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ORIGINAL ARTICLE

Evaluation of Wound Healing Potential of Ascorbic Acid, Castor Oil, and Gum Tragacanth Formulation in Murine Excisional Wound Model

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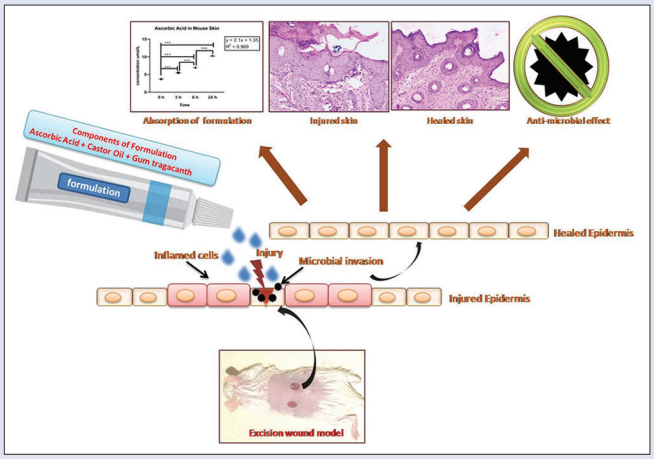
ABSTRACT

Background: Despite remarkable development in wound healing management, there is no ideal drug available to address diverse complexity in wound care. This necessitates the development of a comprehensive wound healing formulation. **Objectives:** The objective of this study was to evaluate the potential of ascorbic acid, castor oil, and gum tragacanth formulation, as an effective wound healing composition. **Materials and Methods:** A formulation containing ascorbic acid (0.5%), tragacanth gum (6%), and castor oil (25%) is prepared and evaluated for its efficacy on full-thickness mouse excisional wound model. Povidone-iodine was used as a positive control for comparative efficacy. Morphological and histopathological studies were carried out for understanding its wound healing potential. Antibacterial action was tested using disk diffusion method. Safety assessment for oral toxicity and eye and skin irritation was investigated in mouse and rabbit models, respectively. Permeability studies of formulation with excised skin have been evaluated by diffusion chamber, and its absorption in skin and serum was measured with high-performance liquid chromatography method. **Results:** Wound treated twice a day with formulation showed up to 24% recovery after 24 h and full skin restoration on the 9th day compared to the untreated wound. The morphological and histopathological examinations of wound indicated noteworthy improvement in recovery. Comparative efficacy showed 98% wound recovery in formulation-treated groups compared to 89% with commercially available povidone-iodine ointment after the 9th day. Permeability data clearly indicate enhanced skin uptake in time-dependent manner. The formulation is found to be safe and effective as demonstrated by antibacterial nature, permeability, and supportive safety studies. **Conclusion:** This study elucidates this formulation possesses strong wound healing potential.

Key words: Ascorbic acid, castor oil, excisional wound, histopathology, tragacanth gum

SUMMARY

- Ascorbic acid, tragacanth gum, and castor oil formulation enhances wound healing in murine excisional model
- Formulation greatly reduces wound size and accelerated collagen formation
- Safety studies showed the formulation as a safe composition
- Formulation also showed a strong antimicrobial property
- The formulation exhibited enhanced permeability for better efficacy.



Abbreviations used: OECD: Organisation for Economic Co-operation and Development; HPF: High-power field; ANOVA: Analysis of variance; SD: Standard deviation; HPLC: High-performance liquid chromatography; HE: Hematoxylin and eosin; MT: Masson's trichrome; ip: Intraperitoneal; AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care; ARRIVE: Animal Research Reporting of *in vivo* Experiments.

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INTRODUCTION

Skin serves as a barrier for chemical, physical, and biological agents.[1] A skin injury may be superficial or deep and can be categorized as acute or chronic depending on the condition of the wound.[2] The development of an effective wound healing drug is still a challenging task even after greater advancement in drug discovery tools. A number of plant extracts, chemical compounds, and various advance materials are under investigation for their ability to heal the injured tissue although only limited success has been achieved in this direction.[3-5] The drugs currently available in market for wound healing are antimicrobial in

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nature addressing infections in wound.[6,7] Recently published reports suggest growth factors, and chemokines could accelerate the wound healing with certain limitations.[8] Wound healing commonly involves hemostasis, inflammation, proliferation, and remodeling phases. The phases are well orchestrated and execute complex interplay of various mediators and molecules which govern the overall outcome of wound healing.[9,10] The interconnected participation of mediators poses a great challenge to develop a drug that accelerates all phases of healing. This necessitates a need to develop a formulation which can control different phases of wound healing apart from antimicrobial action. A formulation has been tested for wound healing properties in mouse excisional wound model consisting of three key components ascorbic acid, tragacanth gum, and castor oil. Ascorbic acid is a water-soluble antioxidant with diverse properties.[11] It acts as an immunomodulator and anti-inflammatory agent.[12] Vitamin C also acts as a cofactor for proline and lysine hydroxylases that stabilize the collagen molecule tertiary structure and promote collagen gene expression and collagen formation in wounded area.[11] Apart from these properties, ascorbic acid has been used in the topical skin cream against ultraviolet (UV) radiation, melanin, and scar formation.[13,14] Reports are available about oral uses of Vitamin C in fast recovery of surgical wounds.[15] Tragacanth is a complex mixture of polysaccharides, which yields upon hydrolysis D-galacturonic acid, D-galactopyranose, L-fucose (6-deoxy-L-galactose), D-xylopyranose, L-arabinofuranose, and a very small amount of L-rhamnose.[16] It is mostly believed that gum tragacanth consists of at least two major components: a water-swellable tragacanthic acid (about 60%–70%) and a water-soluble arabinogalactan.[17] It possesses antibacterial activity and has a wide range of applications in pharmaceutical and cosmetic industries.[18] Tragacanth plays an important role in wound healing as few studies have reported that it helps in homeostasis and modulates proliferation and maturation of keratinocyte.[16] Topical applications of tragacanth help in the acceleration of skin contraction and healing of full-thickness wound in animals.[19] Castor oil is unsaturated fatty acids which mainly consist of ricinoleic acid and linolenic acid. Castor oil possesses bactericidal properties and provides moist bedding to wound.[20] Few publications are available about castor oil and its formulation with other compounds in helping the wound healing in smaller as well as larger animals.[21] Considering the role of these individual compounds in wound healing, a formulation has been prepared for the evaluation of wound healing ability. Mouse excisional model was used for testing of the formulation. Various treatment groups of animals were divided for the evaluation of efficacy of the formulation. Standard parameters such as wound area and contraction were measured. Comparative efficacy studies were performed in various treated groups of the animals using povidone-iodine ointment. Histopathological assessment of skin wound was performed in various experimental animals. Safety studies such as skin irritation test and acute toxicity through oral and eye irritation were investigated for this formulation as per the Organisation for Economic Co-operation and Development (OECD) guidelines. Diffusion-chamber method was adopted for evaluation of permeability of formulation in the skin. Agar disk diffusion assay has been performed for the evaluation of antimicrobial properties of formulation.

MATERIALS AND METHODS

Chemicals

The active compounds include ascorbic acid (A92902-500G; purity 99%), tragacanth gum (G1128-500G; purity 60%–70%), and Castor oil (C9606-500ML; purity 99.9%) were purchased from Sigma-Aldrich, whereas other chemicals used in experiments were sodium chloride (S3014), potassium phosphate monobasic (P9791), sodium fluorescein (F6377-500G), potassium sorbate (40430-100MG), diazolidinyl urea (D5146-250G), Luria broth (L3522-250G), Luria broth with agar (L2897-250G), glycerin (G2289-500ML), formaldehyde (252549-500ML), xylenes (534056-500ML), absolute ethanol (34935-100ML), wax (327212-1KG), hematoxylin (1051752500), eosin (E4382-100G), Masson's trichrome (MT) staining kit (HT15-1KT), and antibiotic-antimycotic solution (100x) (A5955-100ML) also from Sigma-Aldrich. Ketamine (i.p.), Betadine™, and Megaheal™ were procured from the specified manufacturers.

Animal investigations

Mice

Specific pathogen free and 25.0–30.0 g body weight of strain "A" female mice were received from the Institute of Nuclear Medicine and Allied Sciences (INMAS) experimental animal facility at the age of 9 weeks. Six mice per cage were housed under 25°C ± 3°C temperature and relative humidity of 30%–70% in 12 h light/dark cycle with standard food and water were made available *ad libitum*. Mice were housed for a week for acclimatization.

Rabbit

Specific pathogen free and 2–2.5 kg body weight New Zealand white healthy male rabbits were received from the INMAS experimental animal facility at the age of 2.5 years. Rabbits were housed in individual cages with a standard pellet diet, water, and 12 h light/dark cycle under the supervision of a veterinarian. They were given seasonal fresh vegetables daily. Each group contained six rabbits. Each experiment was repeated twice.

Statement of ethics

All experimentation performed in animals were fully comply the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) international guidelines[22] and were approved by the Committee on the Ethics of Animal Experiments of the INMAS, Defence Research and Development Organisation (DRDO), Delhi, India (Institute Animal Ethics Committee number: INM/IAEC/2016/21 valid until February 23, 2017). Animal studies are reported in compliance with the Animal Research Reporting of *in vivo* Experiments guidelines (ARRIVE).[23] The humane endpoint was used for all the experimentation performed for the study.

Formulation preparation

A 6% (w/v) of tragacanth gum was mixed with 25% (v/v) of castor oil to form slurry at a temperature in the range of 25°C–30°C under stirring in the range of 30–40 rpm. Further, the preservatives (potassium sorbate and diazolidinyl urea in a 1:1 ratio) and glycerin moisturizers were added to the slurry at a temperature in the range of 25°C–30°C under stirring in the range of 30–40 rpm to obtain the second mixture. A 0.5% w/v aqueous solution of ascorbic acid was added dropwise at a temperature in the range of 25°C–30°C under stirring in the range of 30–40 rpm to the resulting mixture to obtain the wound healing composition.

Physicochemical evaluations of the formulation

Parameters such as pH, viscosity, spreadability, and extrudability were carried out as described elsewhere.[24]

Wound healing efficacy

Excisional wound model

The excisional wound studies were carried out on mouse model with a reproducible circular wound area of about 20 mm^2 being employed for the purpose of the tests. Excisional wound model was used for the

study of the rate of contraction of wound and epithelization. Before wound creation, animals were anesthetized with 80 mg/kg dose of ketamine (i.p.) and the dorsal body hairs were depilated by shaving. An impression was made on the dorsal thoracic region 1 cm away from the vertebral column using a 5 mm diameter skin biopsy punch (Acuderm Inc., Fort Lauderdale, FL, USA) was used for this purpose to produce full-thickness wound.

The wounds were treated by applying the formulation, and the skin recovery was recorded as a function of time. The formulation was applied in a fixed amount (0.05 g) over the wound periodically for a period of 9 days (twice a day), and the progress was recorded. An alternate excisional wound created on the same mouse was left untreated to observe the natural wound healing as an untreated control.

Measurement of the wound area

The progressive changes in wound area were monitored by a digital camera (Fuji, S20 Pro, Japan) on predetermined days, i.e., 0, 1, 2, 3, 6, and 9 days. Later on, the wound area was measured using ImageJ software (NIH, Bethesda, MD, USA).

Measurement of wound contraction

Wound contraction was calculated as a percentage of the reduction in original wound area size using the formula as reported elsewhere.[24] The area was calculated using the following formula:

Percentage wound contraction = (initial area of the wound − N^{th} day area of the wound)/initial area of the wound × 100

Histopathological examination

A specimen sample of tissue excised from the skin of each group of the mice at 6^{th} and 9^{th} days to evaluate histopathological alterations. Samples were fixed in 10% buffered formalin, processed and blocked with paraffin, and then sectioned into 5 µm and stained with hematoxylin and eosin (HE) and Masson's trichrome (MT). Photomicrographs were captured at a magnification of 40X. Sections were analyzed and scored as mild (+), moderate (++) and marked (+++) for epidermal or dermal re-modeling vis-a-vis necrosis/Ulcer, granulation tissue, fibroplasia, neovascularization and total defect area. At the end of the examination, all the wound healing processes were combined and staged for wound healing phases as inflammation, proliferation, and remodeling in all groups.

Comparative wound healing efficacy

The comparative analysis of the wound healing compositions in excisional wound was carried out on mouse model with a reproducible wound area of about 20 mm^2. The 20 mm^2 excisional wound was created using the protocol as described in the efficacy study of this manuscript. As a comparison in terms of efficacy, the commercially available povidone-iodine was compared with our formulation. The povidone-iodine and formulation were applied over the wound area twice a day, and the progress was recorded until the complete recovery. The progressive changes in wound area were captured with a digital camera at the 0, 1, 2, 3, 6, and 9 days. Later on, the wound area was measured using ImageJ software (NIH, Bethesda, MD, USA). The wound contraction was calculated as a percentage of the reduction in original wound area size using the formula as reported elsewhere.[24]

Skin permeability assay

Although the ascorbic acid has wound healing properties, the only limitation with ascorbic acid formulation is limited skin permeability. Hence, the current study was designed to see the skin permeability of ascorbic acid in the formulation. The full-thickness dorsal abdominal skin from mouse was excised. The skin was suspended in phosphate-buffered saline and stored at 4°C. The experiment was started within 24 h of excision of skin from the animal. The skin was cut into small circular pieces of 2.5 cm diameter and allowed to equilibrate for 1 h at 37°C before experimentation. The skin was mounted carefully onto the diffusion cell with the stratum corneum side facing the donor compartment. The receiver buffer was deaerated before use using a sonicator. A fixed amount (0.5 g) of formulation was applied to each donor compartment. Each receptor compartment was filled with 7 mL of isotonic phosphate buffer (pH 7.4). Permeation experiment was performed at 37°C and stirred at 600 rpm throughout the experiment. After the start of the experiment, 0.2 ml of assay sample was collected from the receptor compartment at 0.25, 0.5, 0.75, 1, 1.5, 2.5, 3, 4, 6, 8, 12, 18, 20, 24, 30, 42, and 48 h and was immediately replaced with an equal volume of fresh buffer equilibrated at 37°C. The obtained samples were centrifuged for 2 min at 14000 rpm, and the supernatant was immediately frozen at −80°C. The samples were analyzed on a liquid chromatographic system using an UV detector. Mobile phase A was 30 mM KH_2PO_4, pH 3.6, and mobile phase B was methanol. The two mobile phases were mixed as per gradient profile.

Absorption of formulation in excisional wound mice

Full-thickness excisional wound was created in the animals divided into different groups. This formulation was applied to the wound, and after 3, 6, and 24 h, animals were sacrificed. Animal serum and skin were used for the measurement of ascorbic acid by high-performance liquid chromatography.

Toxicity study

Acute dermal toxicity

All the experimental procedures performed in this experiment were strictly followed the OECD guidelines for toxicity study (Ref: OECD 404). For this experiment, we have followed the limit test experiment as per the following method. The limit test is a sequential test that uses a maximum of 6 animals. Twenty-four hours before the test (drug application), hair on the back and flanks of each rabbit were closely clipped exposing (approximately 6 cm^2 area) of skin. A 0.5 g sample of drug (formulation) in cream form was evenly applied to a small area (approximately 6 cm^2) of the closely clipped skin of each of the rabbits. Besides the test compound, two positive controls, i.e., povidone-iodine (0.5 g) and Megaheal™ cream (0.5 g), were also applied to the rabbit at two different areas (approximately 6 cm^2 each). The sites of applications were covered with a cotton gauze patch which was kept in contact with the skin by means of semi-occlusive dressing. A negative control, i.e., cotton gauze patch, was also applied to the same rabbit. The same procedures were carried out for all the experimental animals. At the termination of 4 h exposure period, the bandages/gauze was removed and treatment sites were cleaned with wet gauze to remove any residual test substance. The skin condition was scored for edema, erythema, and normal skin irritation at 1, 24, 48, 72 h, 7 day, and 14 day time points.

Acute oral toxicity

All the experimental procedures performed in this experiment were strictly followed the OECD guidelines for toxicity study (Ref: OECD 425). For this experiment, we have followed the limit test experiment as per the following method. The limit test is a sequential test that uses a maximum of 6 animals. A test dose of 2000 mg, or exceptionally 5000 mg/kg, may be used. Animals were made to fast for 3–4 h before administration of

the dose. The drug was administered using a suitable intubation cannula by gavage. The dose and the volume to be administered were calculated with respect to each animal's body weight. It was ensured that none of the administered doses came out after administration. First, mice were administered 2000 mg/kg body weight of the formulation. In this case, no mice died, so we moved forward for 5000 mg/kg body weight of formulations to all six mice as per the guidelines of OECD. The animals were kept under observation to check for mortality. The total body weight of animals were measured on daily basis up to 14 days whereas the morphological analysis of diffident organs and their weight were measured at 14 days after the euthanization of animals.

Acute eye irritation

All the experimental procedures performed in this experiment were strictly followed the OECD guidelines for toxicity study (Ref: OECD 405). A total of six rabbits per test group were subjected to a rigorous study of the ocular structures: cornea, iris, and conjunctiva. A volume of 0.1 ml of extract was instilled to the bottom of the right conjunctival sac, keeping eyelids together over the next 20 min. Both eyes of each animal were examined at the time and 24, 48, and 72 h after always by the same specialist. Corneal damage was determined in a dark room with the use of a solution of 2% sodium fluorescein, and physiological saline was used to remove excess solution from the instilled developer substance. Finally, an UV light was used for observation. Observations were made up to 5 days to assess the reversibility of the effects, and animals were weighed at the end of the study to compare variations in this parameter.

Antimicrobial screening of formulation

Agar disk diffusion testing is a cheap and simply performed method for screening of antimicrobial property of test formulations.[25] This technique relies on the principle that antibiotic inseminated disk, placed on agar previously inoculated with the bacterium, picks up moisture and the antibiotic diffuse radially outward through the agar medium producing an antibiotic concentration gradient. Antibiotic disc made up of Whatman filter was dipped in three individual components of formulation. Antibiotic antimycotic solution containing penicillin, streptomycin, and amphotericin B was used as a positive control. All Petri dishes were incubated overnight in incubator at 37°C. After incubation, plates were observed for clear zone.

Statistical analysis

The data are presented as the mean ± standard deviation of 6 animals from each group for the efficacy of the formulation, comparative efficacy (povidone-iodine), histopathological analysis, skin irritation, oral toxicity and skin permeability assays, absorption of formulation, acute eye irritation, and antimicrobial screening. Comparisons were made among different groups on the basis of selected endpoints. The data were analyzed using one-way analysis of variance, and multiple comparisons among different groups were performed by applying the Bonferroni *t*-test. A probability of <5% was considered significant.

RESULTS

Wound healing efficacy in excisional wound model

The wound area (mm^2) in all animal groups was measured on 0, 1, 2, 3, 6, and 9 days. The untreated group showed the wound area 20 ± 0.16, 17.30 ± 0.14, 12.95 ± 0.11, 8.80 ± 0.11, 6.08 ± 0.15, and 2.92 ± 0.10 mm^2 on respective days [Figure 1]. Whereas, animals treated with topical application of the formulation resulted in highly significant ($P < 0.001$) decrease in wound area compared to untreated group of animals (19.35 ± 0.12, 13.84 ± 0.14, 8.09 ± 0.13, 5.58 ± 0.16, 2.11 ± 0.09, and 0.15 ± 0.08 mm^2 on 0. 1, 2, 3, 6, and 9 days, respectively), and the wound area was completely healed by the 9th day [Figure 1]. Similarly, the percentage reduction in wound area was calculated 24 h after wound creation in all animal groups at different time intervals, i.e., 1, 2, 3, 6, and 9 days [Figure 1]. The topical application with formulation showed a significant increase in percentage reduction in wound area in comparison to untreated group animals (untreated vs. formulation: 13.48 ± 0.6 vs. 28.47 ± 0.56, 35.21 ± 0.49 vs. 58.19 ± 0.44, 55.98 ± 0.56 vs. 71.16 ± 0.93, 69.57 ± 0.61 vs. 89.07 ± 0.50, and 85.41 ± 0.53 vs. 99.20 ± 0.44% on 1, 2, 3, 6, and 9 days, respectively) [Figure 1].

Comparative wound healing efficacy with povidone-iodine

A comparative efficacy study was designed by taking the commercially available iodine formulations (povidone-iodine) with the formulation. Both were applied twice daily topically to the wound area. The wound area was measured in mm^2 to compare the efficacy among the formulation- and povidone-iodine-treated animals on 0, 1, 2, 3, 6, and 9 days [Figure 2]. The formulation showed a significant decrease in wound area when compared with the povidone-iodine at noted time points (formulation vs. povidone-iodine: 19.63 ± 0.13 vs. 19.75 ± 0.16, 14.88 ± 0.17 vs. 16.32 ± 0.14, 9 ± 0.16 vs. 11.02 ± 0.11, 6.16 ± 0.17 vs. 7.65 ± 0.11, 2.59 ± 0.10 vs. 5.43 ± 0.15, and 0.29 ± 0.12 vs. 2.02 ± 0.10 on 0, 1, 2, 3, 6, and 9 days, respectively) [Figure 2]. The commercially available iodine formulation (povidone-iodine)-treated animals showed the percentage of wound area reduction as 18.42% ± 0.14%, 44.87% ± 0.10%, 61.74% ± 0.11%, 72.85% ± 0.15%, and 89.87% ± 0.10% reduction in wound area on 1, 2, 3, 6, and 9 days, respectively [Figure 2]. However, the formulation shows a significant increase in percentage reduction when compared with the povidone-iodine. The formulation showed 24.21% ± 0.17%, 54.15% ± 0.16%, 68.62% ± 0.16%, 86.82% ± 0.10%, and 98.52% ± 0.12% reduction in wound area on 1, 2, 3, 6, and 9 days, respectively [Figure 2]. The results clearly indicate that the formulation is better in efficacy compared to the commercially available povidone-iodine formulation.

Histopathological examination

Measurement of inflammation, proliferation, and epithelization in wound area were measured using various histopathological parameters such as macrophages, necrosis/ulcer, granulation tissue, fibroplasia, neovascularization, and total defect area in control and treated wound at 6th and 9th day time points [Figure 3 and Table 1]. Measurements of these parameters were also performed in normal skin sample as a standard. Significant ($P < 0.001$) changes in all the parameters were seen in the case of wound control samples when compared with the normal skin sample [Figure 3 and Table 1]. However, topical application of the formulation significantly ($P < 0.001$) reduces these parameters when compared with the wound control and takes it to almost normal level by the 9th day [Figure 3 and Table 1].

Skin permeability of ascorbic acid

The skin permeability assay as measured by diffusion-chamber method clearly exhibited time-dependent enhancement in ascorbic acid levels, as shown in Figure 4.

Absorption of ascorbic acid in wound area

The standard controls run for ascorbic acid showed linearity in concentration manner. The serum and skin samples of the animals have exhibited a significant difference in ascorbic acid concentrations studied at 3, 6, and 24 h. The serum level of ascorbic acid was found to be constant and found similar to the control at each time point, whereas in

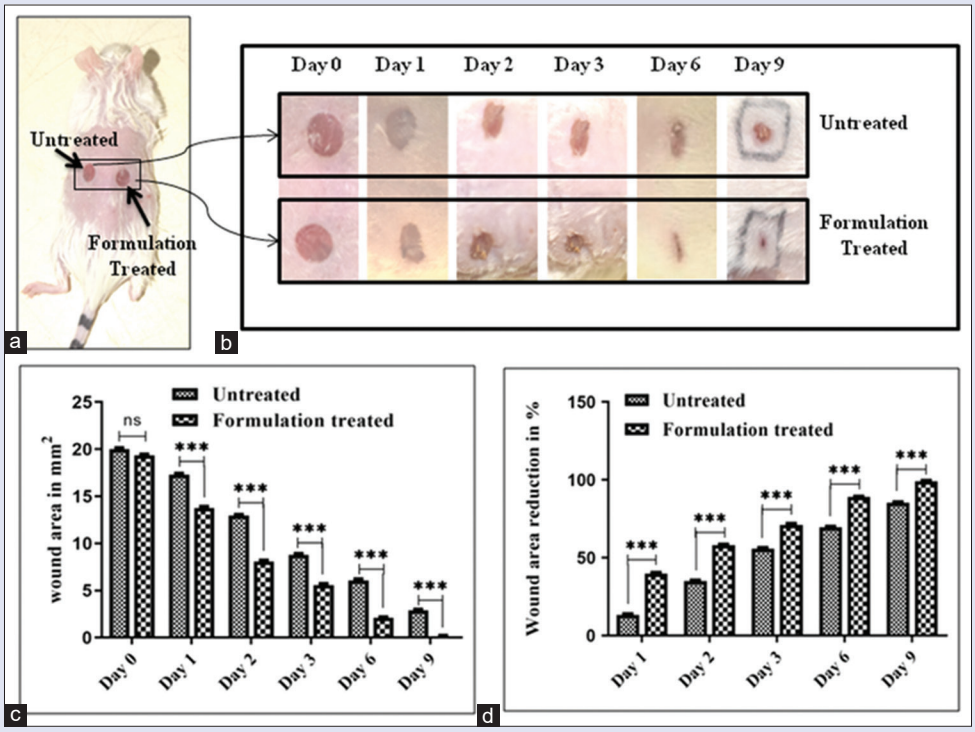

Figure 1: Excisional wound healing ability of formulation in mouse model: (a) Excisional wound in the mouse. (b) Wound healing studies with formulation at different time intervals. (c) Wound area in mm^2 in mouse model at the selected time points. (d) Percentage reduction in wound area in untreated and formulation treated groups at the noted time points. Each experiment consisted of 6 animals and was repeated twice. The bars represent the mean ± standard deviation of both experiments. A value of $P < 0.05$ is considered statistically significant. ***: $P < 0.001$, ns: Not significant ($P > 0.05$)

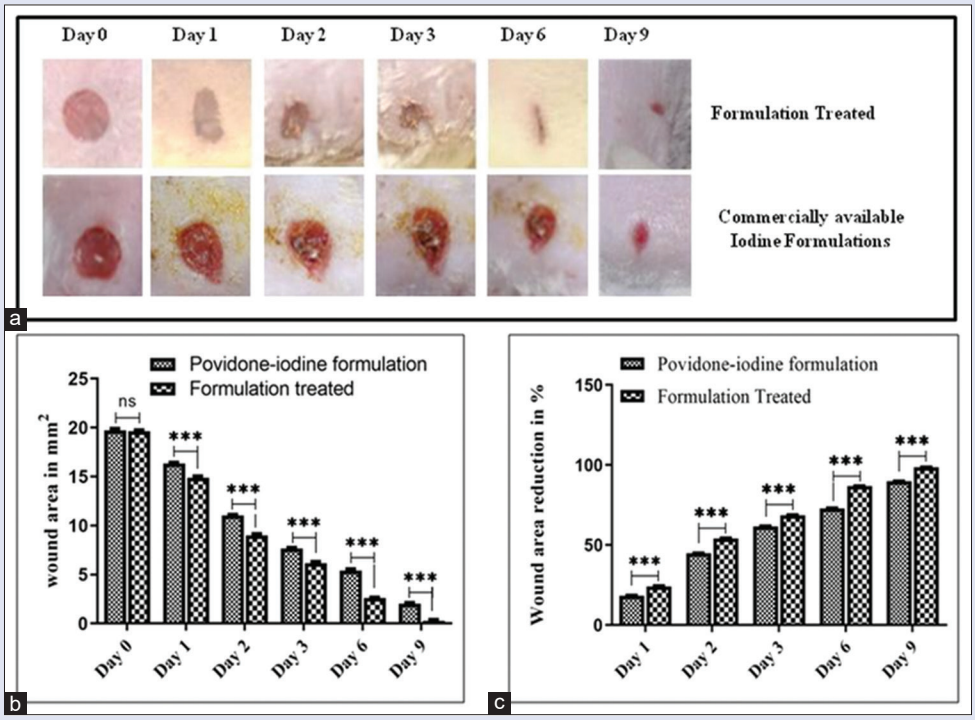

Figure 2: Comparative wound healing ability of formulation and povidone-iodine in mouse model: (a) Excisional wound in different treatment groups at different time intervals. (b and c) The graphs show wound area in mm^2 and percentage reduction in wound area of formulation and povidone-iodine in mouse model at the noted times. Each experiment consisted of 6 animals and was repeated twice. The bars represent the mean ± standard deviation of both experiments. A value of $P < 0.05$ is considered statistically significant. ***: $P < 0.001$, ns: Not significant ($P > 0.05$)

Table 1: Histopathological analysis of skin samples of different treatment groups

Parameters	Normal skin	Wound control (6 days)	Wound with ascorbate formulation (6 days)	Wound control (9 days)	Wound with ascorbate formulation (9 days)
Size of tissue (cm)	0.3×0.2×0.2	0.6×0.5×0.3	0.6×0.5×0.1	0.6×0.5×0.2	0.5×0.4×0.3
Macrophages	Not identified	Present - 5-10/HPF	Present - Rare, 1-5/HPF	Present, 5-10/HPF	Not identified
Necrosis/ulcer	Nil	Present with dense suppurative exudate	Present with surface fibrinopurulent exudate	Focal crusting present on skin surface	Not identified
Granulation tissue	Nil	++	++	++	+++
Fibroplasia	Nil	++	++	++	+++
Neovascularization	Nil	+	++	++	++
Total defect area (mm)	Nil	4×2	3×2	3×2	2×2

+: Mild; ++: Moderate; +++: Marked; HPF: High-power field

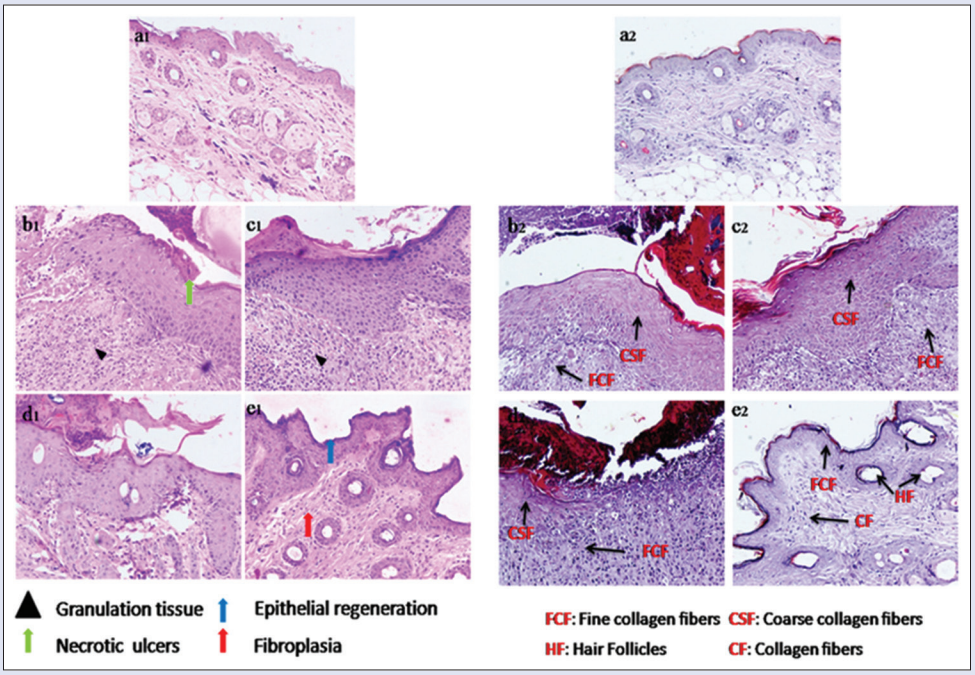

Figure 3: Histopathological analysis of mouse skin samples: Representative image of H and E skin samples (a1) normal, (b1) untreated at the 6th day, (c1) formulation treated at the 6th day, (d1) Untreated at the 9th day, and (e1) formulation treated at the 9th day. Representative image of Masson's trichrome of skin samples (a2) normal, (b2) untreated at the 6th day, (c2) formulation treated at the 6th day, (d2) untreated at the 9th day, and (e2) formulation treated at the 9th day. Each experiment consisted of 6 animals and was repeated twice. A value of $P < 0.05$ is considered statistically significant

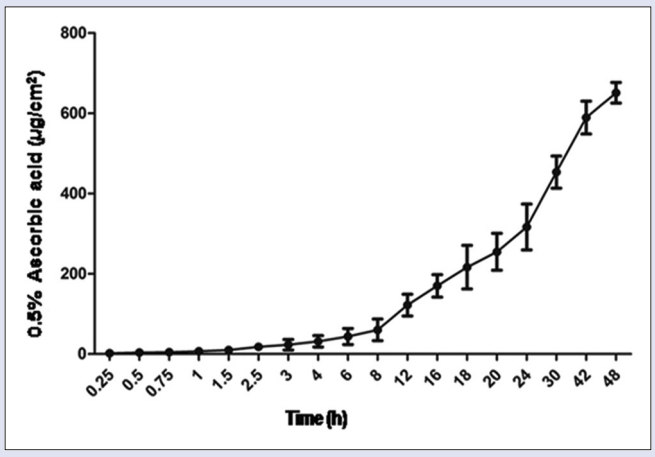

Figure 4: Skin permeability of ascorbic acid: The results showed skin permeability of 0.5% of ascorbic acid increased with due course of time. Each experiment consisted of 6 animals and was repeated twice. A value of $P < 0.05$ is considered statistically significant

case of skin samples, the level of ascorbic acid showed time-dependent enhancement from 3.7 to 10.2 µmol/L in 24 h [Figure 5].

Toxicity study of formulation

Acute oral toxicity

The acute oral toxicity studies with formulation showed that it is safe as no mortality was observed even at 5 g/kg body weight. The animals were kept under observation to check for mortality up to 14 days. No significant abnormality was observed in body weight, organ weight and organ morphology [Figures 6 and 7].

Acute dermal toxicity

The application of formulation to New Zealand white rabbit skin revealed no appreciable clinical signs throughout the observation period of 14 days, and there was no mortality seen. In the dermal toxicity test, no erythema, eschar, edema, or any other reactions were observed in intact sites of all rabbits which were treated with either formulation or distilled water [Table 2]. There was no significant change in body weight of the rabbit from the application of formulation during the observation

Table 2: Summary of clinical signs of dermal toxicity in New Zealand white rabbits

Sex	Drug	Dosing phase	Test site		
			Erythema and eschar	Edema	Skin irritation
Male	Control	1 day to ≤2 weeks	0	0	0
	Povidone-iodine formulation		0	0	0
	Ascorbate formulation		0	0	0
	Megaheal™		0	0	0

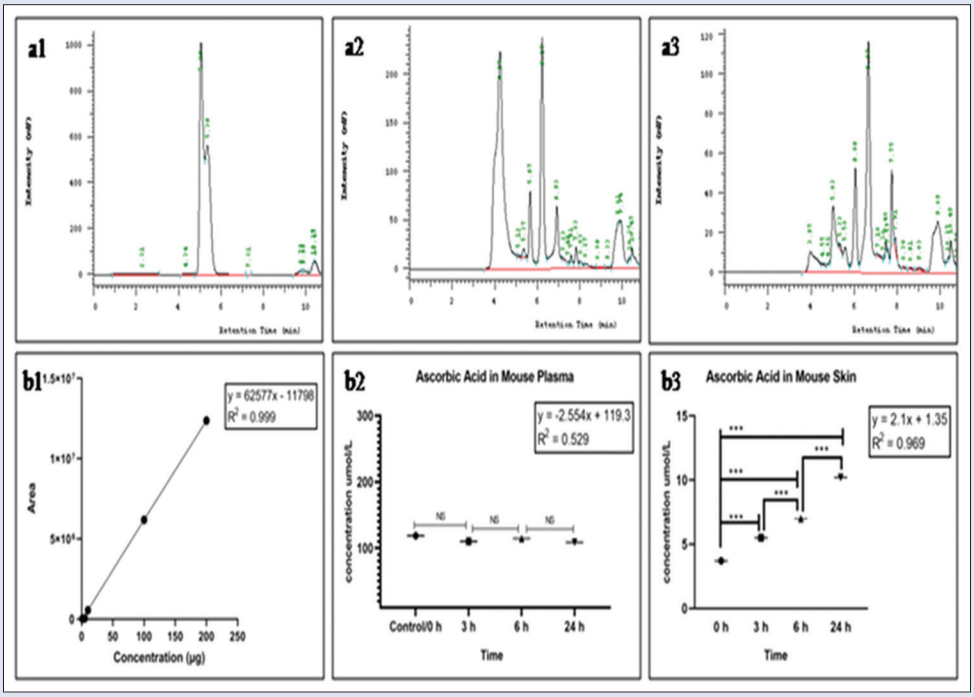

Figure 5: Ascorbic acid absorption in mouse skin and plasma: (a1 and b1) Ascorbic acid chromatogram and linearity. (a2 and b2) Representative chromatogram and serum concentration of ascorbic acid. (a3 and b3) Representative chromatogram and ascorbic acid measurements in wound skin at selected time points. Each experiment consisted of 6 animals and was repeated twice. A value of $P < 0.05$ is considered statistically significant. ***: $P < 0.001$, ns: Not significant ($P > 0.05$)

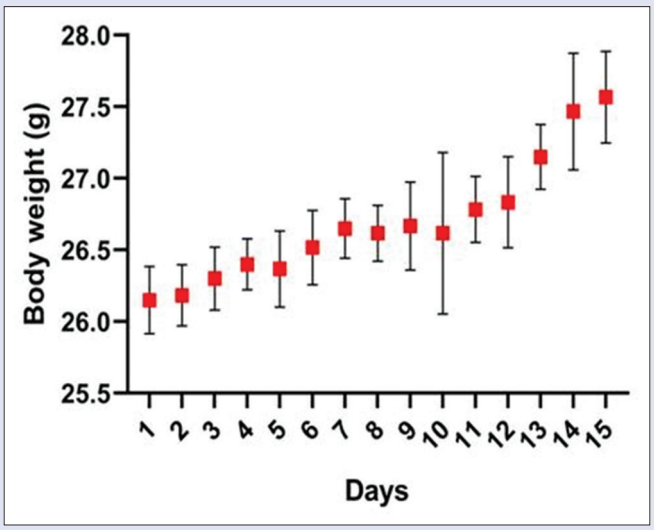

Figure 6: Body weight of animals orally administered with formulation: Measurement of body weight of mice at various time points that were orally administered 5000 mg/kg of formulation. Each experiment consisted of 6 animals and was repeated twice. A value of $P < 0.05$ is considered statistically significant

period. Furthermore, there were no prominent gross lesions observed in all animals [Figure 8].

Eye irritation study

Results obtained with the eye irritation test showed no severe ocular effect. No observable conjunctival redness or edema was seen in all six rabbits at 1, 24, 48, and 72 h after application. Iridal congestion was not observed in all six rabbits throughout the 3-day observation period. On the other hand, no ocular lesions were observed even after 7 days of formulation application. No other clinical signs were observed during the period of eye irritation study.

Antimicrobial property of formulation

In disk diffusion assay, gum tragacanth and castor oil have shown antimicrobial properties by forming zone of inhibition against microbial load [Table 3]. However, the absence of zone of microbial inhibition was observed in the ascorbic acid-treated dishes. Positive control antibiotic antimycotic solution exhibited clear zone.

DISCUSSION

Acute or chronic wound etiology differs significantly that imparts a greater challenge in their treatment modalities. The prevalence of chronic wounds is very high even after the advancement in treatment

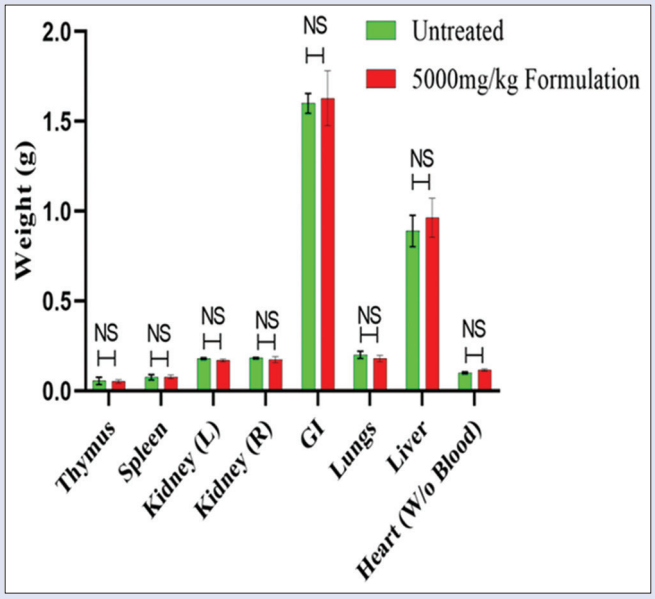

Figure 7: Organs weight of mice orally given formulation: Data shown are the comparison of organ weight of untreated with formulation treated animals. Each experiment consisted of 6 animals and was repeated twice. A value of $P < 0.05$ is considered statistically significant

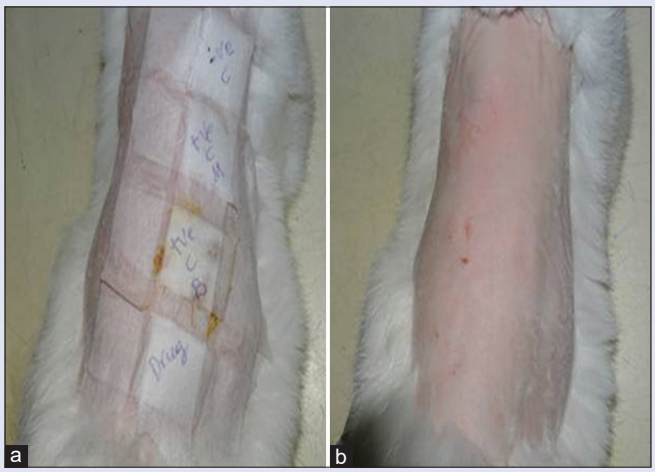

Figure 8: Comparative study of acute dermal toxicity of formulation with Povidone-iodine and Megaheal™ in rabbit: (a) Representative image of negative control, positive control (Povidone-iodine and Megaheal™) and formulation treated skin of rabbit at the 1st day. (b) Representative image of rabbit's skin after 14-day treatment. No prominent gross lesions observed in animal skin. Each experiment consisted of 6 animals and was repeated twice. A value of $P < 0.05$ is considered statistically significant

Table 3: Antibacterial activity by zone of inhibition assay

Micro organism	Antibiotic antimycotic solution	Tragacanth gum	Castor oil	Ascorbic acid
Staphylococcus aureus	+++	+++	++	-

++: Moderate sensitive; +++: Highly sensitive; –: Absences of zone of inhibition/ resistant

modalities. Besides, wound fatalities are common in war, accidents as well as in operative care. Currently, available formulations such as Betadine, Acticoat 7, Silverseal, Urgotul SSD, Manuka IG, and Algivon are being used in the treatment of a wound based mainly on their antiseptic and antimicrobial properties.[26-32] The antimicrobial activity is one of the important parameters for wound care, and a large number of formulations are introduced in the market to overcome the microbial infestation in the wound. However, on the other hand, wound regeneration and repair are equally important parameters that need to be addressed.

The wound healing process is a complex cascades of biochemical events that are set in motion as part of wound healing.[33,34] The typical phases include blood clotting (hemostasis), inflammation, tissue growth (proliferation), and tissue remodeling (maturation).[35,36] This necessitates the development of a formulation that can address various aspects at different stages of the wound healing process.

A proof-of-concept study on the local therapeutic activity in the treatment of wounds has been investigated in mouse model with topical application of this formulation along with comparative efficacy study with commercially available povidone-iodine formulation. These experiments clearly illustrated the difference in wound area and its percentage reduction. Contraction of wound is an important step in wound healing as it helps in the restoration of damaged tissues back to healthy condition and it starts with fibroblastic phase in which wound area shrinks.[37] The sharp decrease in wound area with high significant value ($P < 0.001$) was seen in untreated versus formulation-treated wound from the 2nd day of treatment, i.e., 17.30 ± 0.14–13.84 ± 0.14 mm^2 and percentage reduction of 35.21 ± 0.49 versus 58.19 ± 0.44 and 6.08 ± 0.15–2.11 ± 0.09 mm^2 and percentage reduction of 69.57 ± 0.61 versus 89.07 ± 0.50% on the 6th day, and this increased to 99% in treatment group on the 9th day, as shown in Figure 1. This clearly shows the higher efficacy potential of formulation.

For further confirmation of the efficacy of designed product, histopathological analysis of wound skin was done which revealed the restoration of cellular structure of damaged tissue. A histological result at different time intervals using HE and MT was done. It revealed a difference of untreated versus treated tissue samples at the 6th and 9th day after treatment at cellular level. According to histological analysis that was done under high-power field (HPF), macrophages were not identified in normal as well as in treated tissues after the 9th day in granulation tissue, and there was a unique relation observed with decrease in macrophage number whereas increase in granulation tissue. It was also found that wound treated with formulation contained more collagen tissue with increased angiogenesis, epithelialization, and reduced scar tissue. Necrosis was present with dense suppurative exudate in untreated wound which changed to fibrin a purulent exudate in treated wound with formulation on the 6th day and became clearly absent on the 9th day with almost clear boundary of epidermis layer.

Overall, formulation-treated groups have shown a significant wound healing activity as observed in marked changes in granulation, collagen formation, neovascularization, and epithelization than untreated wound skin on the 9th day. The histological results exhibited the role of formulation in all the phases of wound healing. It showed an anti-inflammatory potential by indicating lower number of macrophages and promoted repair and regeneration as observed in higher neovascularization, fibroplasias, and epithelization in treated animals when compared to control groups. Hair follicle formation at the wound site in treated groups further confirms its wound healing potential as it was not observed in wound controls [Table 1].

During the proliferative phase of wound healing, fibroblasts play an important role by promoting collagen fiber synthesis. The collagen synthesis mainly depends on the availability of dietary nutrients such

Table 4: Proposed mechanism of action of wound healing activity exhibited by formulation

Ascorbic acid	Castor oil	Tragacanth gum
Anti-Inflammation	Chemical debridement	Hydrogel formation
Collagen formation initiation	Endothelial cells: Angiogenesis	Extracellular matrix regeneration and cell migration
		Wound contraction and scar formation

as Vitamin C.[38] The most essential role of ascorbic acid is due to its influence on collagen synthesis and angiogenesis process. It has also been shown that scorbutic individuals suffer a delay in healing and tend to have weaker scar integrity and abnormal capillary formation, confirming a consensus opinion that ascorbic acid is the only true vitamin deficiency to impair wound healing.[39,40]

Ascorbic acid is one of the principal components in our formulation which is highly labile due to its easy oxidation by the enzyme dehydroascorbate reductase.[41] Its continuous availability at the site of the wound is extremely necessary. Our results show that there is an enhanced permeability of ascorbic acid with due course of time using the formulation [Figure 4]. Serum concentration of ascorbic acid did not change much which may be considered as a positive aspect for a topical cream.

The stable concentration of ascorbic acid in skin thereby helps in enhanced repair and regeneration of damaged skin in formulation-treated animals. Although it is very difficult to stabilize ascorbic acid, only a few attempts were able to develop a stable topical formulation.[42] Similarly, our formulation is able to provide stable skin concentration with enhanced ascorbic acid permeability**.** According to the various reports, available ascorbic acid is recognized potent antioxidant and collagen enhancement properties.[11,12,43-46] It is also shown to have significant skincare value and provides photoprotection, skin lightening, wound healing, and skin rejuvenation properties.[47,48] Similarly, the tragacanth gum has myofibroblast contraction, coagulation of surface proteins, and preventing wound infection properties, which helps in accelerating the wound healing process.[49] Furthermore, castor oil is also known to have natural antibacterial, antifungal, and antimicrobial properties.[50-52] The wound healing properties of the formulation could be based on various individual properties of ascorbic acid, tragacanth, and castor oil which have been represented in Table 4.

The toxicological parameters are the mandatory studies for drug development process as safety is equally important as efficacy.[53] The safety parameters such as oral toxicity, dermal toxicity, eye irritation, and body and organ weight measurement studies were conducted with formulation as per the OECD guidelines. All these parameters have indicated the safe nature of this formulation.

The antibacterial testing method used in this research was the agar diffusion method for evaluation of the inhibition zone against bacteria around the paper disc. Inhibitory zone diameter has increased in castor oil and gum tragacanth treatments. This finding is in corroboration with earlier reports where both compounds have shown antimicrobial properties.[54] The ascorbic acid did not inhibit the microbial growth in our experiments. There is non-availability of publication data for its antimicrobial nature. The results clearly showed the antimicrobial action of formulation.

CONCLUSION

Our overall experimental results support the potential role of our formulation in wound healing. It quickly heals the wound area by possibly participating in different phases of wound healing, i.e., inflammatory, proliferative, and regenerative phases by reducing inflammation, infection, and accelerating repair and regeneration of skin tissue in the wound area as evidenced by our studies. Further studies are needed to confirm the role of individual components of formulation in conferring its role in wound healing. A large-scale study is in process to understand the mechanism of action using different molecular parameters.

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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