsions (E1-E5) prepared using EEO (oil phase), water (aqueous phase), Twenn-85 (surfactant) and Transcutol (cosurfactant).

formation of macroemulsion, cracking or coalescence etc. Formulations which were stable at centrifugation were subjected to heating & cooling cycles. Three heating & cooling cycles were performed between 50°C and 4°C for the period of 48 h for each cycle and observed again for phase separation, formation of macroemulsion, cracking or coalescence etc. Formulations which were stable at heating & cooling cycles were subjected to freeze-pump-thaw cycles. Three freeze-pump-thaw cycles were performed between -21°C and 25°C for the period of 24 h for each cycle and observed again for phase separation, formation of macroemulsion, cracking or coalescence etc. Overall, formulations those were found to be stable at all three steps of physical stability tests were selected for further studies.

2.5 Self-nanoemulsification efficiency tests

The aim of “self-nanoemulsification test” was to assess any precipitation/phase separation upon mild agitation/dilution with three different diluents including “water, acid buffer (0.1 N HCl) and phosphate buffer (pH 7.4)”. This test was conducted by diluting 1.0 mL of each nanoemulsion (E1-E5) with “water, 0.1 N HCl and phosphate buffer (pH 7.4)”. The dilution ratio of each nanoemulsion to particular diluent was kept constant at 1:500. The self-nanoemulsification power of each nanoemulsion was evaluated visually using A-E grading systems as described below^{16, 30}:

- Grade A: Rapidly forming clear/transparent nanoemulsion
- Grade B: Rapidly forming bluish slightly less clear nanoemulsion
- Grade C: Slowly forming milky/turbid emulsions
- Grade D: Dull, grayish slowly forming milky/turbid emulsions
- Grade E: Milky/turbid emulsions with the presence of oil globules at the surface

2.6 Physicochemical evaluation of EEO nanoemulsions

Developed nanoemulsions of EEO were evaluated for

various physicochemical parameters including “droplet diameter, polydispersity index (PDI), zeta potential (ZP), refractive index (RI), the percentage of transmittance (% T) and surface morphology using transmission electron microscopy (TEM)”. The average droplet diameter, PDI and ZP of prepared nanoemulsions (E1-E5) were measured using “Malvern Particle Size Analyzer (Holtville, NY)” at 25.0 ± 1.0°C. The scattering angle for this analysis was set at 90°. The detailed procedure for these measurements is given in our publications¹⁶. The RIs of EEO nanoemulsions (E1-E5) were measured using “Abbes type Refractometer (Precision Testing Instruments Laboratory, Germany)” at 25 ± 1°C as proposed in literature¹⁶. The % T of EEO nanoemulsions (E1-E5) was measured spectrophotometrically at the wavelength of 550 nm as proposed in literature¹⁶.

The surface morphology and droplets diameter of optimized EEO nanoemulsion (E1) was studied using “JEOL TEM technique (JEOL JEM-2100 F, USA)”. TEM analysis was performed under light microscopy which was operated at 100 KV. The detailed descriptions for TEM analysis are given in our previous publication²⁹.

2.7 Wound healing evaluation in rat model

Based on various physicochemical parameters such as minimum droplet diameter (32.45 nm), least PDI (0.153), optimal ZP value (-34.25 mV), optimal RI value (1.335) and maximum % T value (99.1%), nanoemulsion formulation E1 was optimized and selected for wound healing activity in rats. For wound healing study, female Albino Wistar rats (weighing from 200 to 250 g) were taken from the “Experimental Animal Care Center at King Saud University, Riyadh, Saudi Arabia”. All the rats were provided controlled environmental conditions in terms of temperature and humidity with free access of water and standard pellet diet. This study on rats was approved by “Research Ethics Committee of College of Pharmacy, King Saud University, Riyadh, Saudi Arabia” and their guidelines were followed. Wound excision rat model was used for this purpose. The rats were divided randomly into four different groups con-

taining six rats in each group. The rats were anaesthetized using diethyl ether and depilated at the predetermined site before wounding. An excision wound was produced by cutting away approximately 500 mm² full thickness of the area on the anterior-dorsal side of each animal³¹. Rats were treated by oral administration of various samples.

Group I (control group) rats were treated by oral administration of an optimized nanoemulsion E1 without EEO; group II animals were treated by oral administration of pure EEO (25 mg/kg of body weight); group III animals were treated by oral administration of an optimized nanoemulsion E1 with EEO (containing 25 mg/kg of EEO) and group IV animals were treated by oral administration of gentamycin suspension (25 mg/kg) which was taken as positive control group. Different formulations were administered orally to respective group once daily for the period of 24 days. Wound healing property of each group was evaluated in terms of the percent of wound contraction and closure time. The wound area was determined every third day³². The time for wound closure was recorded when total wound healed.

2.8 Collagen determination

The skin pieces from the wound area of animals belong to each group were taken at the end of the experiment (after 24 days) and studied for the collagen content in each group. For collagen determination in skin, 5 mL of TES buffer and 0.1 mL of acid hydrosolate were taken in the tube, mixed well and incubated at 37°C for 6 h. The contents were then filtered using a syringe filter into clean tube and allowed to stand. About 0.2 mL of test filtrate was taken and 2 mL of ninhydrin reagent was added into filtrate and boiled for about 30 min. After cooling at ambient temperature, 10 mL of 1-propanol was added and the absorbance was taken spectrophotometrically at the wavelength of 570 nm^{33, 34}. The collagen content in terms of mg/g of tissue was then calculated from the spectrophotometric absorbance. The skin pieces were also taken from untreated rats (control) and subjected for the analysis of leucine content. The leucine content in untreated rats was considered as negative control.

2.9 Histopathology of tissues

For histopathology studies, the healed tissues from the animals of each group were procured at the end of the experiment, fixed with 10% formalin, dehydrated using alcohol and embedded in paraffin blocks. Tissue sections were deparaffinized using xylene dye. Serial sections with specific diameter were cut using microtome and stained with hematoxylin-eosin (HE) dye. The tissue sections were studied using light microscopy. The severity of the inflammation in the healed areas was investigated by counting the inflammatory cell infiltration per field in each group. The presence of "epithelization, inflammatory cell infiltra-

tion, fibroblast proliferation, neovascularization and collagen deposition" on healed area of skin were measured using a modified 0-5 numerical scale stated below¹²:

- Score 0: Absence
- Score 1: Occasional presence
- Score 2: Light scattering
- Score 3: Abundance
- Score 4: Confluence of cells
- Score 5: Fibres

2.10 Data analysis

The values of in vitro evaluation/physicochemical investigation are presented as mean $\pm$ standard deviation. However, the values of wound healing activity are presented as mean $\pm$ standard error of mean (SEM). The statistical significance was analyzed using ANOVA in which $p < 0.05$ was taken as statistically significant.

3 RESULTS

3.1 Construction of pseudo-ternary phase diagrams and preparation of EEO nanoemulsions

Pseudo-ternary phase diagrams were constructed using EEO, Tween-85, Transcutol and water for the development of EEO nanoemulsions. The results of phase diagram constructions are shown in Fig. 2. The results summary of aqueous phase titration is furnished in Table 2. From results recorded, it can be seen that S_{mix} ratio of 1:0 (surfactant alone) presented very low nanoemulsion zones (Fig. 2a). The highest amount of EEO/oil phase that was found to be solubilized by this mass ratio was recorded as 13% w/w using 62% w/w of S_{mix} . When the amount of Transcutol/cosurfactant was increased with respect to Tween-85/surfactant i.e. S_{mix} ratio of 1:2, the nanoemulsion zones were found to be increased as compared to 1:0 ratio (Fig. 2b). The highest amount of EEO that was solubilized by 1:2 ratio was 22% w/w using 52% w/w of S_{mix} . However, when the concentration of Tween-85 and Transcutol was kept equal i.e. S_{mix} ratio of 1:1, the nanoemulsion zones were increased significantly as compared with 1:0 and 1:2 S_{mix} ratios (Fig. 2c). The highest amount of EEO that was solubilized by this ratio was obtained as 36% w/w using 55% w/w of S_{mix} (Table 2). On the other hand, when the S_{mix} ratio of 2:1 was studied, the nanoemulsion zones were found to be decreased again in comparison with 1:1 S_{mix} ratio (Fig. 2d). The maximum concentration of EEO that was solubilized by this ratio was obtained as 28% w/w using 41% w/w of S_{mix} (Table 2). When the concentration of Tween-85 was increased further with respect to Transcutol i.e. S_{mix} ratio of 3:1, the nanoemulsion zones were found to be decreased again as compared to 1:1 and 2:1 ratios (Fig. 2e). The highest amount of EEO that was solubilized by this ratio was obtained as 24% w/w using 56% w/w of

Fig. 2 Pseudo-ternary phase diagrams constructed via aqueous phase titration method for nanoemulsion zones (dotted area) for EEO (oil phase), water (aqueous phase), Tween-85 (surfactant) and Transcutol (cosurfactant) at S_{mix} ratios of a. 1 : 0, b. 1 : 2, c. 1 : 1, d 2 : 1 and e. 3 : 1.

Table 2 Results summary for aqueous phase titration of nanoemulsions.

Figure	S_{mix} ratio	Surfactant	Cosurfactant	Nanoemulsion zones	Oil phase solubilized (% w/w) ^a	S_{mix} solubilized (% w/w) ^b
1a	1:0	Tween-85	Transcutol	Low	13	62
1b	1:2	Tween-85	Transcutol	Higher than 1a	22	52
1c	1:1	Tween-85	Transcutol	Highest	36	55
1d	2:1	Tween-85	Transcutol	Lower than 1c	28	41
1e	3:1	Tween-85	Transcutol	Lower than 1c & 1d	24	56

^a The maximum amount of EEO (oil phase) that was solubilized

^b The maximum amount of S_{mix} (Tween-85:Transcutol) phase that was solubilized with respect to maximum amount of oil phase

S_{mix} (Table 2).

3.2 Physical stability tests

The aim of physical stability tests was to eliminate any metastable or unstable nanoemulsions. Therefore, prepared EEO nanoemulsions were subjected to different physical stability tests. The qualitative results of physical stability tests are furnished in Table 3.

3.3 Self-nanoemulsification tests

Prepared nanoemulsions of EEO (E1-E5) were further evaluated for self-nanoemulsification test²⁹. The qualitative results of this test are also furnished in Table 3. It was observed that nanoemulsion formulations (E1-E3) passed this test with grade A and formulations E4 and E5 passed this test with grade B in the presence of all three diluents.

3.4 Physicochemical evaluation of EEO nanoemulsions

The results recorded for physicochemical evaluation of prepared EEO nanoemulsions (E1-E5) are furnished in Table 4. It was observed that the droplet diameter of EEO nanoemulsions (E1-E5) was obtained in the range of 32.45–142.35 nm (Table 4). The PDIs of EEO nanoemulsions (E1-E5) were obtained as 0.153–0.278 (Table 4). The least PDI was obtained in EEO nanoemulsion E1 (0.153), indi-

cating the highest uniformity of droplet diameter as compared to other formulations.

The ZP values for nanoemulsions (E1-E5) were obtained as -38.25 to -34.25 mV (Table 4). The least ZP value was obtained in nanoemulsion E1 (-38.25 mV). However, the maximum ZP value was obtained in nanoemulsion E5 (-34.25 mV).

The RIs for EEO nanoemulsions (E1-E5) were obtained as 1.335–1.341 (Table 4). The maximum RI value was obtained in EEO nanoemulsion E5 (1.341 ± 0.10). However, the minimum RI value was obtained in EEO nanoemulsion E1 (1.335 ± 0.04).

The % T of EEO nanoemulsions (E1-E5) was obtained as 91.1–99.1% (Table 4). EEO nanoemulsion E1 showed the maximum % T value ($99.1 \pm 0.2\%$). However, EEO nanoemulsion E5 showed the minimum % T value ($91.1 \pm 0.8\%$).

The TEM images of optimized formulation E1 were taken and interpreted for surface morphology and droplet diameter (Fig. 3).

3.5 Wound healing evaluation

The results of effect of oral administration of pure EEO and optimized nanoemulsion E1 as compared with standard gentamycin on rats are shown in Table 5 and Fig. 4.

Table 3 Qualitative results of physical stability and self-nanoemulsification tests.

Code	*Self-nanoemulsification test grade	Thermodynamic stability tests		
		Cent.	H&T	FPT
E1	A	✓	✓	✓
E2	A	✓	✓	✓
E3	A	✓	✓	✓
E4	B	✓	✓	✓
E5	B	✓	✓	✓

✓ (Passed the particular test), cent. (centrifugation), H&T (heating and cooling cycles), FPT (freeze-pump-thaw cycles), * (all the formulations passed this test with grade A or B in the presence of water, 0.1 N HCl and phosphate buffer)

Table 4 Different physicochemical parameters of EEO nanoemulsions (E1-E5).

Code	Physicochemical parameters				
	$\Delta_{\text{dm}} \pm \text{SD}$ (nm)	PDI	ZP (mV)	RI $\pm$ SD	% T $\pm$ SD
E1	32.45 ± 2.84	0.153	-34.25	1.335 ± 0.04	99.1 ± 0.2
E2	46.24 ± 4.28	0.174	-36.21	1.336 ± 0.06	98.6 ± 0.5
E3	61.52 ± 5.84	0.202	-37.32	1.337 ± 0.07	97.8 ± 0.6
E4	124.32 ± 8.74	0.242	-38.01	1.340 ± 0.09	92.4 ± 0.7
E5	142.35 ± 10.58	0.278	-38.25	1.341 ± 0.10	91.1 ± 0.8

Mean droplet diameter (Δ_{dm}), polydispersity index (PDI), % transmittance (% T), zeta potential (ZP), refractive index (RI), standard deviation (SD)

Fig. 3 TEM images of optimized EEO nanoemulsion (E1) with spherical shaped droplets.

In the research study, the rats were anaesthetized using diethyl ether due to its major advantages over other anaesthetic agents. The % wound contraction was found to be enhanced till day 24 in both test samples (pure EEO and

nanoemulsion E1) and standard gentamycin treated rats. Pure EEO and optimized nanoemulsion E1 facilitated wound contraction significantly from day 12 to 24 as compared with control ($p < 0.05$). The difference in wound contraction between optimized nanoemulsion E1 and standard was not statistically significant ($p > 0.05$). However, the wound healing abilities of optimized nanoemulsion E1 and standard were statistically significant as compared with pure EEO ($p < 0.05$). The results of effect of oral administration of tests (pure EEO and nanoemulsion E1) as compared with standard on epithelization period are furnished in **Table 6**. The time for the complete epithelization was obtained as 13.40 ± 0.79 , 12.40 ± 0.82 , 9.00 ± 0.56 and 7.80 ± 0.48 days for control, pure EEO, optimized nanoemulsion E1 and standard, respectively. Epithelization time was significantly lower in standard and optimized nanoemulsion E1 treated rats as compared with pure EEO and control group rats ($p < 0.05$). However, epithelization period was not significant in optimized nanoemulsion E1 and standard ($p > 0.05$). These results suggested the potential of nanoemulsion for greater wound healing effects upon oral administration as compared with pure EEO.

3.6 Collagen content determination

Leucine content in terms of mg/g of tissue in the granulation tissues of the animals on day 1 and day 10 after oral administration of pure EEO, optimized nanoemulsion E1

Table 5 Effect of oral administration of pure EEO and an optimized nanoemulsion E1 in comparison with standard gentamycin on circular excision wound model in rats at different days of treatment.

Formulations	Wound area of contraction $\pm$ SEM (%)						
	0 Day	4 th Day	8 th Day	12 th Day	16 th Day	20 th Day	24 th Day
Control	0.00 ± 0.00	16.14 ± 2.32	30.54 ± 2.26	41.87 ± 1.74	61.18 ± 1.23	80.24 ± 0.68	98.72 ± 0.16
Pure EEO	0.00 ± 0.00	17.82 ± 2.34	34.51 ± 2.58	48.28 ± 1.47	67.72 ± 1.16	84.31 ± 0.12	99.84 ± 0.09
E1	0.00 ± 0.00	42.88 ± 0.72	54.10 ± 0.40	75.15 ± 0.81	88.29 ± 0.56	99.49 ± 0.16	100.00 ± 0.00
Gentamycin	0.00 ± 0.00	61.03 ± 0.73	78.58 ± 0.43	90.25 ± 0.67	95.46 ± 0.73	99.53 ± 0.14	100.00 ± 0.00

Fig. 4 Wound healing effects of pure EEO, optimized nanoemulsion (E1) and gentamycin as compared with control after 0, 4, 8, 12, 16, 20 and 24 days of inducing wound in rats.

Table 6 Effect of oral administration of pure EEO and an optimized nanoemulsion E1 in comparison with standard gentamycin on wound epithelization period in excision wound model in rats.

Formulations	Period of epithelization $\pm$ SEM (Days)
Control	13.40 $\pm$ 0.79
Pure EEO	12.40 $\pm$ 0.82
E1	9.00 $\pm$ 0.56
Gentamycin	7.80 $\pm$ 0.48

and standard are furnished in Table 7. The leucine levels of nanoemulsion E1 and standard antibiotic treated rats were found to be enhanced significantly on day 10 as compared with pure EEO treated rats and negative control ($p < 0.05$).

3.7 Histopathology of healed tissues

The photomicrographs of histopathological examination of control, tests (pure EEO and nanoemulsion E1) and standard antibiotic treated rats are shown in Fig. 5. Histopathological examination of skin at day 12 with H&E suggested the sign of “ulceration, edema, epithelization, granulation and abundance of mononuclear cells infiltration” in control group rats (Fig. 5a). However, the photomicrographs of pure EEO treated animals showing the sign of “less ulceration, edema and large amount of granulation as well as sign of healed skin structure with well-formed, near to normal epidermis, restoration of adnexa, and extensive fibrosis and collagen tissue” within the dermis (Fig. 5b). On the other hand, optimized nanoemulsion (E1) treated animals showed large amount of granulation tissue, small number of mononuclear inflammatory cells, and restoration of adnexa and extensive fibrosis and no sign of ulceration and edema (Fig. 5c). The animals treated with standard showed healed skin structures with well-formed, near to normal epidermis, restoration of adnexa, and extensive fibrosis and collagen tissue within the dermis (Fig. 5d). The results of histopathological examinations are furnished in

Fig. 5 Histopathology of rat skin at day 12 stained with H&E (100x); (a) control group rats; (b) pure EEO treated rats; (c) EEO nanoemulsion (E1) treated rats; (d) gentamycin treated rats.**Table 8.**

4 DISCUSSION

The results of aqueous phase titration suggested that the maximum zones of nanoemulsion were represented by S_{mix} ratio of 1:1 (Fig. 2c) and hence various nanoemulsions for EEO were selected from Fig. 2c. With respect to internal phase concentration (EEO), the entire zones in phase diagram were considered in Fig. 2c. In selected nanoemulsions (E1-E5), the concentration of EEO was varied from 12–28% w/w and the concentration of S_{mix} was kept constant at 24% w/w. It was observed that all nanoemulsions were physically stable at three different steps of these tests. The presence of cosurfactant (Transcutol) along with surfactant (Tween-85) in nanoemulsions might be the main reason for physical stability of nanoemulsions²⁸. The surfactant alone is not capable to reduce interfacial tension between oil phase and aqueous phase up to the required value²⁹. However, the presence of cosurfactant along with

Table 7 Estimation of collagen content after oral administration of pure EEO and an optimized nanoemulsion E1 in comparison with standard gentamycin on circular excision wound model in rats at 1st and 10th day of treatment.

Formulations	Collagen $\pm$ SEM (mg of leucine/g of tissue)		
	1 st day	10 th day	Negative control
Pure EEO	0.22 $\pm$ 0.07	0.50 $\pm$ 0.03	0.33 $\pm$ 0.04
E1	0.25 $\pm$ 0.05	0.65 $\pm$ 0.02	0.33 $\pm$ 0.04
Gentamycin	0.28 $\pm$ 0.03	0.98 $\pm$ 0.03	0.33 $\pm$ 0.04

Table 8 The median histopathologic scores for wound healing effects determined after oral administration of pure EEO and an optimized nanoemulsion E1 in comparison with and control and standard gentamycin by using a modified 0 to 5 numerical scale; the scores were 0 for absence, 1 for occasional presence, 2 for light scattering, 3 for abundance, 4 for confluence of cells and 5 for fibres.

Formulations	Epithelialisation	Inflammatory cell infiltration	Fibroblast proliferation	Neovascularization	Collagen deposition
Gentamycin	5.22 ± 0.34	0.89 ± 0.51	5.40 ± 0.38	4.10 ± 0.23	5.27 ± 0.61
E1	3.80 ± 0.52	1.71 ± 0.35	3.58 ± 0.67	2.34 ± 0.56	3.64 ± 0.57
Pure EEO	2.48 ± 0.41	3.67 ± 0.64	2.53 ± 0.71	1.45 ± 0.34	2.24 ± 0.54
Control	0.88 ± 0.43	5.52 ± 0.27	0.72 ± 0.01	0.92 ± 0.31	1.05 ± 0.36

surfactant could reduce the interfacial tension up to negative value and systems become highly stable²⁸⁻³⁰. The objective of self-nanoemulsification test was to evaluate any precipitation/phase separation upon mild agitation or dilution with “water, acid buffer (0.1N HCl) and phosphate buffer (pH 6.8)”^{16,30}. The results of this test suggested that EEO was maintained in solubilized form at molecular level in developed nanoemulsions and its self-nanoemulsification behavior was independent of pH³⁵. The droplet diameter of nanoemulsions was found to be decreased significantly with decrease in the concentration of EEO in formulation. The results suggested that EEO/oil phase had significant impact on droplet diameter of EEO nanoemulsions. The largest droplet diameter was obtained in formulation E5 (142.35 ± 10.58 nm) that was due to the presence of highest concentration of EEO (28% w/w) in formulation E5. However, the droplet diameter of EEO nanoemulsion E1 was obtained as lowest (32.45 ± 2.84 nm) that was due to the presence of lowest concentration of EEO (12% w/w) in formulation E1. The highest PDI value was obtained in EEO nanoemulsion E5 (0.278). Overall, the PDIs were recorded as <0.3 in all nanoemulsions, suggesting uniformity of droplet diameter in all nanoemulsions. The determination of ZP is useful in evaluation of surface charge and stability of prepared nanoemulsions. The results of ZP determination suggested stable formation of EEO nanoemulsions. The determination of RI is helpful in evaluation of transparent nature and nanoemulsion type. The RIs values of all nanoemulsions were much closed with that of water (1.333), suggesting transparent nature and oil-in-water type behavior of all EEO nanoemulsions. The determination of % T is also helpful in evaluation of transparent nature of nanoemulsions. The results of % T suggested transparent behavior of all EEO nanoemulsions. TEM analysis was conducted for the evaluation of the surface morphology of optimized EEO nanoemulsion E1. The size of all internal droplets of formulation E1 was obtained within nanometer range as shown in Fig. 3. The shape of nanoemulsion droplets was recorded as spherical. The enhanced wound healing effects of optimized nanoemulsion E1 were possible due to nanosized droplet diameter of nanoemulsion

and the presence of solubilizers including Tween-85 and Transcutol in optimized nanoemulsion E1. The potential of producing wound contraction by EEO and EEO loaded nanoemulsion obtained in this study suggested that *Eucalyptus* plant possesses a definite prohealing action because most of the wound healing occurred due to wound contraction³⁶. The wound healing effects of pure EEO and EEO nanoemulsion could be due to definite enhancement in the proliferation of epithelial cells as proposed in literature³⁷.

Many essential oils have been studied for their wound healing effects on different animal models³⁸⁻⁴¹. Nanoemulsions are known to enhance oral absorption and therapeutic efficacy of drugs¹¹⁻¹⁵ and essential oils have potential for wound healing effects³⁸⁻⁴¹. Therefore, nanoemulsions of EEO were prepared in order to enhance wound healing effects of EEO in this study. It was proposed that the restoration and the functional integrity of the wound tissue involve various processes including “inflammation, wound contraction, angiogenesis, extracellular matrix deposition and tissue remodeling”. Either single or multiple mechanisms could be responsible in different phases of wound healing which can contribute to the overall outcome of the wound healing process^{42,43}. At day 4 post-wounding, a significant reduction in swelling and exudates in rats treated with an optimized nanoemulsion E1 was recorded. These effects were comparable to standard antibiotic treated rats and higher than control and pure EEO as shown in Fig. 4.

It has been proposed that the internal oil phase/lipoidal portion of nanoemulsions enhance the intestinal lymphatic uptake of lipophilic compounds which could further help in avoidance of pre-systemic metabolism of these compounds⁴⁴. This potential of nanoemulsions could definitely results in rapid absorption of drugs from nanoemulsions and finally results in enhanced oral bioavailability and therapeutic efficacy of such lipophilic compounds^{44,45}. Hence, the enhanced wound healing effects of EEO nanoemulsion E1 were possibly due to enhancement in lymphatic uptake of EEO from nanoemulsions and avoidance of pre-systemic metabolism. In the present study, leucine assay was used for collagen determination in wound tissues^{46,47}. The collagen accumulation is the sum of synthesis and destruction

which occur simultaneously during wound healing process⁴⁸). Therefore, the enhanced levels of leucine in optimized nanoemulsion E1 and standard antibiotic treated rats suggested enhanced collagen content in these sample matrices. However, the level of leucine was much lower in case of negative control which was possible be due to a prolonged inflammatory phase in negative control. Overall, the formulation of EEO in terms of nanoemulsion was advantageous in terms of collagen content because the level of collagen in rats treated with optimized nanoemulsion E1 was much higher as compared with pure EEO and negative control. The results of histopathological evaluation suggested that the wound healing and repair are accelerated by EEO in the form of nanoemulsion.

5 CONCLUSIONS

Different nanoemulsion formulations of EEO were prepared and investigated for its wound healing potential in this study. Nanoemulsions of EEO were prepared using aqueous phase titration method and characterized for various physicochemical parameters. Based on various physicochemical parameters including minimum droplet diameter, least PDI, optimal ZP value, optimal RI value and maximum % T value, nanoemulsion formulation E1 was optimized and selected for wound healing activity in rats. Wound healing potential of optimized nanoemulsion was found to be significant as compared with pure EEO and control. However, it was comparable with standard antibiotic. Optimized nanoemulsion formulation also showed significant enhancement in the level of collagen as compared with pure EEO and negative control. Histopathological examinations of optimized nanoemulsion treated rats showed no signs of inflammatory cells which suggested that prepared nanoemulsion was safe and nontoxic to animals. The results of this study suggested the potential of nanoemulsion for oral delivery of EEO for enhancing its wound healing potential.

CONFLICT OF INTEREST

"The authors declare that they have no conflict of interest with this manuscript."

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