Wound Medicine 26 (2019) 100168

Contents lists available at ScienceDirect

Wound Medicine

journal homepage: www.elsevier.com/locate/wndm

Original research article

A topical ointment formulation containing leaves extract of *Aegialitis rotundifolia* Roxb., accelerates excision, incision and burn wound healing in rats

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ARTICLE INFO

Keywords:
Aegialitis rotundifolia
Mangrove
Ointment formulation
Wound healing
In-vivo antioxidant activity
Burn wound

ABSTRACT

Background: *Aegialitis rotundifolia* Roxb., is a small mangrove tree or shrub used traditionally as a potent cure for pain arising from various injuries. Presently, there is a single scientific report on the wound healing property of this mangrove species which has been performed *in-vitro* but there is no *in vivo* scientific evidence of the wound healing properties. Further, few reports have claimed that reduction of pain could accelerate wound healing process and thus, the present work deals with the development of a topical ointment formulation incorporated with *Aegialitis rotundifolia* Roxb., ethanolic leaves extract (ARELE) which could potentially accelerate healing of excision, incision and burn wound models in Wistar rats.

Methods: Topical ointment containing ARELE was first tested for their stability (90 days) and possible dermal toxicity using standard procedures. In the excision wound model, parameters such as *in-vivo* antioxidant, acute inflammatory marker (myeloperoxidase), wound microbial load, connective tissue parameters, and histopathology of healed skin were performed. Incision and burn (thermal and chemical) wounds were conducted following the standard methods.

Results: The ointment formulations were found to be stable and dermatologically non-toxic. In the excision wound model, a significant increase in percent wound contraction was observed for ARELE ointment treated groups which are substantiated by strong *in-vivo* antioxidant activity, increased collagen formation, almost normal skin histology, and reduced myeloperoxidase and microbial colonies. Strong wound breaking strength was observed in incision wound repair and a significant increase in percent wound closure in both thermal and chemical burn wound model was recorded for ARELE ointment treated groups.

Conclusion: Therefore, the topical application of ARELE ointment formulations showed remarkable excision, incision and burn wound healing in Wistar rats thus showing its potential as a promising wound healing agent.

1. Introduction

Wound can be defined as a disruption in the anatomical and cellular architecture and physiological function of tissue which includes skin, mucous membrane, deeply lying tissues or surface of internal organs ranging from an incision, laceration, puncture, abrasion, and closed wounds such as hematoma, contusion, and crush [1]. Upon disruption of such tissues, intricate and complex processes are initiated to repair the damaged tissue which involves a complex cascade of cellular events resulting in reconstitution, resurfacing and restoration of the tensile strength of the injured skin [2]. Burns are wound injuries to tissues caused by excessive heat, electricity, radioactivity or corrosive chemicals that destroy protein in the exposed cells. They are considered one of the most distressing injuries which gives rise to many physical, mental and serious complications if they are not treated at the right time [3].

The healing of wound is a dynamic process which can be divided into three phases like inflammatory phase, proliferation phase, and the maturation phase. The inflammatory phase is the body's natural response to injury, where the blood vessels in the wound bed contract and a clot is formed after initial wounding. Once haemostasis has been achieved, blood vessels then dilate to allow essential cells, antibodies, white blood cells, growth factors, enzymes and nutrients to reach the wounded area. It is at this stage that the characteristic signs of inflammation can be seen such as erythema, heat, oedema, pain and functional disturbances. During proliferation, the wound is rebuilt with

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https://doi.org/10.1016/j.wndm.2019.100168
Received 17 July 2018; Received in revised form 22 April 2019; Accepted 22 July 2019
Available online 26 July 2019

new granulation tissue which is comprised of collagen and extracellular matrix and into which a new network of blood vessels develops, a process known as angiogenesis. Epithelial cells finally resurface the wound, a process known as epithelialisation. Maturation is the final phase and occurs once the wound has closed. This phase involves the remodelling of collagen [4].

Wound care is considered a biomedical burden and the promotion of wound healing has become a key objective of medical treatment, especially in the developing countries [5]. The skin plays a vital role in physiological functions such as perspiration, heat and pain sensation and thus any damage caused to the skin may lead to severe consequences such as pain and trauma, ill health and even death in severe cases [6]. Thus, there is an urgent need to develop effective and low cost remedies which would minimise the physical and financial burden upon human society. In today's scenario, the synthetic drugs used in the treatment of wounds are considered less effective and various toxic side effects are reported which prompted many scientists to develop plant based medicines for wound healing which would provide a much more effective treatment with minimal side effects at low cost [7,8].

Aegialitis rotundifolia Roxb., (Plumbaginaceae) is a small mangrove tree or shrub which usually grows up to a height of 2–3 m and is available in shorelines of the Andaman Sea and the Bay of Bengal and are endemic to the coastal parts of South Asia. In Orissa it is locally known as Banrua [9,10]. This mangrove species is reported to produce one of the best quality honey [11]. Traditionally the leaf is used in the treatment of various injuries accompanied by pain and inflammation and is locally utilized as an anti-ache agent [12]. Further, the leaf of the plant is pounded with oil to make a paste which acts as an antidote for insect bites [13]. According to the present literature, there have been very few scientific reports of pharmacological screening conducted such as analgesic, antipyretic [13], *in-vitro* antioxidant [14], antimicrobial [15,16], anti-inflammatory [13,17], *in-vitro* thrombolytic activity, antibacterial [17], and anticancer activity [18]. Previously, we have reported the presence of gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, rutin, coumarin, and quercetin by performing quantitative high performance liquid chromatography (HPLC) analysis and an organosilicon compound, (-)-spiro[1-[(tert-Butyldimethylsiloxy)methyl]-3,5,8-trimethyl-bicyclo[4.3.0]non-2-en-5,7-diol-4,1′-cyclopropane] was detected in gas chromatography-mass spectrometry (GC–MS) analysis as the most abundantly found compound [10]. Recently, we have reported the wound healing properties of this mangrove species which was performed *in-vitro* on human dermal fibroblast (HDF) cell migration using wound scratch model [19]. According to these finding and hypotheses, the present work deals with the detailed study of the wound healing properties of *Aegialitis rotundifolia* leaves in the form of a topical ointment formulation which has still not been scientifically studied *in vivo*. The wound healing activity has been investigated using three different models *viz.*, excision, incision and burn (thermal and chemical) wound models in Wistar albino rats.

2. Materials and methods

2.1. Chemicals and reagents

Ethanol 99.9% was procured from Changshu Hongsheng Fine Chemicals Co. Ltd., China. All major chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck Specialities Pvt. Ltd., (Mumbai, India). The solvents used were of high purity and HPLC grade. All other chemicals and reagents used in the whole study were of analytical grade.

2.2. Collection and authentication of plant materials

The fresh leaves of *Aegialitis rotundifolia* Roxb., was collected from healthy fully-grown plants in the month of May 2016 from Bichitrapur mangrove located in Kharibil, Orissa, India (21°34′54.0″N - 87°25′25.4″E). The plant materials were then authenticated from Botanical Survey of India (BSI), Central National Herbarium, Botanic Garden, Howrah, West Bengal, India and was assigned with a Voucher no. CNH/Tech.II/2016/11a and specimen no. DG-01.

2.3. Preparation of extracts

The collected plant materials were gently washed in tap water to remove dirt and then they were shade dried in the laboratory under room temperature (24 ± 2 °C) for 3–4 weeks. After complete drying, the dried plant materials were pulverized by using a mechanical grinder followed by sieving to obtain a coarse powder. The powdered plant material was then extracted with ethanol (99.9%) using reflux technique. The crude extract solution obtained was filtered using Whatman No. 42 filter paper after which the excess solvents were evaporated by rotary vacuum evaporator (Evator, Media Instrument Mfg. Co., Mumbai, India) and concentrated on water bath to obtain *Aegialitis rotundifolia* Roxb., ethanolic leaves extract (ARELE). The crude ethanol extract obtained was stored at 4 °C before analysis.

2.4. Preparation of topical formulations

Topical ointment formulations containing ARELE were prepared to investigate its wound healing efficacy, in comparison with povidone-iodine ointment. Simple ointment base was prepared as per British Pharmacopoeia [20]. Briefly, 5 g wool fat, 5 g hard paraffin, 5 g cetostearyl alcohol, and 85 g soft white paraffin was taken in a beaker and heated at 65 °C in a water bath until all the ingredients were melted. The mixture was then allowed to cool and was homogenized by a homogenizer at 1500 rpm for 10–15 min. Different ointment formulation, ARELE 2.5% w/w and ARELE 5% w/w were prepared by incorporating the required amount of extract (ARELE) into 100 g of simple ointment base. The formulations were freshly prepared every third day of the study period.

2.5. Stability studies of formulations

The prepared ointment formulations were studied for their stability according to the guidelines of the International Conference on Harmonization (ICH) [21]. The ointment formulations were first assessed for their physical stability by testing the following organoleptic parameters: appearance, colour, odour, phase separation and consistency. The formulation samples were then kept at different temperatures (4 °C, 24 °C, and 40 °C) for 90 days and observed periodically for any abnormal changes like the development of any objectionable odour, colour, or any phase separation. Accelerated deterioration of the ointment formulations was tested by centrifugation of samples at 10,000 rpm for 10 min. [22]. The formulations which passed all the physical stability tests including centrifugation were selected for further tests to study the in-depth stability of the ointment formulations. Tube extrudability was determined by measuring the weight (g) required to extrude 0.5 cm ribbon of ointment in 5 s [23]. The pH of the ointment was measured using a pH meter (Elico, LI 120, India) according to the standard methods [23]. Spreadability of the formulations was determined by a modified wooden block apparatus which was set up for the experiment according to standard protocol [24]. The spreadability was calculated using the following formula:

$$S = \frac{M \times L}{T}$$

Where S is spreadability in g/s, M is the weight in grams tied to upper slide, L is the length of the glass slide, and T is the time in seconds.

2.6. Preliminary phytochemical tests

ARELE was analysed for the presence of various phytochemical

groups such as alkaloids, flavonoids, cardiac glycosides, triterpenoids, saponins, tannins, proteins, carbohydrates and sterols using standard procedures [25,26].

2.7. Experimental animals and housing conditions

Adult healthy male Wistar albino rats (age, 8–12 weeks: body weight, 150–200 g) were used to investigate the wound healing activity. Three rats were housed per polypropylene cages. The animals were housed for at least one week in the laboratory animal room prior to testing and maintained in clean polypropylene cages with stainless steel top grill at standard conditions (Temp, 25 ± 2 °C; relative humidity, 65%; 12/12 h light/dark cycle). Fresh paddy husk was used as the bedding material. The animals were fed with standard rat pellet feed (M/s Hindustan Lever Ltd., Mumbai) and drinking water was provided *ad libitum* in clean polypropylene bottles with stainless steel sipper tubes. The cage beddings and water bottles were cleaned on a daily basis.

2.8. Ethical approval

All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of GITAM Institute of Pharmacy, Visakhapatnam, Andhra Pradesh, India (CPCSEA regd. no.: 1287/PO/Re/S/09/CPCSEA and Approved proposal no.: IAEC/GIP-1287/DB-S/Approved/11/2017-18). Experiments were performed according to the guide for the care and use of laboratory animals.

2.9. Acute skin irritation test

Before commencing wound healing study ARELE ointment formulation was investigated for possible acute skin irritation test which was performed according to Gfeller et al. [27] on Wistar albino rats. An area of 500 mm^2 was marked on the dorsal side and the dorsal hairs in that area were shaved and cleaned. Then the ointment formulations (2.5% and 5%) were applied to the shaved area of different groups of animals. After application of the ointment formulations, those animals were observed for 4 h for any sign and symptom of inflammation or other abnormal changes on their skin.

2.10. Evaluation of wound healing activity

2.10.1. Experimental grouping and dosing

The rats were acclimatised for one week prior to use in the experimental models. The selected animals were divided into four groups of six animals each (n = 6 per group) for each wound healing models *viz.*, excision, incision, thermal burn and chemical burn wound models. The grouping of animals was done as follows: Group I: treated with simple ointment (vehicle), Group II: treated with a reference standard. Group III: treated with ARELE 2.5% ointment formulation. Group IV: treated with ARELE 5% ointment formulation. Povidone-Iodine ointment (5% w/w) was used as the reference standard for excision and incision wound model, whereas silver sulfadiazine ointment (1% w/w) was used as the reference standard for chemical and thermal burn injury model.

2.10.2. Excision wound model

Wound healing activities were evaluated using the excision wound model on Wistar rats according to the methods described by Morton and Malone [28]. Animals were anesthetized prior to the creation of the wounds with ketamine hydrochloride (100 mg/kg, i.m.). An impression was made on the dorsal thoracic region 1 cm away from vertebral column and 5 cm away from the ear on the anaesthetized rat. The dorsal fur of the animals was shaved with an electric clipper and the anticipated area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless-steel stencil. A full thickness of the excision wound of circular area of 500 mm^2 and 2 mm depth was created along the markings using toothed forceps, scalpel and pointed scissors. Haemostasis was achieved by blotting the wound with cotton swab soaked in the normal saline. The entire wound was left open. All surgical procedures were performed under aseptic conditions. All the wounded rats were kept separately in polypropylene cages and the wounds were left untreated for a period of 24 h. The extracts and reference drugs were applied topically at the wound site once daily.

The wound closure rate was assessed by tracing the wound on 0^{th}, 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 15^{th} and 18^{th} post-wounding days using transparent paper and a permanent marker. The tracing paper was then placed on a 1 mm^2 graph sheet and the wound area was retraced. Changes in wound area were calculated, giving an indication of the rate of wound contraction. The percentage of wound closure was calculated using the formula.

$$\%\text{wound closure} = \frac{\text{wound area on day 0 - wound area on day n}}{\text{wound area on day 0}} \times 100$$

where n = 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 15^{th}, and 18^{th} post-wounding days

The period of epithelialization was also calculated as the number of days required for falling of the dead tissue remnants without any residual raw wound.

Table 1
Extrudability, pH and spreadability of ARELE ointment formulations kept at room temperature for 90 days.

Ointment formulation	Day	Extrudability	pH	Spreadability
2.5%	0^{th}	0.82 ± 0.07	6.90 ± 0.08	20.95 ± 1.90
	30^{th}	0.88 ± 0.04	6.78 ± 0.10	22.62 ± 2.14
	60^{th}	0.93 ± 0.07	6.75 ± 0.10	28.36 ± 3.15
	90^{th}	0.94 ± 0.05	6.69 ± 0.09	28.78 ± 5.33
5%	0^{th}	0.85 ± 0.05	6.83 ± 0.09	19.47 ± 1.50
	30^{th}	0.86 ± 0.03	6.76 ± 0.07	21.93 ± 3.12
	60^{th}	0.96 ± 0.08	6.70 ± 0.04	27.67 ± 3.22
	90^{th}	0.95 ± 0.08	6.61 ± 0.07	29.76 ± 2.58

Table 2
Preliminary phytochemical test of ethanol extract of *A. rotundifolia* Roxb. leaves.

Sl no.	Phytochemicals	Tests performed	Inference
1.	Alkaloids	Mayer's test	+
		Dragondorff's test	+
		Wagner's test	+
		Hagers's test	+
2.	Carbohydrates	Molisch's test	+
		Fehling's test	+
		Benedict's test	+
3.	Proteins and amino acids	Biuret test	–
		Ninhydrin test	–
		Xanthoproteic test	–
		Millon's test	–
4.	Tannins	Ferric chloride test	+
5.	Steroids and sterols	Liberman Burchard test	+
		Salkowski's test	+
6.	Triterpenoids	Sulphuric acid test	+
7.	Cardiac glycoside	Keller killiani test	–
8.	Saponins	Foam test	+
9.	Flavonoids	Shinoda test	+
		Ferric chloride test	+
		Lead acetate test	+
		Zn dust test	+

(-) Absent, (+) Present.

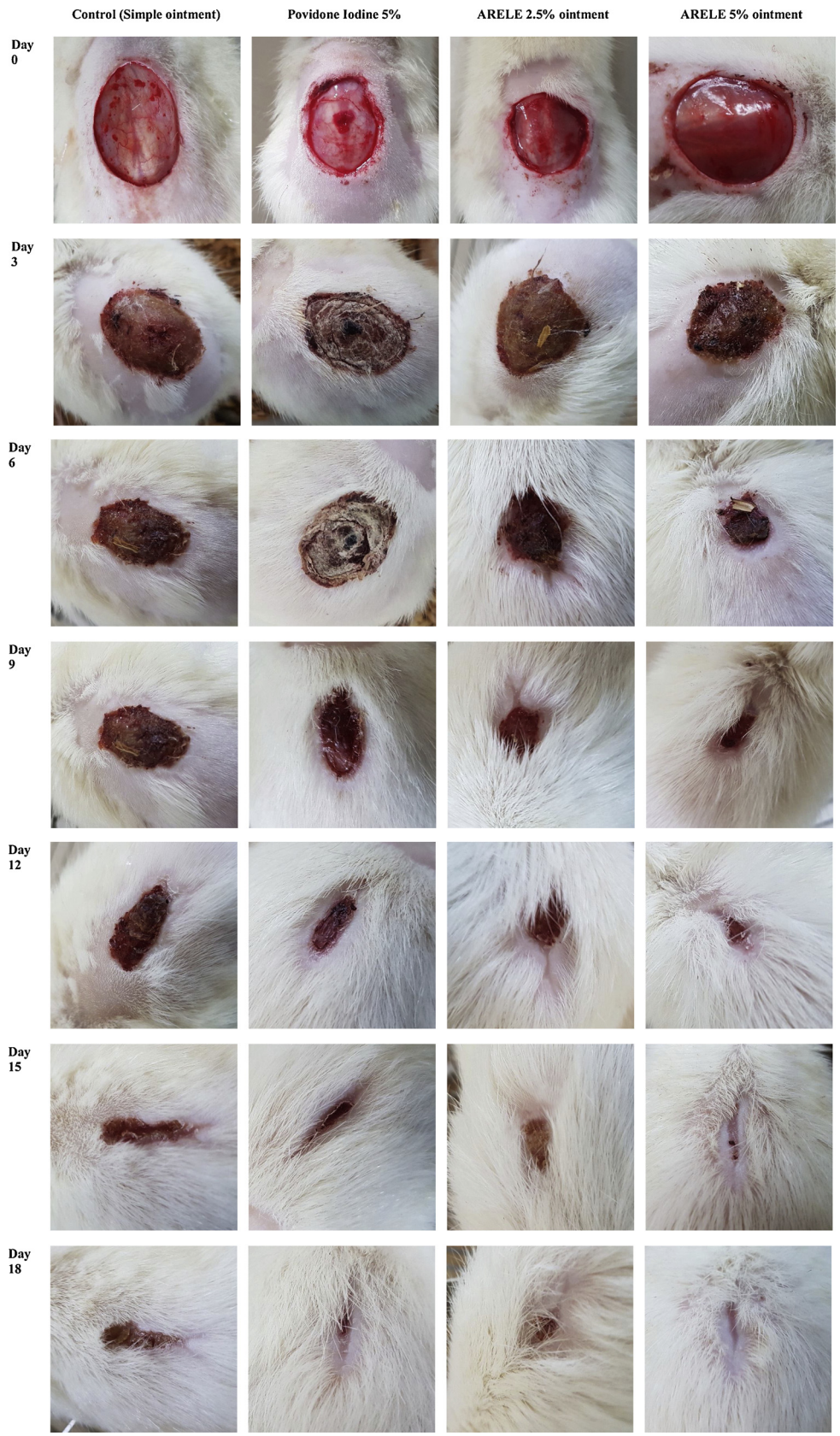

Fig. 1. The gross appearance of excision wound healing on three days interval during the 18-day study period.

Determination of the connective tissue parameters, *in-vivo* antioxidant activity, wound microbial load and histopathology was also performed from the excision wound model.

2.10.3. Estimation of granulation tissue free radicals, antioxidants and myeloperoxidase (MPO)

The granulation tissue free radicals, antioxidants and

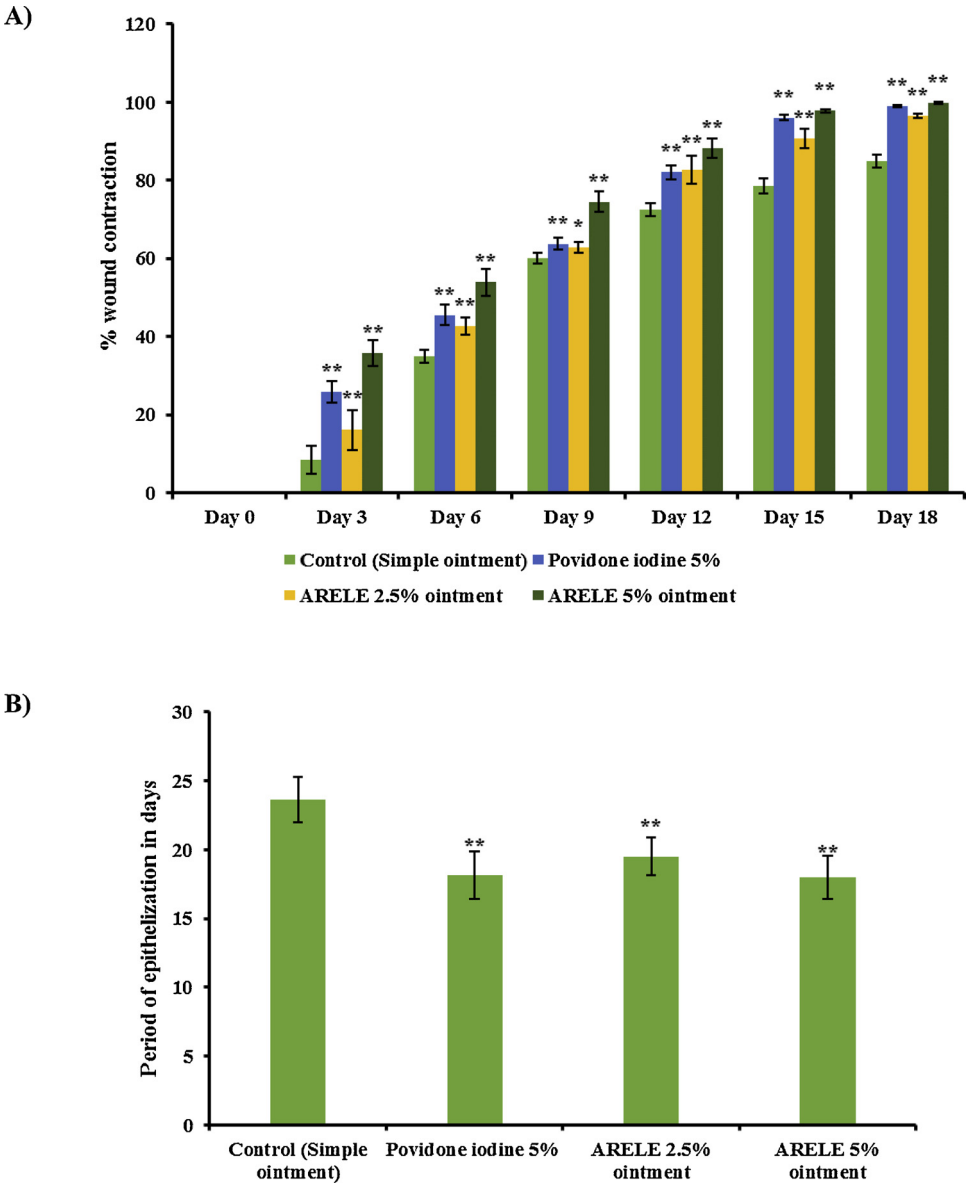

Fig. 2. Effect of ARELE ointment formulations on excision wound healing (A) Wound contraction percentage. (B) Period of epithelisation. Values are expressed as mean ± S.D. (n = 6). Statistical analysis done by one way ANOVA followed by Dunnet's t-test. **P < 0.01 compared to control group. *P < 0.05 compared to control group.

myeloperoxidase (MPO) were estimated on the 10th day of post wounding by obtaining the wet granulation tissue samples from the respective excised wound patches of the animals. The tissues were homogenized using phosphate buffer saline (PBS, 10% homogenate) at 4 °C after which the mixture was centrifuged at 40,000 × g for 30 min. The supernatant obtained was analysed for lipid peroxidase (LPO) [29], nitric oxide (NO) [30], reduced glutathione (GSH) [31], catalase (CAT) [32], and superoxide dismutase (SOD) [33]. Total protein content in the wet granulation tissue was also determined according to standard procedure [34]. For determination of acute inflammatory marker myeloperoxidase (MPO), homogenization of granulation tissue (5% w/v) was done in 0.5% hexadecyltrimethylammonium bromide with 50 mM potassium phosphate buffer (pH: 6). The homogenate was freeze-thawed three times, sonicated for 10 s, and then centrifuged at 4 °C for 45 min. at 14,000 × g. The resulting supernatant was taken for the estimation of MPO and calculated using standard procedures [35].

2.10.4. Estimation of connective tissue parameters

For the estimation of connective tissue parameters, the wet tissue sample was taken on the 10th post wounding day from the respective excised wound patches of the animals and was dried for 24 h at 60 °C. The dry tissue was then weighed and about 40 mg of dry tissue sample was transferred to each test tubes with glass stopper. Then to each tube, 1 mL of 6 N HCl was added and the tubes were kept on boiling water bath for 24 h for hydrolysis. Then the hydrolysate was cooled and 10 N NaOH using phenolphthalein as an indicator was added to neutralize excess acid. The neutral hydrolysate was diluted with distilled water to a concentration of 20 mg/mL which was used for the estimation of hydroxyproline [36], hexosamine [37], and hexuronic acid [38].

2.10.5. Determination of wound microbial load

Wound microbial load was measured at 4, 8, and 16 days post treatment. Wound swabs from each rat belonging to different groups were taken in duplicate using sterile swab sticks. The total viable count of the microbial colonies present in wound samples was determined using standard procedures [39]. Briefly, stock solutions which were used for double (10^{-2}) fold dilution were prepared by inoculating each wound swab in sterile nutrient broth. Then using a sterile pipette,

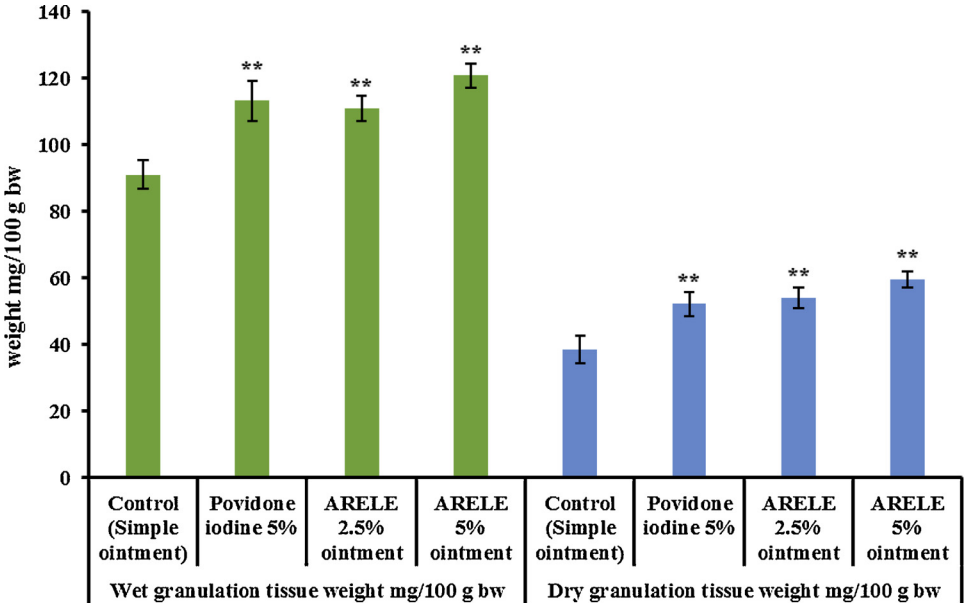

Fig. 3. Effect of ARELE ointment formulation on wet and dry weight of granulation tissue collected during evaluation of excision wound healing. Values are expressed as mean ± S.D. (n = 6). Statistical analysis done by one way ANOVA followed by Dunnet's t-test. **P < 0.01 compared to control group. *P < 0.05 compared to control group.

0.1 mL of the double fold dilution was poured into the surface of sterile nutrient agar. A sterile glass spreader was used for even distribution of the inoculum. The inoculated plates were then incubated for 24 h at 37 °C and the colonies formed after 24 h were counted using a colony counter.

2.10.6. Histomorphological analysis of wound tissue from healed excised wound

The cross-sectional full thickness skin specimens (wound tissues) were cut on the 18th day of post-wounding from all the groups to study the histopathological changes. The tissue samples were then fixed in Bouin's solution and were dehydrated through increasing grades of ethanol and then embedded in paraffin wax. The tissues were then cut to 5 µm sections with a rotary microtome, deparaffinised, mounted on clean glass slides and stained with haematoxylin and eosin (H & E) [40]. The glass slides were then observed under the microscope for histomorphological changes.

2.10.7. Incision wound model

Incision wound model was performed according to the methods described by Ehrlich and Hunt [41]. The rats were anaesthetized with ketamine hydrochloride (100 mg/kg, i.m.) prior to and during creation of the wounds. The dorsal fur of the animals was shaved with an electric clipper and a longitudinal paravertebral incision of 5 cm long was made through the skin and cutaneous tissue on the back. After the incision, the parted skin was sutured 1 cm apart using a surgical thread and curved needle. The wounds were left undressed. The ointment formulations were topically applied over the wound once daily. The sutures were removed on 8th day of post wound and the application of the formulations to the wounds continued till 10th day of post wounding. The wound breaking strength was measured on the 10th day evening after the last application. For determination of wound breaking strength, the rats were anesthetized and secured to the operation table and a line was drawn 3 mm away from the edge of the wound on either side. Two forceps were firmly applied on to the line facing each other on the opposite side of the incision wound. One of the forceps was fixed on stands, while the other was connected to a freely suspended light-weight polypropylene graduated container through a string run over to a pulley. Standard weights were put steadily and carefully into the container. Addition of weights in the container gradually increases the pressure on the wound site pulling apart the wound edges. As and when the wound just opened up, the addition of weights into the container stopped and the total weights put into the container was weighed and noted.

2.10.8. Chemical and thermal burn wound models

The hairs of the dorsal skin were shaved mechanically 24 h before induction of burn. Then the shaved area was disinfected with ethanol (70% v/v). Chemical burn injuries were induced in animals by spreading few drops of concentrated hydrochloric acid over the shaved skin on 5 x 5 cm area. Thermal burn injuries were induced on the dorsal skin of the rat by pressing the skin with a metal rod (10 mm diameter) heated over the open flame for 30 s. Then the wound area was dressed with sterile gauge and animals were housed separately after complete recovery from anaesthesia. Drugs were applied once daily upon the burn. The wound closure rate was assessed on 0th, 6th, 12th, and 18th days of post wounding using transparent paper and a permanent marker [42]. The percentage of both chemical and thermal burn wound healing was calculated according to the following formula.

$$\%\text{wound closure} = \frac{\text{wound area on day 0 - wound area on day n}}{\text{wound area on day 0}} \times 100$$

where n = 6th, 12th, and 18th days of post wounding

The period of epithelialization was also calculated as the number of days required for falling of the dead tissue remnants without any residual raw wound.

2.11. Statistical analysis

The results were calculated and expressed as Mean ± Standard deviation. The data obtained in the studies were subjected to one way of analysis of variance (ANOVA) for determining the significant difference. The inter group significance was analysed using Dunnet's t-test. A p-value < 0.01 was considered to be significant. All the statistical analysis and data presentation were done using GraphPad InStat Version 3.06 (GraphPad Software, Inc. La Jolla, CA, USA) and Microsoft excel 2013 standard (Microsoft Corp., Redmond, WA, USA).

3. Results

3.1. Stability of the formulation

The stability of the formulations was assessed by evaluating various parameters. The organoleptic parameters showed that ARELE ointment

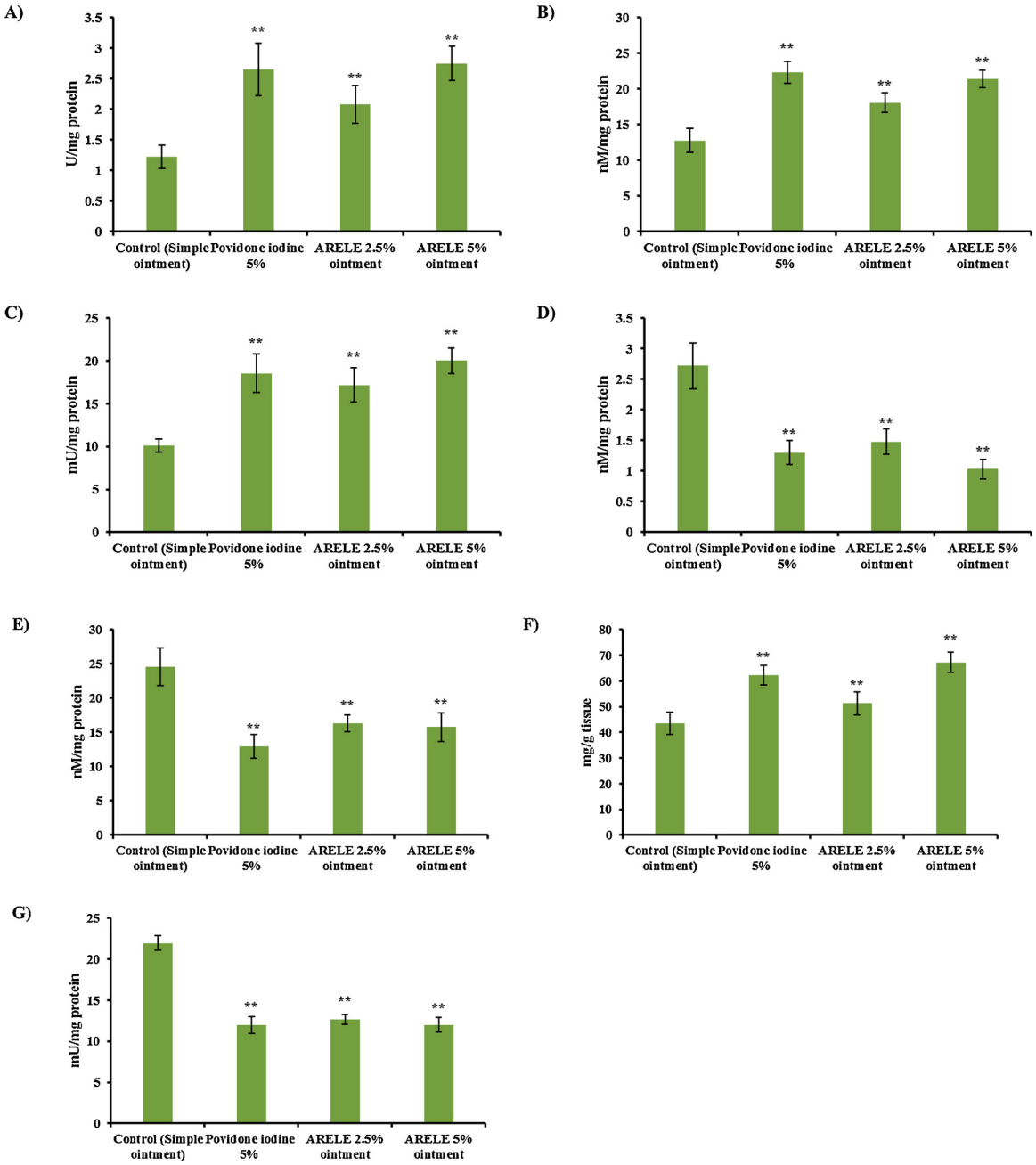

Fig. 4. Effect of topical application of ARELE ointment formulations on different antioxidants and free radicals (A) Superoxide dismutase (B) Reduced glutathione (C) Catalase (D) lipid peroxidase (E) Nitric oxide (F) Total protein (G) Myeloperoxidase. Values are expressed as mean ± S.D. (n = 6). Statistical analysis done by one way ANOVA followed by Dunnet's t-test. **P < 0.01 compared to control group; *P < 0.05 compared to control group.

formulation at 5% and 2.5% showed dark greenish brown and light greenish brown, respectively in colour which remained unchanged throughout the 90 days of storage at various temperatures. The consistency of the ointment formulations was uniform and smooth on the initial day and throughout the accelerated stability study period. No severe objectionable odour was developed during the stability study period, however, a very slight unpleasant smell was noticed for ARELE ointment formulation at 2.5% on the 90th day at both 24 °C and 40 °C. ARELE ointment formulation at 5% also developed slight foul smell at 40 °C on the 90th day of the stability studies. No phase separation was recorded for all formulations during the study period. Further, the formulations also passed the centrifugation test with no signs of deteriorations. The extrudability, pH, and spreadability of the formulations are given in Table 1, which shows that these parameters stayed almost normal over the course of the study period (90 days) at room temperature.

3.2. Preliminary phytochemical screening

Preliminary phytochemical screening of ethanol extract from *A. rotundifolia* leaves revealed the presence of major phytochemical groups such as alkaloids, carbohydrates, tannins, steroids and sterols, triterpenoids, saponins and flavonoids as shown in Table 2.

3.3. Acute skin irritation test

ARELE ointment formulation at 5%, and 2.5% showed no signs of irritation, redness, swelling, inflammation or any other unusual

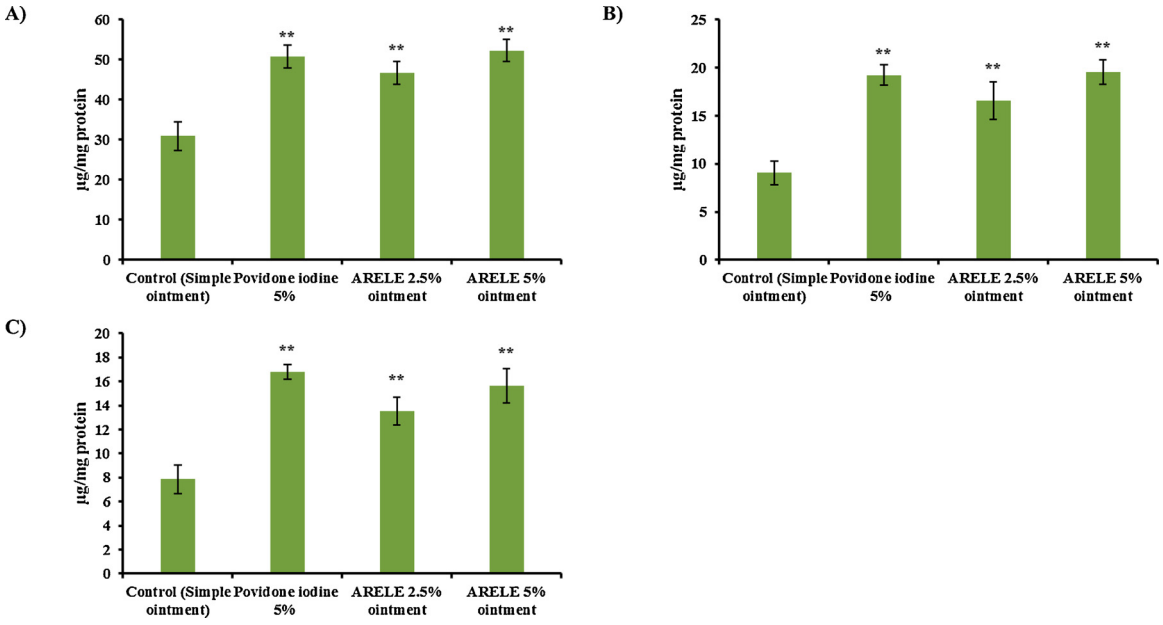

Fig. 5. Effect of topical application of ARELE ointment formulations on different connective tissue parameters. (A) Hydroxyproline. (B) Hexosamine. (C) Hexuronic acid. Values are expressed as mean ± S.D. (n = 6). Statistical analysis done by one way ANOVA followed by Dunnet's t-test. **P < 0.01 compared to control group; *P < 0.05 compared to control group.

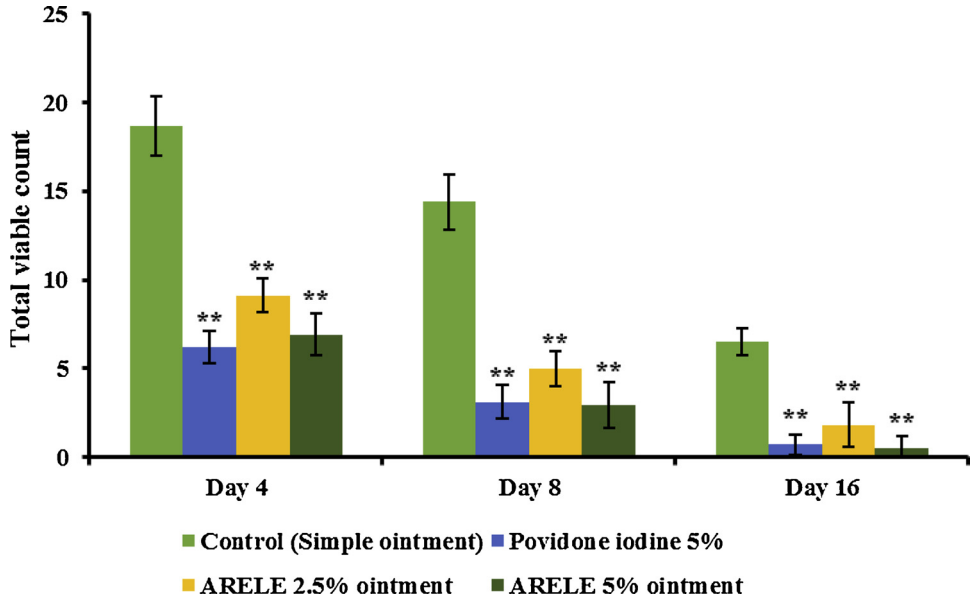

Fig. 6. Effect of ARELE ointment formulations on wound microbial load. Values are expressed as mean ± S.D. (n = 6). Statistical analysis done by one way ANOVA followed by Dunnet's t-test. **P < 0.01 compared to control group; *P < 0.05 compared to control group.

changes on the exposed skin of rats. Thus, the formulations can be considered safe for applying on the rat's skin.

3.4. Effect of ARELE ointment formulation on wound contraction and epithelisation time in excision wound model

The gross morphology of healing of excision wounds has been given in Fig. 1, whereas wound contraction percent and period of epithelization is presented in Figs. 2A and B. The observations revealed that wounds treated with ARELE ointment formulation or with the reference standard (povidone iodine, 5%) healed considerably faster in comparison with the normal control (simple ointment) treated group. The percentage wound closure rate increased significantly (P < 0.01) for ARELE (2.5% and 5%) ointment and reference standard povidone iodine 5% during the 18 days study when compared with the control group. Maximum wound closure rate percent was observed in ARELE 5% ointment treated group on the 18th day which was 99.76 ± 0.29 followed by reference standard povidone iodine 5% and ARELE 2.5% ointment treated group which was 98.93 ± 0.25 and 96.50 ± 0.52, respectively on the 18th day. Control group rats treated with simple ointment revealed very slow wound contraction rate with 84.87 ± 1.59 on the final day. Thus, it can be said that the wound contraction rate was dose dependent as ARELE 5% ointment showed better wound contraction than ARELE 2.5% ointment when compared with the control. Average number of days that took for the shedding of eschar without leaving any residual raw wound (epithelization period) was shortest for rats treated with ARELE 5% ointment followed by reference standard povidone iodine 5% and ARELE 2.5% ointment. The

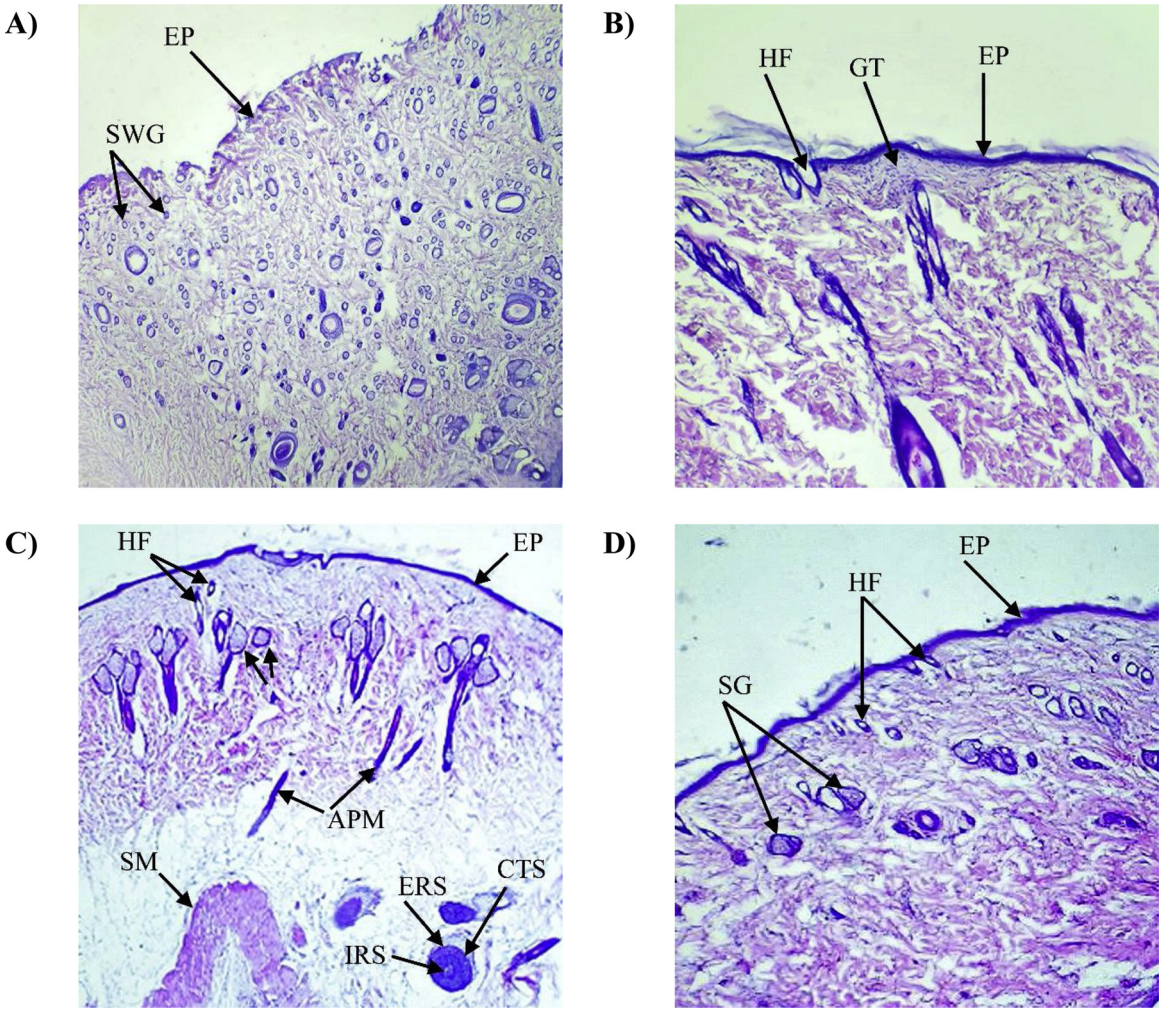

Fig. 7. Histological examination of healed excised wound treated with (A) Control (Simple ointment) (B) Povidone iodine 5% (C) ARELE ointment 2.5% (D) ARELE ointment 5%. Abbreviations: EP: Epidermis; SWG: Sweat glands; HF: Hair follicles; GT: Granulation tissue; APM: Arrector pilli muscle; SM: Skeletal muscles; SG: Sebaceous glands; IRS: Internal root sheath; ERS: External root sheath; CTS: Connective tissue sheath.

epithelisation period was longest for rats treated with simple ointment (control group).

3.5. Effect of ARELE ointment formulation on wet granulation tissue antioxidants, free radicals, total protein and myeloperoxidase

The results revealed that the wet granulation tissue weight in ARELE ointment formulation and reference standard treated groups increased significantly ($P < 0.01$) when compared with the control group (Fig. 3). The maximum wet granulation tissue weight was recorded for ARELE 5% ointment followed by reference standard and ARELE 2.5% ointment treated groups. The result of the antioxidant, free radicals, protein content and myeloperoxidase are presented in Fig.4. A significant ($P < 0.01$) increase in antioxidant marker enzymes such as SOD, GSH and CAT was observed in both ARELE (5% and 2.5%) ointment and reference standard povidone iodine 5% treated groups when compared with the control (simple ointment) group. Wet granulation tissue collected from ARELE 5% ointment treated rats showed maximum SOD and CAT activity whereas maximum GSH activity was shown by reference standard povidone iodine 5% treated group. Free radicals such as LPO and NO decreased significantly ($P < 0.01$) when compared with the control group. Maximum reduction of LPO and NO was observed in ARELE 5% ointment treated group and reference standard povidone iodine 5% treated group, respectively. The total protein content increased significantly in povidone iodine and ARELE ointment treated groups when compared with the control (simple ointment). Significant ($P < 0.01$) reduction of myeloperoxidase (MPO) was observed in both ARELE (5% and 2.5%) ointment formulation and reference standard povidone iodine 5% treated groups when compared with the control (simple ointment) group and was maximum in povidone iodine 5% treated group.

3.6. Effect of ARELE ointment formulation on dry granulation tissue weight, HPR, HXA and HUA

The results revealed a significant ($P < 0.01$) increase in dry weight of granulation tissue in ARELE (5% and 2.5%) ointment and povidone iodine 5% treated group when compared with the control group (Fig. 3). Maximum dry granulation weight was observed in ARELE 5% ointment treated group. The levels of HPR, HXA and HUA increased significantly ($P < 0.01$) in ARELE (5% and 2.5%) ointment and reference standard povidone iodine 5% treated groups. The HPR and HXA content was highest in ARELE 5% ointment treated group whereas HUA content was maximum in povidone iodine 5% treated group (Fig. 5). The increase in connective tissue parameters *i.e.* HPR, HXA and HUA increased in a dose dependent manner as ARELE 5% ointment showed much increase in connective tissue parameters than ARELE 2.5% ointment when compared with the control group.

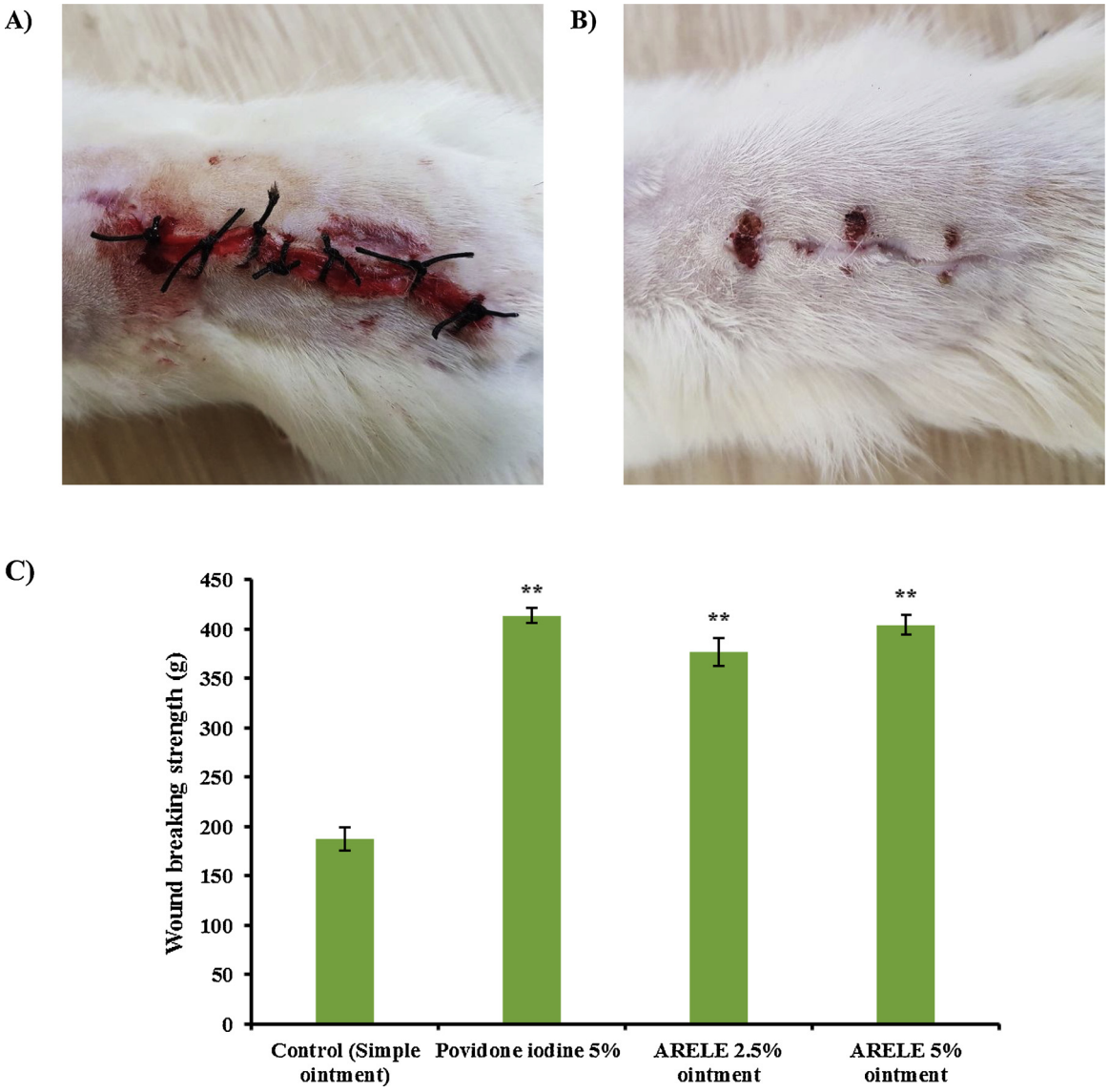

Fig. 8. Effects of topical application of ARELE ointment formulations on incisional wound (A) Incisional wound created (Day 0) (B) Incisional wound healing (Day 10) (C) wound breaking strength in incision wound healing. Values are expressed as mean ± S.D. (n = 6). Statistical analysis done by one way ANOVA followed by Dunnet's t-test. **P < 0.01 compared to control group; *P < 0.05 compared to control group.

3.7. Effect of ARELE ointment formulation on wound microbial load

The total viable bacterial count obtained from the wound samples is presented in Fig. 6. The results revealed that total microbial colonies formed decreased significantly (P < 0.01) in wound samples of rats treated with ARELE ointment formulations and reference standard (povidone iodine) when compared with the normal control group after 4, 8 and 16 days of treatment. The reference standard showed the least number of microbial colonies formed after the 4th day of treatment, whereas ARELE 5% ointment formulation showed the least number of microbial colonies after 8th and 16th post treatment. The reduction of microbial colonies was dose dependent as ARELE 5% ointment showed much reduction in the number of microbial colonies than ARELE 2.5% ointment when compared with the control group.

3.8. Histology of healed skin

Microscopic images of H & E stained excision biopsy of skin at day 18 at 4 × magnification is given in Fig. 7. Histological studies of the tissue sections revealed a maximum amount of scarring with signs of inflammatory cells infiltration, few blood vessels and fibroblast and sparing amount of collagen for the control group treated rats. Rat's treated with ARELE (5% and 2.5%) ointment formulation and reference standard povidone iodine 5% showed almost healed skin structures with normal epithelization, restoration of adnexa, reduction of scarring and inflammatory cells, an increase of fibroblast, blood vessels and collagen deposition. However much less scarring and inflammatory cells infiltrations were observed in ARELE 2.5% ointment treated group when compared with the control, reference standard and ARELE 5% ointment treated groups.

3.9. Incision wound model

The healing of incision wound measured in terms of the tensile strength of the healing skin administered with different ARELE ointment formulations on 10th day of post-wounding is shown in Fig. 8. The result revealed that the wound breaking strength of the healing skin of rats treated with ARELE ointment formulation (2.5% and 5%) and reference standard povidone iodine 5% increased significantly (P < 0.01) when compared with the control group on 10th day of post-

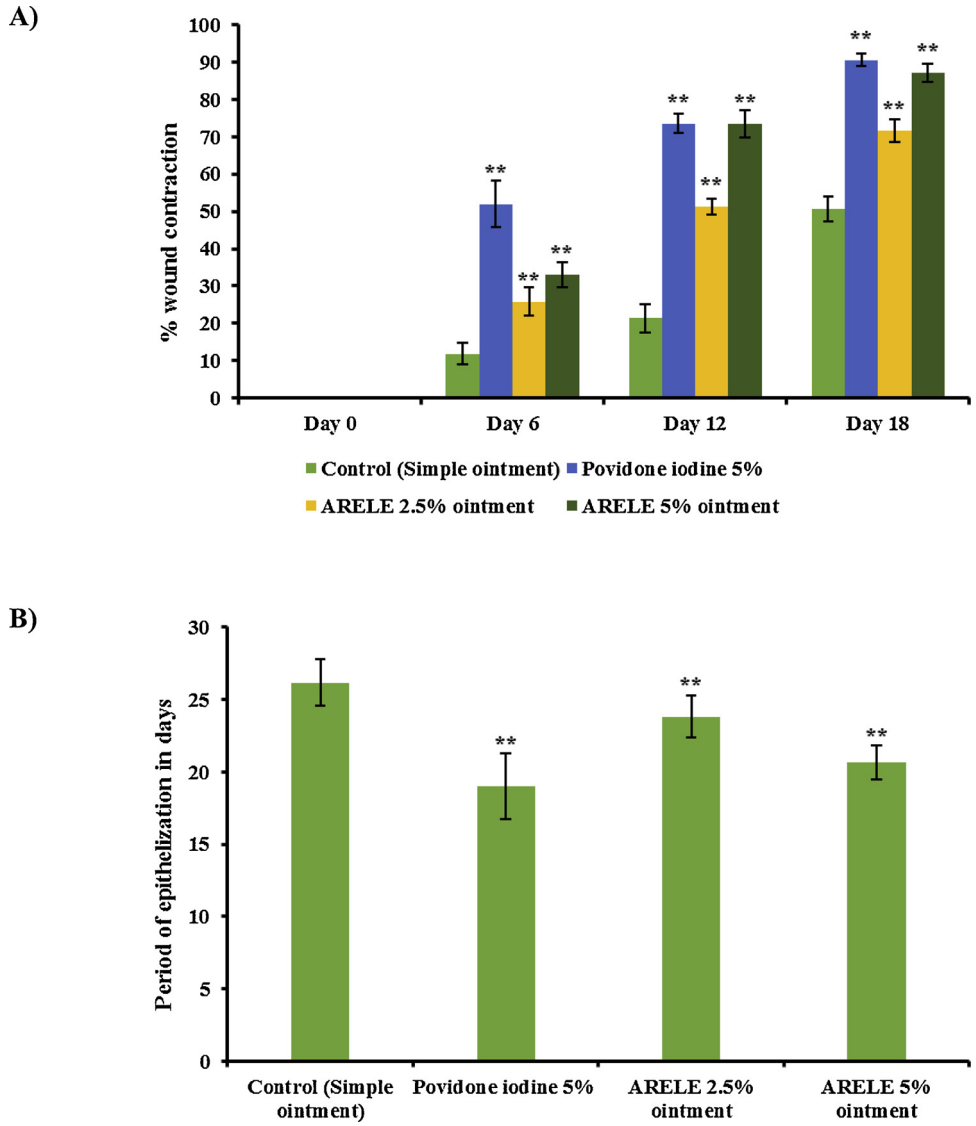

Fig. 9. Effect of ARELE ointment formulations on thermal burn wound healing (A) Wound contraction percentage. (B) Period of epithelisation. Values are expressed as mean ± S.D. (n = 6). Statistical analysis done by one way ANOVA followed by Dunnet's t-test. **P < 0.01 compared to control group; *P < 0.05 compared to control group.

wounding. The wound breaking strength (WBS) for reference standard povidone iodine 5% was 413.83 ± 7.54 g with a percentage tensile strength of 54.71 ± 2.12, whereas the WBS of ARELE ointment formulation at 2.5% and 5% doses was 376.75 ± 14.26 and 404.33 ± 10.09, respectively with the percentage tensile strength of 50.25 ± 1.98 and 53.64 ± 2.35, respectively. The wound breaking strength for the control group was 187.5 ± 11.91.

Maximum WBS was observed for povidone iodine 5% followed by ARELE 5% ointment and then ARELE 2.5% ointment formulation and the least WBS was showed by the control group. This also shows that the increase in WBS in the ARELE ointment formulation treated groups was dose dependent when compared with the control group.

3.10. Effect of ARELE on chemical and thermal burn wounds

The percentage contracting ability and period of epithelisation of thermal wounds is given in Fig. 9A and B, whereas for chemical wound it is presented in Fig. 10A and B. The result shows that in both chemical and thermal induced burn wound, a significant (P < 0.01) increase in percent wound closure was observed in ARELE (2.5% and 5%) ointment and silver sulfadiazine 1% treated groups when compared with the control group. In the total study period of 18 days, maximum wound closure percent in both the chemical and thermal induced burn model was observed in rats treated with silver sulfadiazine 1% followed by ARELE 5% and ARELE 2.5% ointment treated group. In chemical induced burn wound the period of epithelization recorded for silver sulfadiazine 1% was 23.16 ± 1.16 days and that of ARELE 2.5% and 5% ointment was 27.66 ± 1.36 and 24.16 ± 2.40 days respectively. In thermal induced burns, the period of epithelization recorded for silver sulfadiazine 1%, ARELE 2.5% and 5% ointment was 19.00 ± 2.28, 23.83 ± 1.47 and 20.66 ± 1.21 days, respectively. In both chemical and thermal burns, the increase in wound contraction was dose dependent as ARELE 5% ointment showed higher wound contraction than ARELE 2.5% ointment when compared with the control group.

4. Discussion

Herbal medicines and its derivatives have been used in many developing countries as an alternative to allopathic medicines in the treatment of various diseases [43]. According to the traditional knowledge, this mangrove species is a potent cure for pain, and used as a pain-relieving agent by various local healers in mangrove areas [10].

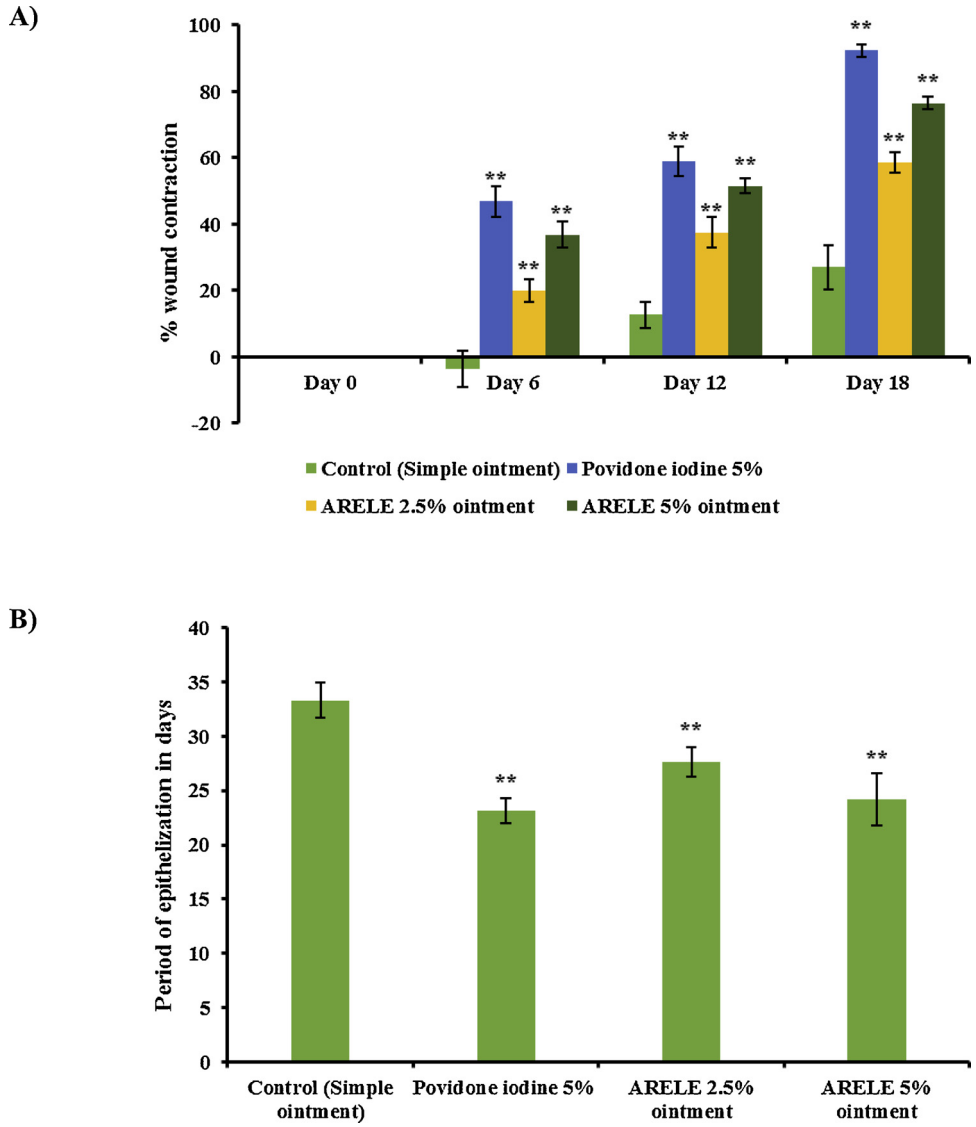

Fig. 10. Effect of ARELE ointment formulations on chemical burn wound healing (A) Wound contraction percentage. (B) Period of epithelisation. Values are expressed as mean ± S.D. (n = 6). Statistical analysis done by one way ANOVA followed by Dunnet's t-test. **P < 0.01 compared to control group; *P < 0.05 compared to control group.

In a report published by Mudge and Orsted [44], the pain is often associated with wounds which may arise due to damage of tissues (nociceptive pain) or abnormal functioning of the nervous system (neuropathic pain). According to McGuire et al. [45], pain is also responsible for delayed wound healing process as they are known to dysregulate neuroendocrine and immune function which are known to play a vital role in the wound repair mechanism. Thus, herbal preparations with potent pain-relieving activity can be potentially employed for accelerating the wound healing process. According to the current literature, there is no investigation done on the *in vivo* wound healing properties of this rare mangrove plant, however it's *in vitro* wound healing properties has been recently reported by Ghosh et al. [19]. The plant's strong ameliorating effect on pain and inflammation has also been scientifically reported previously by Raju et al. [13]. Hence, the present research will provide the first information report of the *in vivo* wound healing properties of this scientifically unexplored mangrove plant which is traditionally used by the local healers for curing pain caused by various injuries and as an antidote for insect bites. The wound healing properties have been evaluated comprehensively using three *in-vivo* models *viz.*, excision, incision and burn wound models.

Herbal ointments containing extracts of different medicinal plants have been used in folk medicine and are said to enhance wound healing by minimising pain, discomfort and scarring [46]. Selection of appropriate ingredients and their correct amounts during the preparation of different pharmaceutical formulations is important. Various factors such as formulation appearance, colour, pH, odour, chemical, and physical stability should be taken into consideration. Sometimes it is necessary to use several different excipients so that a vehicle of desired physicochemical properties can be obtained. It is required to use different excipients for making any kind of formulations (here an ointment base) as it is impossible for a single excipient to produce an ideal vehicle, whereas combinations of different excipients give an increased bioavailability and drug potency [47,48]. Ointment formulation was chosen as it was more convenient dosage form for topical application and its stability and different physicochemical parameters were studied to find out its suitability for topical usage. Our stability study of the ointment formulations showed that all parameters were normal during the 90-day study, however the very minimal uncharacteristic smell was noticed on the 90th day of the ARELE 5% and 2.5% ointment formulations.

The preliminary phytochemical tests help in determining the class

of chemical compound present in the extracts which may lead to their quantitative estimation and identifying the source of pharmacologically active phyto-constituents [49]. The results of the preliminary phytochemical test of ARELE revealed the presence of alkaloids, carbohydrates, tannins and phenolic compounds, steroids and sterols, triterpenoids, saponins and flavonoids. The presence of these phytochemical groups will provide the basis of the class of chemical compound present in ARELE.

Wounds are common in both animals and humans due to their exposure to chemical, physical and thermal injuries. The damage caused to the tissues if not managed could lead to severe infections, chronic inflammation, *etc.* [50]. In excision wound, ARELE ointment formulation and reference standard (povidone iodine) treated groups showed enhanced wound closure and epithelization period than the control (simple ointment) group. Wound contraction is the process of mobilizing the healthy skin around the wound to cover the denuded area. It usually involves complex and superbly orchestrated interaction of cells, extracellular matrix, and cytokines. The activities of myofibroblast may be responsible for this centripetal movement of the wound margin [51]. Our results showed that the wound contraction rate was enhanced by ARELE ointment application and maximum contraction was seen for the wounds treated with ARELE 5% ointment which was even more than the reference standard povidone iodine 5%. This was may be due to the enhanced contractile property of myofibroblast or increased number of myofibroblast entering the wound area of the wounded rats treated with the test ointment at high dose. According to a previous report by Thomas et al. [52] povidone iodine is responsible for the reduction of fibroblast cell migration and proliferation thus retarding the healing process. This might be the cause of the inferior wound contraction rate of the povidone iodine 5% treated group than the ARELE 5% ointment treated group.

Collagen is an extracellular protein constituent in the granulation tissue formed during the healing of a wound. After an injury, there is a rapid increase in synthesis of collagen and thus its break down liberated free hydroxyproline and its peptides. Measurement of hydroxyproline gives an indication of the rate of collagen synthesis and fibre stabilization. Thus, higher the concentration of hydroxyproline higher would be the rate of wound healing [53]. Our research showed a significant increase in hydroxyproline content in granulation tissue collected from rats treated with ARELE ointment and standard (povidone iodine) when compared with the control. ARELE 5% ointment formulation showed the highest concentration of hydroxyproline which indicates that test ointment formulation was able to synthesize more collagen and promote fibre stabilization on the wound site. Hexuronic acid and hexosamine are matrix molecules which acts as the ground substratum for the production of extracellular proteins. Both hexosamine and hexuronic acid are components of glycosaminoglycans which stabilizes the collagen fibres by increasing the ionic and electrostatic interactions and also control their characteristic size and alignment [54]. A significant increase in hexosamine and hexuronic acid was observed in rats treated with ARELE ointment and reference standard when compared with the control group treated rats. Oxidative stress is caused by overproduction of reactive oxygen species (ROS), and an increase in MPO activity thereby causing delay in the wound healing process. Therefore in the healing of chronic wounds, elimination of ROS and reduction of MPO activity could be an important strategy and estimation of antioxidants (SOD, CAT, and GSH) and free radicles (LPO and NO) in granulation tissue becomes relevant as they help accelerate the process of wound healing [55,56]. In our study, ARELE ointment treated groups showed potent *in-vivo* antioxidant activity by reducing free radicals stress and thus ARELE could help to prevent oxidative damage and promote the healing process.

We further illustrated the healing mechanism by determining wound microbial load and the result showed that ARELE ointment formulations significantly reduced the microbial colonies formed. Post-operative wounds are usually prone to microbial infections which delays the wound healing process and results in scratching/biting on the wound site [57,58]. Thus, it is important to control the microbial growth on the wounded site for better and faster healing.

To further substantiate the results of the excisional wound healing properties, histopathology of the skin area of the excised wound on 18th day of post-wounding were performed which showed normal epithelization, restoration of adnexa and fibrosis within the dermis and prominent layer of epidermis in both ARELE ointment and standard (povidone iodine) treated groups.

Healing of incisional wounds is measured by the wound breaking strength which is the amount of force required to disrupt or break the wound. The breaking strength of wounds increases when there is an increase in the concentration of collagen and stabilization of fibres [59]. The synthesized collagen molecules during the wound healing process are amalgamated at the wound site and are cross-linked to form fibres. The remodelling of collagen and formation of stable inter- and intramolecular crosslink fibres results in a strong wound breaking strength [60]. In our study, ARELE ointment formulation showed a significant increase in wound breaking strength when compared with the control.

In burn wounds, the repair process is more complicated as there is an extensive loss of cells and tissues compared to an incision wound. Thermal burn is caused due to contact of the skin with a hot object like boiling water, hot oil, fire, *etc.*, whereas chemical injury is caused when a living tissue is exposed to any corrosive material such as strong acid or base. Chemical burns can be extremely painful and sometimes diffuse into skin tissues and damage the structures underneath the skin without immediately damaging the skin surface. An injury caused by a burn produces an alternation in the skin in the form of a lesion in the corneal strata which is enough to cause the skin to lose its capacity to act as a barrier [42]. The result of our study showed that ARELE ointment and reference standard (silver sulphadiazine 1%) treated groups produced a significant increase in wound contraction rate when compared to the control group. Even though silver sulphadiazine 1% produced a slight better burn wound healing effect than the test ointment treated groups it also known for delaying the healing process [61]. Thus, ARELE ointment was moderately effective in contraction of burn wounds.

Several phytochemical groups such as flavonoids, tannins, glycosides, alkaloids, saponins, sterols, terpenoids, *etc.*, are known to accelerate the wound healing process [62], and our study revealed the presence of such phytochemical groups in the extract (see Table 2). Also, in our previous work on this mangrove plant [10], polyphenolic compounds like gallic acid, chlorogenic acid, rutin, and quercetin were detected in *A. rotundifolia* leaves all of which possesses potent wound healing activity [63–66]. The presence of these phytochemicals in the extract guarantees a potential activity of curbing ROS and promoting the process of wound healing since a high amount of ROS are produced at the site of wounding [67].

5. Conclusion

This study revealed that topical application of ARELE ointment formulation showed strong wound healing activity in all the three models *viz.*, excision, incision and burn wound when compared to the control (simple ointment). In excision wound model, ARELE ointment formulation at high dose showed strong wound contraction rate which may be due to increase in *in-vivo* antioxidant activity, reduction of myeloperoxidase, increased collagen deposition and reduction of microbial colonies. The result of the excision wound is further supported by the histological findings of the healed excised skin after 18th day which showed almost healed skin structures with normal epithelisation. In incision wound model, ARELE ointment formulation at high dose showed a significant increase in wound breaking strength. In both thermal and chemical burns, ARELE ointment formulation at high dose showed moderate burn wound healing activity, however, the test ointment formulation was more effective in thermal burn than chemical

burn. This work gives the first report of the *in vivo* wound healing potential of *A. rotundifolia* leaves which was applied topically in the form of an ointment formulation. Stability studies on the ointment formulations were performed before commencing the wound healing studies and the results revealed that they were stable at different conditions. This study can also be taken as a benchmark for further investigation to identify the phyto-constituents which are responsible for the wound healing activities.

Ethical statement

All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) of GITAM Institute of Pharmacy, Visakhapatnam, Andhra Pradesh, India (CPCSEA regd. no.: 1287/PO/Re/S/09/CPCSEA and Approved proposal no.: IAEC/GIP-1287/DB-S/Approved/11/2017-18). Experiments were performed according to the guide for the care and use of laboratory animals.

Financial disclosure

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. We are thankful to GITAM (Deemed to be University), Visakhapatnam, Andhra Pradesh, India for providing facilities to carry out this research.

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