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ORIGINAL ARTICLE

Development and Evaluation of Sea Buckthorn (*Hippophae rhamnoides* L.) Seed Oil Nanoemulsion Gel for Wound Healing

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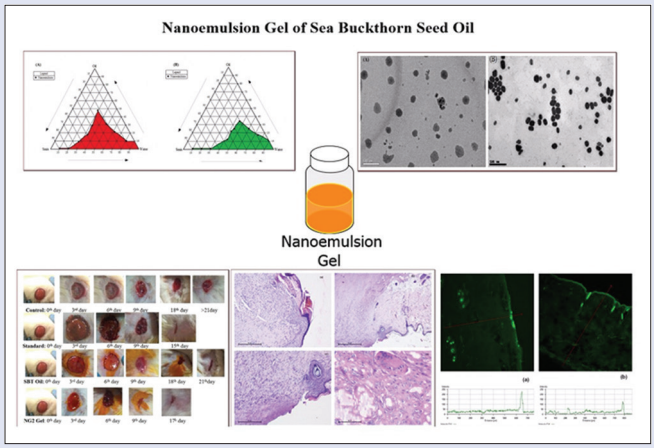
ABSTRACT

Background: Sea buckthorn (SBT) seed oil is reported to have significant wound-healing activity. However, the oil presents with problems such as poor skin permeation and retention in the deeper layers of skin, as well as leakage, dripping, and spreading during application. These issues can be overcome by formulating nanoemulsion (NE) gel of SBT seed oil. **Objective:** The present study involves the development of SBT seed oil NE gels for wound healing. **Materials and Methods:** The NE formulations were prepared by the spontaneous emulsification method. Based on the results of pseudoternary phase diagrams, different NE formulations were prepared using SBT seed oil, surfactant/cosurfactant, and water. The selected NE formulation NEA1 was used to prepare two NE gels containing 0.5% w/w (NG1) and 1% w/w (NG2) of carbopol 940. **Results:** The optimized formulation NEA1 was selected on the basis of stability and conductivity studies. The NG2 NE gel was selected as the final formulation on the basis of viscosity, spreadability, extrudability, and texture analysis. This optimized NG2 formulation was further evaluated for *in vivo* wound-healing activity and *ex vivo* skin penetration studies. A significant improvement was found in NG2-treated group of animals in comparison with SBT seed oil-treated animals w.r.t wound contraction, hydroxyproline, hexosamine content, breaking strength, tensile strength, and epithelialization time of the wound. The optimized formulation also showed improved antibacterial and antifungal activity. **Conclusion:** The formulated NE gel of SBT seed oil showed better wound-healing, antibacterial, and antifungal activity in comparison to pure SBT seed oil.

Key words: *Hippophae rhamnoides* L., nanoemulsion gel, sea buckthorn, spontaneous emulsification, wound healing

SUMMARY

- The objective of the present research work was to develop nanoemulsion (NE) gel of sea buckthorn (SBT) seed oil which can be effectively used for wound healing. This is significant because SBT seed oil is reported to have significant wound-healing activity. However, the oil presents with problems such as poor permeation, leakage, and dripping during application. These issues can be overcome by formulating NE gel of the SBT seed oil. The increased penetration across the skin leads to improve wound-healing, antibacterial, and antifungal activity in comparison to pure SBT seed oil.



Abbreviations used: CPCSEA: Committee for the Purpose of Control and Supervision of Experiments on Animals; DMSO: Dimethyl sulfoxide; FITC: Fluorescein isothiocyanate; FAME: Methyl esters formation of fatty acids; GC: Gas chromatography; IAEC: Institutional Animal Ethics Committee; MIC: Minimum inhibitory concentration; MHA: Mueller-Hinton agar media; MHB: Mueller-Hinton broth; MDS: Mean droplet size; NIPER: National Institute of Pharmaceutical Education and Research; NEs: Nanoemulsions; KOH: Potassium hydroxide; PDI: Polydispersity index; RPMI-1640: Roswell Park Memorial Institute-1640 medium; STZ: Streptozotocin; SBT: Sea buckthorn; S_{mix}: surfactant/cosurfactant; TEA: Triethanolamine; TEM: Transmission electron microscopy; YPD: Yeast extract-peptone-dextrose.

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INTRODUCTION

Hippophae rhamnoides L. (Family *Elaeagnaceae*) is commonly known as sea buckthorn (SBT) which is a branched and thorny nitrogen-fixing deciduous shrub, native to Europe and Asia. It grows at an altitude of 2500–4300 m.[1,2] It is a unisexual plant which has been used for several years in the traditional system of medicines for its antimicrobial, immunomodulatory, antioxidant, hepatoprotective, cytoprotective, and tissue regenerative properties.[3] The SBT seed oil is one of the versatile natural oils containing large quantities of unsaturated fatty acids, viz.,

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palmitic, stearic, linoleic, and α-linolenic acids, tocopherols, carotenoids, and flavonoids. These constituents are known to have significant wound-healing, antibacterial, antiatherogenic, and cardioprotective activities.[4,5]

Wound healing is a tangled step-by-step process of repair that involves the stages of hemostasis, inflammation, proliferation, and remodeling. The aim of wound management is to heal the wound in the shortest time, possible with minimal pain, discomfort, and scarring to a patient.[6] *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa, Streptococcus pneumoniae,* and *Klebsiella pneumonia* are some of the important bacterial organisms of wound infection.[7] *Candida albicans* is one of the normal flora pathogens in the skin and mucous membranes, which is responsible for wound infection.[8,9] Although there are various advantages of herbal medicines such as SBT seed oil, these also have some limitations, including dripping, spreading, low viscosity, inconvenience in application, poor permeation, and retention in the deeper layers of the skin. Development of the novel formulations such as nanoemulsion (NE) gel of SBT seed oil is expected to overcome these issues by improving the contact time, permeation, and retention, which would effectively improve its wound-healing potential.[10]

NEs are isotropic mixture of oil, surfactant/cosurfactant (S_{mix}), and water with droplet diameter approximately in the range of 10–200 nm.[11] The present work involves the development of NE gel of SBT seed oil for topical application and evaluates its wound-healing potential.

MATERIALS AND METHODS

Materials

H. rhamnoides L. (SBT) seed oil was obtained from Katyani Exports and Manufactures, Pitampura, Delhi. α-linolenic acid (omega-3) fatty acid and linoleic acid (omega-6) fatty acid were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Labrasol®, Transcutol® P, and Cremophor® RH 40 were supplied as a gift sample by Gattefosse Pvt. Ltd. and BASF Pvt. Ltd., Mumbai, India. Tween 80 and triethanolamine (TEA) were purchased from Loba Chemie Pvt. Ltd., Mumbai, India. Span 80, carbopol 940, streptozotocin (STZ), yeast extract-peptone-dextrose (YPD), Mueller-Hinton Agar (MHA) media, Mueller-Hinton broth (MHB), and Roswell Park Memorial Institute-1640 medium (RPMI 1640) were purchased from HiMedia Laboratories Pvt., Ltd., Mumbai, India. All other solvents and chemical used in the research work were of analytical grade.

Cultures

Bacterial cultures, viz., *S. aureus* (MTCC 740), *P. aeroginosa* (MTCC 424), and *E. coli* (MTCC 52) Gram-negative bacteria, and cultures including yeast, i.e., *Candida albicans* (ATCC 90028), were used for *in vitro* antibacterial and antifungal activity. These were obtained from the repository of the Department of Molecular Immuno-Parasitology Laboratory and Yeast Biology Laboratory, Shoolini University, Solan, Himachal Pradesh.

Methods

Derivatization of fatty acids present in sea buckthorn seed oil

SBT seed oil diluted with methanol was refluxed in the presence of catalytic amount of concentrated sulfuric acid, and then, reaction mixture was neutralized with the addition of potassium hydroxide (KOH) pellet.[12] After the completion of reflux reaction, the organic layer was separated from the aqueous layer using dichloromethane with the help of fractionation technique. Dichloromethane was removed completely using a rotary evaporator at 40°C to obtain esterified SBT seed oil which was confirmed by Fourier-transform infrared results.

Esterified compound from the SBT seed oil was obtained doing column chromatography. The column was run using a solvent system of hexane-ethyl acetate in different ratios. The esterified compound obtained was further tested by gas chromatography (GC) for the estimation of methyl esters formation of fatty acids (FAME).

Gas chromatography

Esterified compound of SBT seed oil (0.24 g) was accurately weighed and transferred to a volumetric flask. Then, 5 ml of hexane was added followed by the addition of 2 N KOH solution (250 µL) into the volumetric flask which was further mixed by shaking for 2 min. After settling, the supernatant was used for GC analysis.[13]

Chromatographic system

The GC (2014) was equipped with a flame ionization detector and a 0.25 mm × 30 m fused silica capillary column coated with a 0.25 µm FAMEWAX™ column (cat # 12497). The temperature of the detector was maintained at 230°C and that of the injection port at 225°C. The column temperature program was initially set up at 165°C for the first 30 min and then increased at a rate of 1.5°C/min to 220°C, where it remained for the last 15 min. The carrier gas was helium with a split flow of a ratio of 1:40 at the linear velocity of 40 cm/s, and a flow rate of about 0.5 mL/min was measured at the initial temperature.

Standard esters of omega-3 and omega-6 fatty acids were also analyzed by the same procedure, and the retention time in the SBT seed oil was compared with the chromatogram of their standard fatty acids.

Determination of nanoemulsion-forming zones by pseudoternary phase diagrams

For the pseudoternary phase diagrams, S_{mix} mixtures of tween 80/span 80, Cremophor® RH40/span 80, Labrasol®/Transcutol® P, Cremophor® RH40/Transcutol® P, and Cremophor® RH40/Labrasol® at different mass ratios (1:1, 1:2, 2:1, 1:3, and 3:1) were prepared. These pseudoternary phase diagrams were composed of a fixed ratio of SBT seed oil and S_{mix} in the ratio of 1:9, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 9:1 and water concentration varying with all these combinations. The mixtures of S_{mix} ratio were selected on the basis of their combined hydrophylic-lipophylic balance (HLB) values.[14]

Different ratios of SBT seed oil, S_{mix}, and water were used to prepare pseudoternary phase diagrams. All the compositions were prepared by low-energy emulsification technique, i.e., spontaneous emulsification method.[15] According to the method, SBT seed oil and the S_{mix} were mixed together at 40°C to obtain the organic phase. Then, the water maintained at 40°C was added drop wise to the organic phase while mixing using a vortex shaker until the onset of turbidity. The endpoint was visually monitored against light and dark background by illuminating the samples with light. The compositions were studied for 24 h at room temperature and were remonitored visually for transparency, miscibility, and flowability. From the results of different ratios of SBT seed oil, S_{mix}, and water, the corresponding percentages were calculated and plotted on triangular coordinates to construct pseudoternary phase diagrams.

Preparation of nanoemulsion formulations

Pseudoternary phase diagrams that resulted in a maximum NE formation zone were selected for the preparation of NEs. Composition of NEs in terms of SBT seed oil: S_{mix}: water ratios was selected from the NE-forming zones obtained from the pseudoternary phase diagrams. All the NE formulations were prepared by the spontaneous emulsification method as already discussed above. These NE formulations were also characterized for compatibility, stability, and conductivity.

Stability studies of prepared nanoemulsions

Stability studies of the prepared NEs were carried out by heating-cooling cycle, centrifugation, and freeze-thaw cycle.[16]

i. Heating-cooling cycle
   Six cycles between refrigerator temperature 4°C and 45°C with storage at each temperature for 48 h were conducted and the formulated NEs were examined for stability (transparent with no phase separation)
ii. Centrifugation test
   The formulated NEs were centrifuged at 3500 rpm for 30 min observed for transparency and absence of phase separation
iii. Freeze-thaw cycle
   Three freeze-thaw cycles of the NEs between −21°C and +25°C for 48 h were performed and observed for transparency and absence of phase separation.

Conductivity study

Electrical conductivity of the formulated NEs was measured using conductivity meter (ECTestr 11+, USA) at 25°C in triplicate.[17]

Characterization of selected nanoemulsion formulations

NE formulations that passed the stability studies and conductivity test were selected for further characterization. NEs were characterized for following parameters:

Percent transmittance

Percent transmittance of NEs was measured using ultraviolet (UV) visible double beam spectrophotometer keeping distilled water as blank at 560 nm.[17]

Globule size and polydispersity index measurement

Mean droplet size and polydispersity index (PDI) of NEs was determined using dynamic light scattering zeta sizer instrument (Malvern Zeta Sizer; Nano-ZS90). The evaluation was carried out at 25°C at an angle of 90°. Samples were analyzed in triplicate.[15]

Transmission electron microscopy

To perform the transmission electron microscopic (TEM) observations, all the optimized batches were placed on a carbon-coated copper grid (200 mesh/inches) and then stained with 1% phosphotungstic acid. The excess phosphotungstic acid on the sample was gently wiped off using filter paper and examined for morphological aspects after drying for about ½ h at room temperature.[18]

Preparation of sea buckthorn seed oil nanoemulsion gels

NE gels were prepared for optimized NE selected on the basis of above characterization parameters. Two different SBT seed oil NE gels were prepared by adding carbopol 940 in different concentrations of 0.5% and 1% (w/w). Methylparaben sodium 0.2% (w/w) and propylparaben sodium 0.05% (w/w) were also added as preservatives in the formulation by constant stirring. On formation of homogeneous dispersion, the pH was adjusted to 7.0 using TEA to form the NE gel.[17]

In vitro evaluation of nanoemulsion gels

Spreadability test of nanoemulsion gels

A sample of 0.5 g of each developed NE gel formulations was pressed between two slides and left for about 5 min where no more spreading was expected. The results obtained were average of three determinations.[19] The formula used for the determination of spreadability was given below in equation:

$$S = ML/T$$

where S indicated the spreadability (g.cm/s), M indicates the mass (g), L indicates the length (cm), and T indicates time (s).

Extrudability test of prepared nanoemulsion gels

The developed NE formulations were filled in a clean, lacquered aluminum collapsible one-ounce tube with 5 mm opening. It was then placed in between two glass slides and was clamped. Extrudability was determined by weighing the amount of gels extruded through the tip when a constant load of 500 g was placed on the slides, and the amount of gels extruded was collected and weighed.[19] The percentage of the extruded gel was calculated (>90% extrudability: excellent, >80% extrudability: good, >70% extrudability: fair).

Rheological studies

Rheological investigations are basically concerned with the determination of the relationship between shear stress, stress rate, and viscosity. A large number of equations have been suggested to describe various non-Newtonian flow characteristics of gels.[20] Of these, Power law equation is by far the most and widely applicable. It is given as:

$$\gamma = K\tau^n$$

Taking logarithm on both the sides

$$\text{Log } \gamma = \text{Log K} + n \text{ Log } \tau$$

where τ is the shear stress, γ is shear rate, K is consistency index, and n is flow index. Consistency index and flow index are calculated by plotting graphs of log shear rate versus log shear stress. The antilog of intercept is the consistency index and the slope of the line is flow index. Flow index (dimensionless) is a measure of the deviation from Newtonian behavior ($n = 1$), $n < 1$ indicates shear thinning (pseudoplastic behavior) and $n > 1$ shear thickening (dilatant behavior).

The viscosity was measured by RheolabQC rheometer using Rheoplus/32, v 3.40 software (Anton Paar, Graz; Austria) at 25°C ± 2°C. The viscosities of all prepared NE gel formulations were measured using DG 26.7 geometry with shear rate ranging from 2 to 100 for 3 min using 4 g of gel. Temperature control was achieved using a fluid bath surrounding the outer cylinder.

Texture analysis

Texture profile analysis was carried out using texture analyzer; the use of a texture analyzer eliminates manual variables and provides a recordable, mechanized means to assess the semi-solid formulations. The "peak" or maximum force is considered as a measurement of firmness, i.e., the higher the value, the firmer the sample is. The area of the curve up to this point is taken as a measurement of consistency, i.e., the more the value, the thicker the consistency of the sample.[21]

Animal studies

Wistar rats of either sex about 10-12 weeks old (150–250 g) were procured from Animal House Facility, National Institute of Pharmaceutical Education and Research, Mohali, and were housed at Animal House Facility, Shoolini University, Solan, Himachal Pradesh (India). The rats were kept under laboratory conditions: temperature 25°C ± 2°C, relative humidity 45% ±5%, and a photoperiod of 12 h. The studies were conducted after obtaining ethical clearance from the Institutional Animal Ethics Committee (IAEC), Shoolini University, Bajhol, Solan-vide protocol approval no. IAEC/SU-17/02 and conducted according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (Government of India), New Delhi guidelines.

Acute skin irritation test

The study was carried out according to the reported method on rats. About 500 mm^2 area on the dorsal fur of each animal in different groups was shaved. The shaved area was clean and was treated with SBT seed oil in one group and with the optimized NE gel in the second group. The control group was left as untreated. After 4 h of topical application, the skin of each

animal present in different groups was observed for the inflammation signs.

In vivo wound-healing activity

Excision and diabetic incision wound-healing models were used to evaluate the wound-healing activity of optimized SBT seed oil NE gel. Animals were divided into four groups of four animals each as follows:

- Group I: Control group untreated
- Group II: Standard group treated with 5% (w/w) povidone-iodine ointment
- Group III: SBT seed oil treated group with SBT seed oil
- Group IV: Optimized NE gel treated group.

Excision wound model

The excision wound model was used to study the wound-healing activity in various divided groups of rats. Animals were anesthetized with chloral hydrate (300 mg/kg, i.p.) before the creation of experimental wound.[22] The dorsal fur of the animals was shaved and the excision wound of 500 mm^2 was created along the marking using surgical blade, toothed forceps, and pointed scissors. The healing of wound was assessed by tracing the wound on 0^{th}, 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 15^{th}, 18^{th}, 21^{st} postwounding days and recorded; wound areas were measured graphically and observed for various parameters including rate of wound contraction, epithelialization time, biochemical markers, i.e., hydroxyproline as well as hexosamine content, and for histology.

Rate of wound contraction

The rate of wound contraction of control as well as treated groups was measured as a percentage reduction of wound size.[22] The percentage (%) wound contraction was calculated using equation:

$$\% \text{ Wound contraction} = \frac{\text{Wound area on the } 0^{th} \text{ day} - \text{Wound area on the } n^{th} \text{day}}{\text{Wound area on the } 0^{th} \text{ day}} \times 100$$

Epithelialization time

Falling of eschar without any raw wound area was considered as complete healing of wounds, and the number of days required for wound was calculated as a period of epithelialization.[23]

Biochemical parameters

Estimation of hydroxyproline

On the 21^{st} day of postwounding day, all the animals of different groups were sacrificed under chloral hydrate (300 mg/kg body weight, i.p.). A piece of skin from the healed wound area was collected and analyzed for hydroxyproline content, which is a basic constituent of collagen. After recording the wet weight, the granulation tissue was dried in a hot air oven at 60°C–70°C to constant weight and was hydrolyzed in 6 N HCl (5.0 mL) at 110°C for 4 h in sealed tubed. The hydrolysate of different groups was neutralized to pH 7.0, and about 200 µL of hydrolysate was mixed with 0.01 M copper sulfate solution (1 mL) followed by 2.5 N NaOH (1 mL) and 6% H_2O_2 (1 mL). Samples were then kept at 80°C for 5 min on a shaking incubator, cooled, and mixed with 4 mL of 3N H_2SO_4 with agitation. Finally, 2 mL of 5% Ehrlich's reagent was added to the mixture, incubated at 70°C for 16 min, and cooled at 20°C, and the absorbance measured at 557 nm using UV spectrophotometer (UV Evolution™ 201 Thermo Scientific, India). The amount of the hydroxyproline present in the sample was calculated from the standard curve prepared with pure L-hydroxyproline.[22]

Estimation of hexosamine

The weighed granulation tissue from different groups was hydrolyzed in 6 N HCl for 8 h at 98°C, neutralized to pH 7.0 with 4 N NaOH, and diluted with distilled water. The diluted solutions were mixed with acetylacetone solution and heated to 96°C for 40 min. The mixture was cooled and 96% ethanol was added, followed by addition of para-dimethyl amino-benzaldehyde solution (Ehrlich's reagent). The solutions were thoroughly mixed and kept at room temperature for 1 h, and the absorbance was measured at 530 nm using UV spectrophotometer (UV Evolution™ 201 Thermo Scientific, India). The amount of hexosamine was determined by comparing with a standard curve prepared using serial dilutions of pure 0.1% N-acetyl glucosamine.[24]

Diabetic incision model

After overnight fasting, diabetes were induced in all groups by a single injection of STZ (50 mg/kg, i.p.) prepared in citrate buffer 0.1 M, pH 4.5. Blood was drawn from the tail on the 4^{th} day after the STZ injection, and the blood glucose levels were estimated using a glucometer (AccuSure™ Glucometer).[25] The blood glucose levels were used to confirm the development of diabetes. Rats with elevated blood glucose levels (≥140 mg/dL) were considered diabetic and were subjected to the incision wound procedure. The blood glucose levels were estimated at the time of the creation of the wounds. Similar to the excision wound model, diabetic rats were anesthetized before and during the creation of the wound. The dorsal furs of all the animals were shaved with an electric clipper. A longitudinal paravertebral long incision of 5 cm length was made through the skin and cutaneous muscle at a distance about 1.5 cm from the middle on the right side of the depilated back. Afterward, wounds were closed by means of interrupted sutures placed at equidistant points of 0.5 cm intervals using sterile surgical thread (No. 000) and a curved needle (No. 11).[26] The wounds were then left undressed. All groups of animals were treated as described above for wound breaking as well as tensile strength and histology.

Wound breaking and tensile strength

The sutures were removed on the 8^{th} postwounding day when the wounds were cured thoroughly and the tensile strength of the skin was measured by tensiometer on the 10^{th} day.[26] Breaking strength of the healed wound is measured as the minimum force required for breaking the incision apart which also acts as an indication of the tensile strength of wound tissues. Tensile strength was also calculated using the following equation:

$$\text{Tensile strength } (kg/cm^2) = \frac{\text{Breaking strength } (g)}{\text{Cross-section area of skin } (mm^2)}$$

Histology

The skin specimens from healed wound areas were fixed in 10% buffered formalin and processed by a paraffin tissue processing machine. The healed skin was assessed by taking a 3–4 µm section, followed by staining with hematoxylin and eosin.[26]

Ex vivo skin penetration studies

The study was carried out visually using a ×10 objective piece to evaluate the skin dye penetration studies of optimized NE gel formulation in comparison to SBT seed oil using confocal laser scanning microscopy (Zeiss LSM 510). Microscopic analysis of the rat skin was carried out for skin penetration study with fluorescein isothiocyanate (FITC) dye-loaded SBT seed oil and with optimized NE gel. Cryosectioning was done to get cross-sections using cryotome (Shandon, UK).[16]

Antibacterial and antifungal studies

The antimicrobial activity was evaluated by the agar diffusion method using 100 µL of suspension containing 10^8 CFU/mL of bacteria spread on MHA media and 10^4 spore/mL of fungi spread on YPD, respectively.

Bacteria were cultured overnight at 37°C in MHB and fungi at 28°C for 72 h in YPD broth and used as inoculum. The disc (6 mm in diameter) was impregnated with 100 µL of conventional formulation pure S_{mix}, SBT seed oil, and optimized NE gel using DMSO as a solvent to make the solution of all these ingredients. The conventional topical formulation was prepared by adding 15% (w/w) of SBT seed oil in a marketed cold cream preparation. Tetracycline (10 µg/disc) was used as positive controls for bacteria and fluconazole (10 µg/disc) for fungus studies.[27] Minimum inhibitory concentration (MIC) values were also studied for microorganisms, which causes infection in wounds by broth microdilution method using 96-well plates in MHB for bacteria (10^8 CFU/mL) and RPMI 1640 medium for fungus (10^3 CFU/mL). MIC was defined as the lowest concentration of drug that inhibited visible growth in broth.[28]

RESULTS AND DISCUSSION

Gas chromatographic analysis of derivatized fatty acids of sea buckthorn seed oil

The fatty acid content of omega-3 and omega-6 in the esterified SBT seed oil used for the formulation and evaluation of NE gel was found to be 28.4% (w/w) and 20.4% (w/w), respectively, from the results of GC analysis. GC of standard omega-3, standard omega-6 fatty acids, and esterified SBT seed oil is shown in Figure 1a-c.

Construction of pseudoternary phase diagrams

The fixed ratio of SBT seed oil and 25 combination ratios of S_{mix} and water in different concentration was used for the preparation of various pseudoternary phase diagrams as discussed above. The required HLB range of S_{mix} combinations was 8–16 for o/w type NE preparation. Pseudoternary phase diagrams with the largest NE-forming zones were selected for the preparation of NEs. It was observed that the S_{mix} components of Cremophor® RH 40:Span 80 in the ratio of 1:1 and S_{mix} components Cremophor® RH 40:Span 80 in the ratio of 2:1 showed the most prominent NE-forming zone as shown in Figure 2a and b. The composition of NEs formulated by selecting different points within the NE-forming zones of selected two ratios of Cremophor® RH 40:Span 80 in the ratio of 1:1 and 2:1 is shown in Table 1. The rest of the S_{mix} compositions were found to be turbid, formed viscous gel, or lead to phase separation. All the NEs were prepared by low-energy emulsification technique, i.e., spontaneous emulsification method. These NE formulations were subjected to stability studies involving heating-cooling cycles, centrifugation, and freeze-thaw to identify and select the most stable NE formulations. In addition, conductivity test was carried out for the stable NE to confirm the o/w behavior of NE formulations.

Stability studies

The NE formulations that were physically instable showed the signs of phase separation and were rejected. The stability studies' results of different NE formulations prepared from Cremophor® RH 40:Span 80 in two different ratios 1:1 and 2:1 are shown in Table 2.

Conductivity test of nanoemulsion formulations

The selected NE formulations batches that passed the stability studies were further subjected to conductivity test. The conductivity values were found to be high for all NE formulations that confirmed that the NE formulations were o/w type as shown in Table 3. NE formulations NEA1 and NEB2 showed the highest conductivity values and were selected for further characterization.

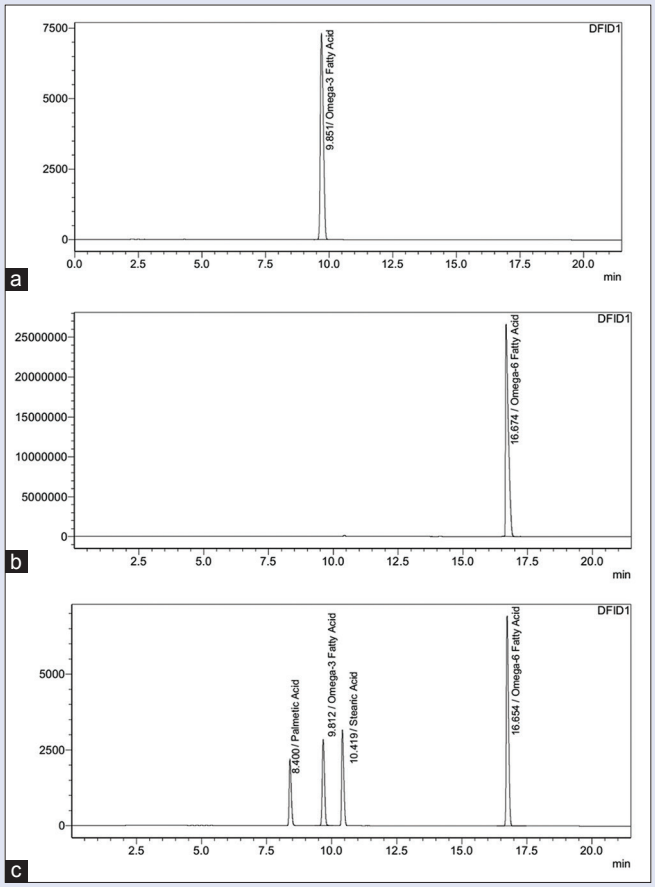

Figure 1: Gas chromatograms of (a) standard omega-3, (b) standard omega-6, (c) esterified sea buckthorn seed oil

Table 1: Selected nanoemulsion forming points within nanoemulsion-forming zones of selected two ratios of Cremophor® RH40:span 80 in the ratio of 1:1 and 2:1

Formulation	S_{mix} ratio (Cremophor® RH40:span 80)	Composition (% v/v)		
		SBT seed oil	S_{mix}	Water
NEA1	1:1	15	40	45
NEA2	1:1	15	65	20
NEA3	1:1	20	50	30
NEA4	1:1	20	55	25
NEA5	1:1	20	60	20
NEA6	1:1	25	45	30
NEA7	1:1	25	55	20
NEB1	2:1	15	75	10
NEB2	2:1	15	60	25
NEB3	2:1	20	50	30
NEB4	2:1	20	55	25
NEB5	2:1	20	58	22
NEB6	2:1	25	45	30
NEB7	2:1	25	50	25

S_{mix}: Surfactant/cosurfactant; SBT: Sea buckthorn

Characterization of selected nanoemulsion formulations

The results of percentage transmittance, globule size, and PDI revealed that the two selected NE formulations NEA1 and NEB2 were nearly transparent and found to be in nanosize with low PDI values as shown in Table 4. However, the percentage transmittance value for NEA1 was

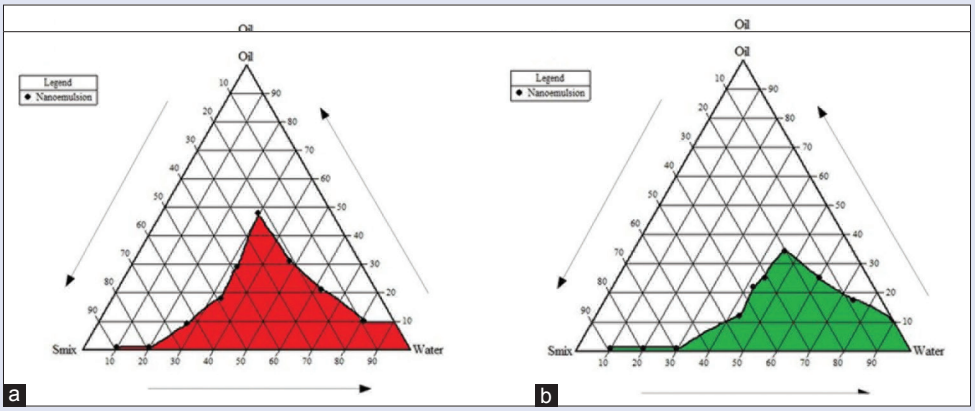

Figure 2: Pseudoternary phase diagrams of sea buckthorn seed oil, indicating o/w nanoemulsion region of sea buckthorn seed (oil), Cremophor® RH 40:Span 80 (S_{mix}) at ratio of (a) 1:1 and (b) 2:1

Table 2: Stability studies of selected nanoemulsion formulations

Formulation	S_{mix} ratio Cremophor® RH 40 : Span 80	Stability Parameters		
		Heating-cooling cycles	Centrifugation	Freeze-thaw
NEA1	1:1	Clear	Clear	Clear
NEA2	1:1	Clear	Clear	Clear
NEA3	1:1	PS*	PS*	PS*
NEA4	1:1	Clear	Clear	Clear
NEA5	1:1	PS*	PS*	PS*
NEA6	1:1	Clear	Clear	Clear
NEA7	1:1	PS*	PS*	PS*
NEB1	2:1	PS*	PS*	PS*
NEB2	2:1	Clear	Clear	Clear
NEB3	2:1	PS*	PS*	PS*
NEB4	2:1	Clear	Clear	Clear
NEB5	2:1	Clear	Clear	Clear
NEB6	2:1	PS*	PS*	PS*
NEB7	2:1	PS*	PS*	PS*

*PS: Phase separation

Table 3: Conductivity of the selected nanoemulsion formulations

Formulation	S_{mix} ratio (Cremophor® RH40:span 80)	Conductivity (µS/cm)
NEA1	1:1	160±0.15
NEA2	1:1	98±0.38
NEA4	1:1	115±0.85
NEA6	1:1	128±0.95
NEB2	2:1	145±0.25
NEB4	2:1	118±0.55
NEB5	2:1	95±0.55
NEB7	2:1	120±0.35

Data are mean±SD. *n*=3. SD: Standard deviation; S_{mix}: Surfactant/cosurfactant

Table 4: Physicochemical characteristics of the selected nanoemulsion formulations

Formulation	Percentage transmittance (%)	Globule size (nm)	PDI
NEA1	97±0.62	52.22±0.62	0.279±0.02
NEB2	90±0.24	148.1±0.70	0.309±0.04

Data are mean±SD. *n*=3. PDI: Polydispersity index; SD: Standard deviation

found to be higher (97% ± 0.62%) as compared to NEB2 (90% ± 0.24%), indicating the higher transparency of the formulation. The formulations NEA1 and NEB2 were found to be in nanosize range with low PDI values. It was observed that the globule size and PDI of formulation NEA1 were lower (globule size - 52.22 ± 0.62 nm; PDI - 0.279 ± 0.02) than NEB2 formulation (globule size - 148.1 ± 0.70 nm; PDI - 0.309 ± 0.04). Lower PDI values indicate the narrow size distribution of the globules, and single peak of NEA1 formulation indicates the uniform distribution of globules within the observed size range [Figure 3]. TEM images at magnification, viz., ×6200 [Figure 4], showed spherical-shaped globules with no aggregation in NEA1 NE formulation in comparison to NEB2 formulation where slight aggregation of globules was observed. Most of the globules in the TEM field for the formulation images were found to be <200 nm. It was observed that the results of percentage transmittance, globule size, and TEM were correlated. Based on the results of characterization parameters of NEA1 and NEB2 NE formulations, the selected final NE formulation was NEA1. The NE gels were prepared using the selected NEA1 formulation.

Preparation of sea buckthorn seed oil nanoemulsion gel

Based on the characterization studies of prepared NEs, it was observed that NEA1 NE was the most stable and optimized formulation and was thus selected for the preparation of NE gels. Two different NE gel formulations of NEA1 containing 0.5% and 1% (w/w) carbopol 940 were prepared and evaluated. The composition for the two prepared NE gels is shown in Table 5. Both the NE gel formulations NG1 and NG2 contained 15% (w/w) SBT seed oil. For the preparation of NE, gel water was divided into two parts (Part-I and Part-II). Part-I containing 20% (w/w) of

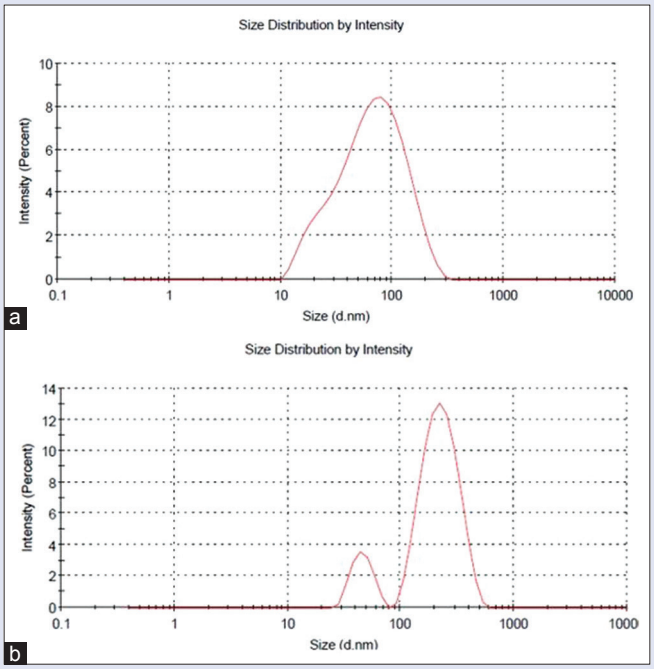

Figure 3: Globule size distribution of nanoemulsion formulation (a) NEA1 and (b) NEB2

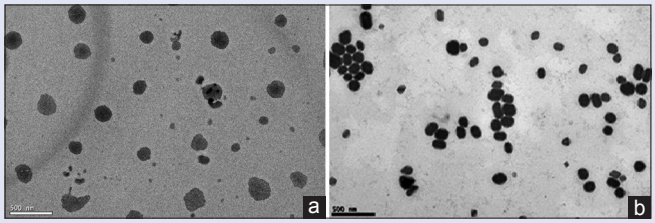

Figure 4: TEM images of nanoemulsion formulation (a) NEA1 and (b) NEB2 with ×6200

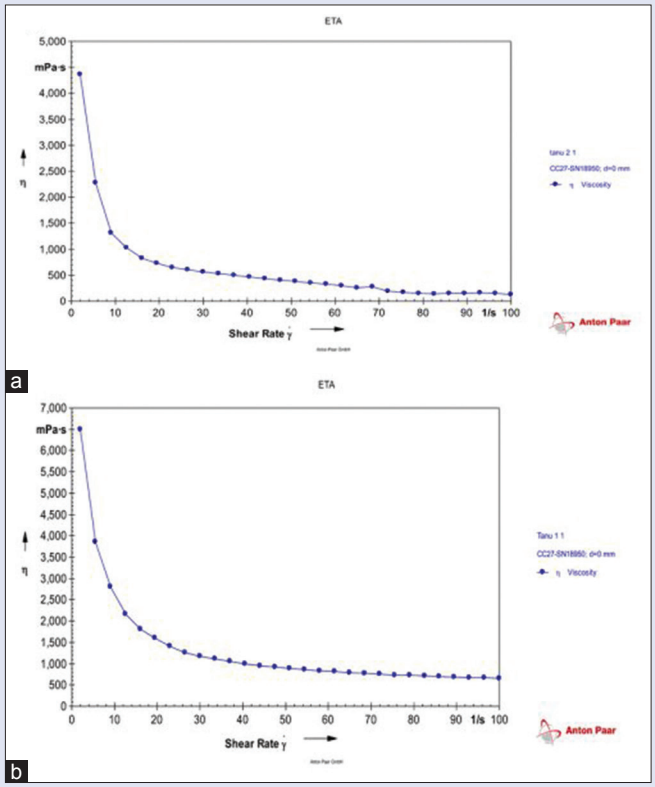

Figure 5: Plot of viscosity (η) versus shear rate (γ) of (a) NG1 and (b) NG2 nanoemulsion gel formulations

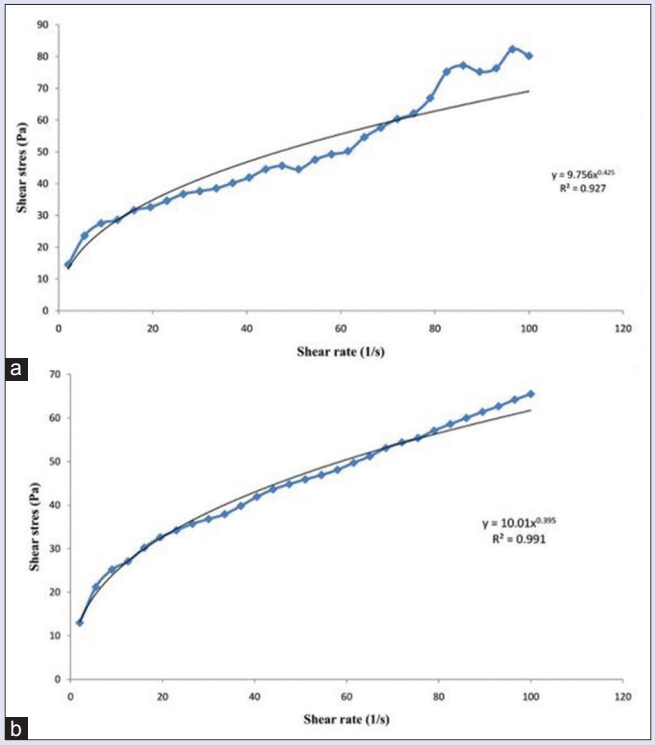

Figure 6: Plot of shear stress (τ) versus shear rate (γ) of (a) NG1 and (b) NG2 nanoemulsion gel formulations

water was added to NEA1. Carbopol 940, propylparaben sodium, and methylparaben sodium were added to the remaining 25% (w/w) of water (Part-II) and kept overnight for swelling. Both the part that was NEA1 and carbopol 940 gels were mixed and the pH was adjusted to 7.0 using TEA. The calculated omega-3 and omega-6 fatty acids content in these formulations was found 4.26% (w/w) and 3.06% (w/w) as per GC analysis. Carbopol 940 was selected as the gelling agent because it is biodegradable, biocompatible, and nontoxic to the human body.[29]

In vitro evaluation parameters of NEA1 nanoemulsion gels

The spreadability and extrudability of NG1 were found to be slightly higher than NG2 formulation due to the lower concentration of carbopol 940 present in the NG1 that also indicate toward its lower viscosity and higher flow which may not be acceptable for a topical gel with good consistency. These observations were confirmed by rheological studies and texture analysis. Figure 5a and b shows the viscosity (η) versus shear rate (γ) graphs of NE gels NG1 and NG2. The viscosity values ranged between 4.37 Pa.s and 0.13 Pa.s for formulation NG1 and between 6.49 Pa.s and 0.65 for formulation NG2. These values indicate a decrease in viscosity on increasing the shear rate from 1 to 100 s^{-1}. This indicates the shear thinning behavior or the pseudoplastic behavior of the prepared

formulations which was confirmed by obtaining plots of shear stress (τ) versus shear rate (γ) and applying the power law to the rheological data as shown in Figure 6a and b.

The consistency index for the formulations of NG1 and NG2 was found to be 9.75 and 10.01, respectively. The flow index of the NG1 and NG2 was found to be 0.42 and 0.39, respectively. The R^2 values for NG1 and NG2 was observed to be 0.9276 and 0.9910, respectively. The R^2 value of 0.9910 for the NG2 NE gel formulation indicated goodness of fit to the Power law equation. The flow index of 0.39 indicated shear thinning and pseudoplastic behavior ($n < 1$) of NG2 formulation. The shear thinning property is important for easy extrudability from the tube and better spreadability during application of the formulation on wounds. The firmness, consistency, cohesiveness, and index of viscosity were determined as texture parameters of the different formulations. All the four parameters of texture analysis were found to be lower for NG1 formulation as compared to NG2 formulation as shown in Figure 7. Based on the results of *in vitro* evaluation of prepared NE gel formulation, NG2 gel was selected as final and optimized formulation and was further evaluated for *in vivo* wound-healing activity, *ex vivo* skin penetration, and antibacterial and antifungal activity to establish its wound-healing potential. Different evaluation parameters of the NE gel formulations, NG1 and NG2, are shown in Table 6.

Acute skin irritation test

The SBT seed oil and its optimized NE gel (NG2) formulation did not show any symptoms of inflammation, swelling, or any other changes on the skin. Hence, the study indicated that the SBT seed oil-based NG2 formulation was nonirritant and can be used for topical applications.

In vivo wound-healing activity

Rate of wound contraction

Wound contraction can be defined as the rate of reduction of the unhealed area during the healing process. A significant increase in wound healing was observed in NG2-treated group in comparison to the group treated directly with SBT seed oil as well as control group. The NG2-treated animals showed a more rapid decrease in wound size compared with the control rats ($^aP < 0.001$) and significant rates of closure of the wound was observed ($^bP < 0.001$) on 6th, 9th, and 18th day in comparison to the rats treated with SBT seed oil, standard-treated group showed a nonsignificant ($P > 0.05$) gradual closure of wound in comparison to NG2 on 9th, 18th, and 21st day of wound healing [Table 7]. The percentage of wound healing of all the treated groups for wound contraction is shown in Figure 8. The photographic presentation of wound contraction is shown in Figure 9. A complete wound closure was observed on the 21st day of treatment in all the treated groups, and still on the 21st day, the control group was not healed properly.

Estimation of epithelialization time, hydroxyproline, and hexosamine content

The period of epithelialization was considered as the day of dropping of eschar. Animals treated with standard, NG2, and

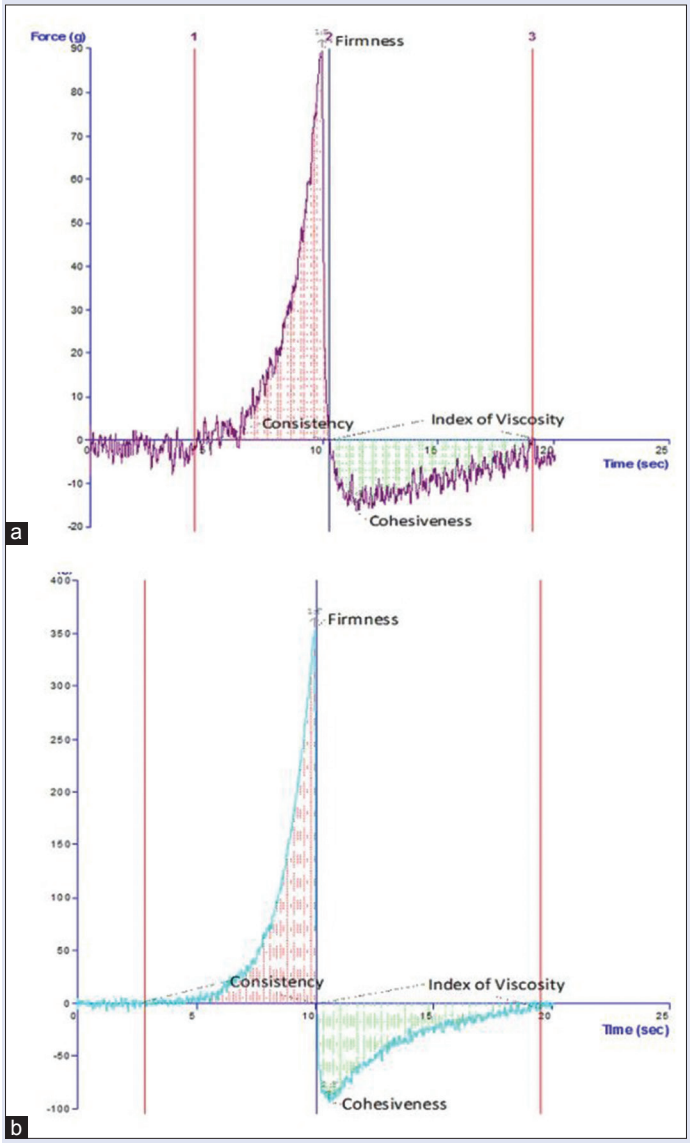

Figure 7: Graph representing texture analysis: (a) NG1 and (b) NG2

Table 5: Composition of selected NEA1 nanoemulsion gels

Ingredients	NG1 (% w/w)	NG2 (% w/w)
SBT seed oil	15	15
Cremophor® RH 40: Span 80 (1:1) S_{mix}	40	40
Carbopol 940	0.5	1
Propyl paraben sodium	0.05	0.05
Methyl paraben sodium	0.2	0.2
Triethanolamine	q.s (pH 7.0)	q.s (pH 7.0)
Distilled water	q.s (100%)	q.s (100%)

S_{mix}: Surfactant/cosurfactant; SBT: Sea buckthorn; NG2: Nanoemulsion gel 2; NG1: Nanoemulsion gel 1

Table 6: Evaluation parameters for prepared nanoemulsion gels

Nanoemulsion gel	Spreadability (g.cm/s)	Extrudability (%)	Texture parameters			
			Firmness (g)	Consistency (g.s)	Cohesiveness (g)	Index of viscosity (g.s)
NG1	13.6±0.20	89.6	89.6±11.5	119.5±53.3	-16.4±8.2	-79.3±4.3
NG2	10.5±0.36	85.2	354.2±8.1	448.4±30.1	-94.5±4.1	-315.9±5.3

Data are mean±SD. n=3. SD: Standard deviation; NG2: Nanoemulsion gel 2; NG1: Nanoemulsion gel 1

SBT seed oil healed significantly earlier than the group treated with control. It was also observed that NG2 formulation showed better ($^bP < 0.05$) wound-healing potential (17 days) than SBT seed oil (20 days) [Figure 10a]. In our study, the hydroxyproline content was found to be significantly ($^bP < 0.05$) higher in the optimized treated NG2 group in comparison to SBT seed oil [Figure 10b]. Although the hydroxyproline content of standard treated group was increased in comparison to NG2-treated group, the level was not statistically significant ($P > 0.05$). Increase in the hydroxyproline content indicates increased collagen synthesis as it is an integral part of collagen fiber. Hexosamine content of the granulation tissue of the wound treated with standard, SBT seed oil, as well as NG2 formulation was significantly ($^aP < 0.05$) higher in comparison to control. There was nonsignificant difference between standard-treated group ($P > 0.05$) in comparison to NG2-treated group. The hexosamine content was found to be significantly ($^bP < 0.05$) higher for NG2-treated group than that of SBT seed oil-treated group as shown in Figure 10c. The results of epithelialization time, hydroxyproline, and hexosamine content are summarized in Table 8.

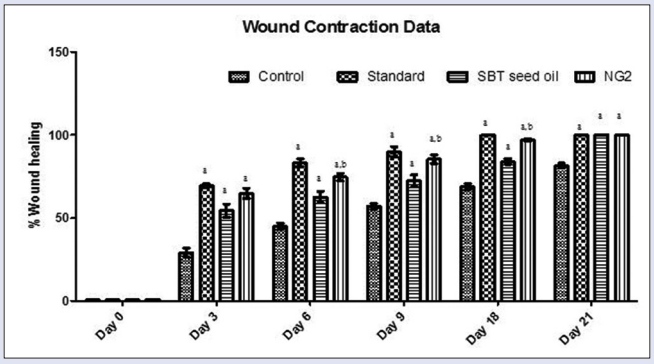

Figure 8: Results of wound contraction for control, standard, sea buckthorn seed oil, and NG2 formulation studied in rats. Values are means ± standard deviation ($n = 4$); $^aP < 0.001$ (all treatment show significant difference in comparison to control), $^bP < 0.001$ (NG2 show significant difference in comparison to sea buckthorn seed oil on day 6th, 9th, and 18th) and $P > 0.05$ (standard show nonsignificant difference in comparison to NG2 on 9th, 18th, and 21th) day of wound healing; two-way ANOVA followed by Bonferroni test

Effect of treatment on wound healing using diabetic incision wound model

The results of breaking and tensile strength observed in different animal groups are shown in Table 9 and Figure 11a and b. From the results, it was observed that the optimized NG2 formulation showed significantly higher breaking strength and tensile strength in comparison to SBT seed oil-treated rats. The animals treated with standard showed significantly ($^aP < 0.05$) higher breaking and tensile strength as compared to the control but showed nonsignificant difference ($P > 0.05$) in comparison to NG2 formulation. From the results of *in vivo* wound-healing activity in excision and diabetic incision wound model, it was observed that the optimized NG2 wound NE gel formulation showed better wound-healing potential in comparison to pure SBT seed oil and also displayed similar activity w.r.t standard preparation used for the treatment of wounds.

Histological study

The histology of the wound tissue was evaluated both in excision wound model and in diabetic incision wound model after 21st day and 10th day

Table 7: Rate of wound contraction in different animal groups

Group (n=4)	3rd day	6th day	9th day	18th day	21st day
Control	29.1±5.43	44.9±3.76	57.1±3.36	68.9±3.41	81.6±2.97
Standard	69.5±2.33	83.3±4.84	89.8±6.18	99.9±0.1	100±0
SBT seed oil	54.3±8.04	62.5±7.14	72.3±6.88	83.9±3.21	100±0
NG2	64.8±6.17	74.6±4.36	85.4±5.21	96.9±1.42	100±0

NG2: Optimized nanoemulsion gel; SBT: Sea buckthorn

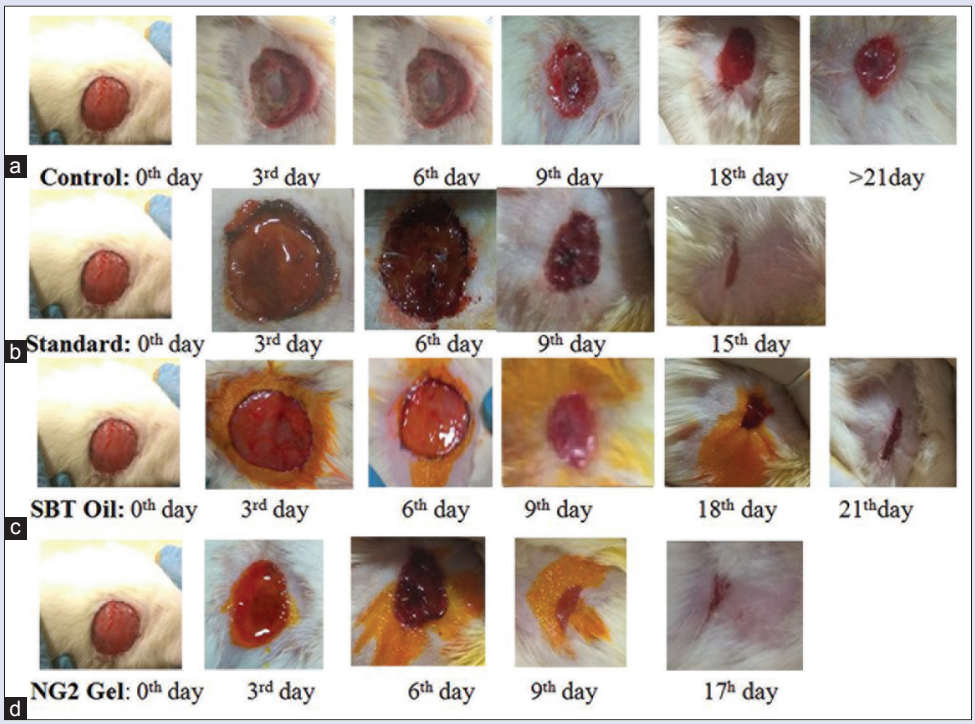

Figure 9: Photographical representation of wound contraction at different time intervals for (a) control, (b) standard, (c) sea buckthorn seed oil, (d) NG2

Table 8: Results for epithelialization time, hydroxyproline, and hexosamine content in different animal groups

Group (*n*=4)	Epithelialization day	Hydroxyproline (mg/g tissue)	Hexosamine (mg/100 mg tissue)
Control	24±1.29	30±4.5	0.38±0.056
Standard	15±2.50	87±7.1	0.88±0.053
SBT seed oil	20±1.02	51±5.4	0.53±0.055
NG2	17±1.70	76±5.6	0.76±0.073

NG2: Optimized nanoemulsion gel; SBT: Sea buckthorn

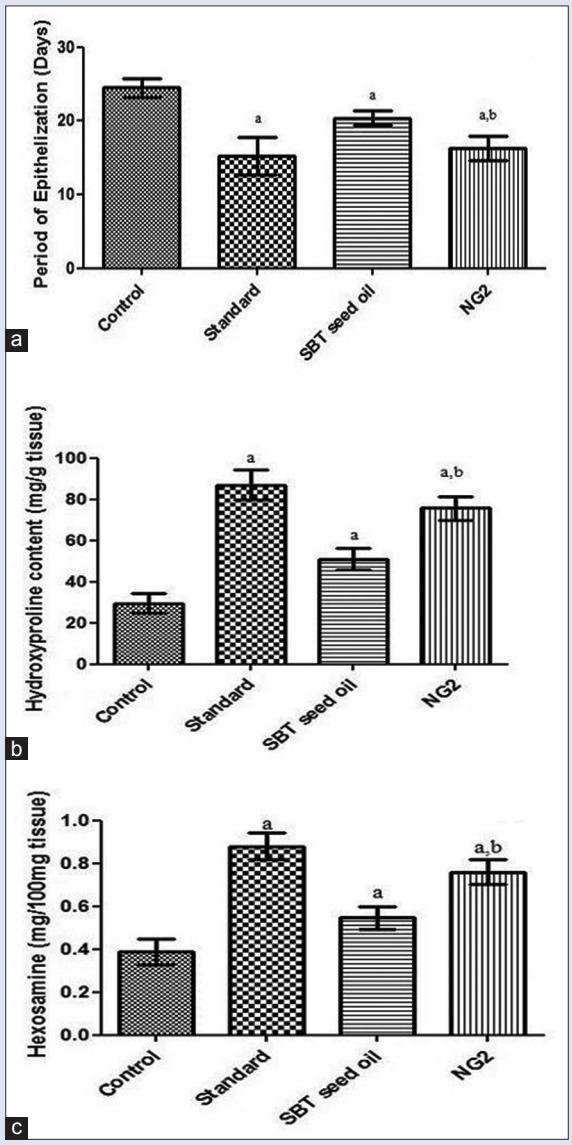

Figure 10: Results of (a) epithelialization time, (b) hydroxyproline, (c) hexosamine content for excision wound studied in rats. Values are means ± standard deviation (n = 4); $^{a}P < 0.05$ (all treatment show significant difference in comparison to control), $^{b}P < 0.05$ (NG2 show significant difference in comparison to sea buckthorn seed oil) and $P > 0.05$ (standard show nonsignificant difference in comparison to NG2); one-way ANOVA followed by Dunnett's test

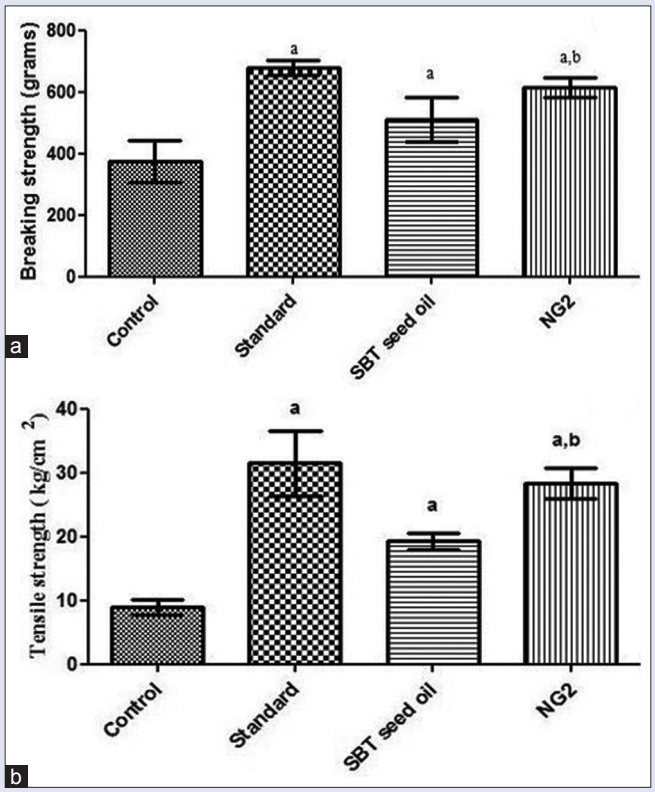

Figure 11: Results of (a) breaking strength (b) tensile strength for diabetic incision wound studied in rats. Values are means ± standard deviation (n = 4); $^{a}P < 0.05$ (all treatment show significant difference in comparison to control), $^{b}P < 0.05$ (NG2 show significant difference in comparison to sea buckthorn seed oil) and $P > 0.05$ (standard show nonsignificant difference in comparison to NG2); one-way ANOVA followed by Dunnett's test

of treatment, respectively. The results revealed evidence of suitability of the optimized NG2 formulation in enhancing the wound-healing activity. The granulation tissue in control-treated animals showed less epithelialization, few inflammatory cells, more aggregation of macrophages with less collagen fibers deposition, indicating incomplete healing of wounds. In standard-treated group, it was found that there was formation of collagen, fibroblasts, and proliferating blood capillaries. The tissue sample obtained from NG2-treated animal group contained fewer inflammatory cells, more collagen with hair follicles, and more epithelialization in comparison to SBT seed oil-treated group, which showed regenerative edges with less collagen formation and epithelialization process. The results of histological studies are shown in Figures 12 a-d and 13 a-d.

Ex vivo studies

Skin penetration studies

Microscopic studies demonstrated that there was decreased green fluorescence signal of FITC within cryotomed skin section treated with SBT seed oil, whereas significantly intense green fluorescence signal was observed in the skin section treated with NG2 formulation. The results from the confocal laser scanning microscopy confirmed that FITC-loaded NG2 formulation was able to penetrate more into skin layers as compared to FITC-loaded SBT seed oil. The results are shown in Figure 14.

Antibacterial and antifungal studies

The results of the zone of inhibition using the agar diffusion method obtained for different treatments including conventional topical preparation containing SBT seed oil, pure S_{mix}, SBT seed oil, and selected NE gel (NG2) are shown in Table 10. Similarly, the MIC values obtained from different treatments are shown in Table 11.

From the results of antibacterial and antifungal activities, it was observed that NG2 gel formulation showed higher zone of inhibition in comparison to conventional formulation containing 15% (w/w) of SBT seed oil for the bacterial strain, viz., *S. aureus, P. aeruginosa,* and *E. coli* as well as for yeast, i.e., *C. albicans*. The zone of inhibition of standard tetracycline and fluconazole was found to be higher in comparison to NG2 formulation. Pure S_{mix} and SBT seed oil did not show any antibacterial activity due to poor diffusion of the SBT seed oil into the agar plate. However, when the SBT seed oil was dispersed in the conventional formulation or NG2 gel, the antibacterial and antifungal activities were observed. The MIC values obtained from different treatment are shown in Table 11. It was observed

Table 9: Results of breaking and tensile strength after incision wound in rats

Group (*n*=4)	Breaking strength (g)	Tensile strength (kg/cm^2)
Control	374.7±67.2	9.005±1.17
Standard	679.9±23.9	31.48±5.13
SBT seed oil	511.7±72.03	19.30±1.29
NG2	614.5±32.12	28.35±2.35

NG2: Optimized nanoemulsion gel; SBT: Sea buckthorn

Table 10: Comparative antibacterial and antifungal activity

Treatment	Zone of inhibition (mm)			
	Bacterial strains			Fungal strain
	Staphylococcus aureus (mm)	*Escherichia coli* (mm)	*Pseudomonas aeruginosa* (mm)	*Candida albicans* (mm)
Conventional formulation + SBT seed oil	7±0.2	8±0.4	10±0.8	8±0.6
Pure S_{mix}	Nil	Nil	Nil	Nil
SBT seed oil	Nil	Nil	Nil	Nil
NG2	13±0.7	12±0.4	14±0.5	15±0.2
Tetracycline	26±0.7	27±0.6	27±0.5	-
Fluconazole	-	-	-	23±0.3

NG2: Optimized nanoemulsion gel; SBT: Sea buckthorn; S_{mix}: Surfactant/cosurfactant

Table 11: Minimum inhibitory concentration obtained from different treatment

Treatment	Bacterial strains			Fungal strain
	Staphylococcus aureus (µg/ml)	*Escherichia coli* (µg/ml)	*Pseudomonas aeruginosa* (µg/ml)	*Candida albicans* (µg/ml)
SBT oil	50	200	100	100
NG2	30	30	15	15
Tetracycline	<10	<10	<10	-
Fluconazole	-	-	-	<10

NG2: Optimized nanoemulsion gel; SBT: Sea buckthorn

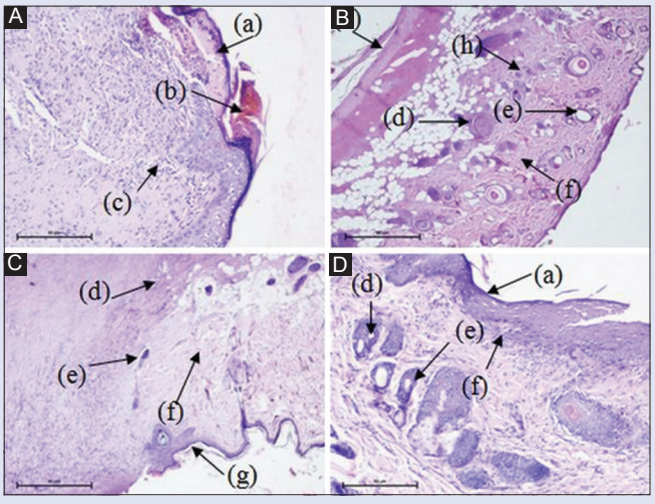

Figure 12: Results of histological studies in excision wound model (A) Control; (B) Standard; (C) SBT seed oil; (D) NG2 optimized formulation. Arrows pointing events during wound healing: (a): epithelialization; (b): inflammation; (c): macrophages; (d): fibroblasts; (e): collagen formation; (f): blood capillaries; (g): regenerative edges; (h): hair follicles

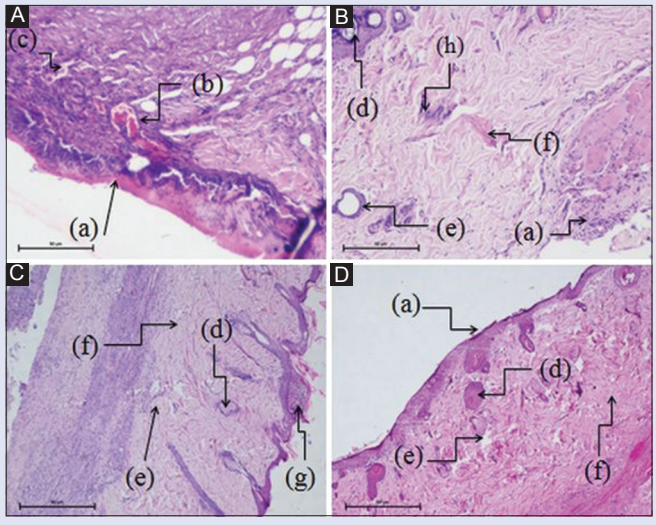

Figure 13: Results of histological studies in diabetic incision wound model (A) Control; (B) Standard; (C) SBT seed oil; (D) NG2 optimized formulation. Arrows pointing events during wound healing: (a): epithelialization; (b): inflammation; (c): macrophages; (d): fibroblasts; (e): collagen formation; (f): blood capillaries; (g): regenerative edges; (h): hair follicles

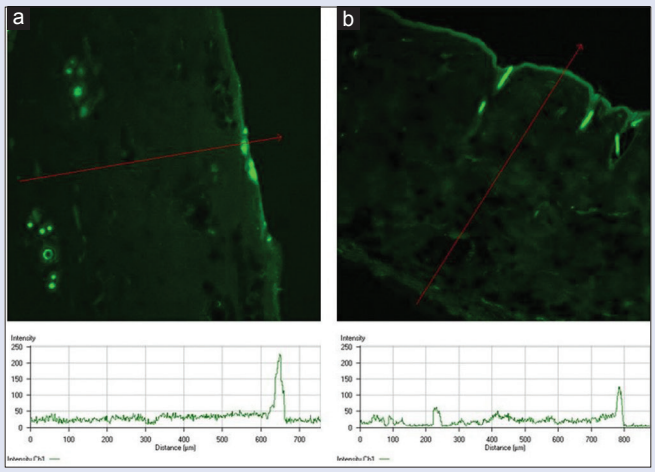

Figure 14: *Ex vivo* skin penetration studies using fluorescein isothiocyanate-loaded (a) NG2 and (b) sea buckthorn seed oil

that the MIC required to inhibit the selected bacterial and fungal strain was lower for NG2 formulation in comparison to SBT seed oil, indicating the improved effectiveness of the NE gel formulation in comparison to pure oil.

CONCLUSION

The NE gel formulation of SBT seed oil was successfully prepared and evaluated for various *in vitro, ex vivo,* and *in vivo* parameters. It was observed that the developed NE gel formulation had nanosized oil droplets, displayed improved viscosity and consistency, and enhanced applicability. The NE gel formulation improved the retention of the oil on the skin and increased its penetration across the skin. It was also observed that NE gel formulation showed better wound-healing, antibacterial, and antifungal activity in comparison to pure SBT seed oil.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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