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Research Article

EXPERIMENTAL EVALUATION OF WOUND HEALING ACTIVITY OF AYURVEDIC ANTIMICROBIAL FORMULATIONS USING IN VIVO ANIMAL EXCISION AND INCISION METHODS

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ABSTRACT

Wound healing comprises of four phases, namely inflammation, proliferation, re-epithelialization and remodelling which re-establish integrity of damaged tissue. This experimental study evaluates wound healing action of two Ayurvedic stem bark formulations, RFNA (containing Neem & Ashoka) and RFUL (containing Udumber & Lodhra) using 5% & 10% aqueous extract concentrations for preparation of ointment for external wound application using excision and incision methods. Phytochemical screening of extracts indicated presence of alkaloids, flavonoids, tannins and carbohydrates while total flavonoid content was 54.76 and 59.14 µg QE/mg and total phenol content was 205.00 and 225.67 µg GAE/mg for RFNA and RFUL. This 14-day study used Swiss albino rats divided into six groups, each group having six animals. While Group A was control group, Group B used Framycetin Sulphate IP as standard drug. Groups C, D, E and F were administered ointment containing 5% and 10% RFNA and RFUL respectively. While excision wound model study evaluated the percentage of wound contraction and amount of pus formation, the incision wound model assessed reduction in wound length and histopathological microscopic examination of wound skin, on the 4th, 7th and 14th day. During this study, 10% RFNA and RFUL exhibited similar therapeutic efficacy as standard drug while 5% concentrations showed a little lower but highly significant properties, possibly due to high concentration of phenolic and flavonoidic compounds. The results showed that highest therapeutic efficacy was shown by 10% RFNA followed by 10% RFUL, 5% RFNA and 5% RFUL groups respectively in both excision and incision models.

KEY WORDS: Ayurvedic formulation, *Azadirachta indica, Saraca asoca, Ficus glomerata, Symplocos racemosa*, Wound healing, excision and incision model

INTRODUCTION

A wound is defined as a disruption of the cellular and anatomic discontinuity of a tissue and may be produced by chemical, physical, thermal or microbial reasons which causes discomfort and possibly infection. Wound healing consists of an orderly progression of events that re-establish the integrity of the damaged tissue. Wound healing consists of four phases: inflammation, proliferation, re-epithelialization and remodelling. Animal models have been developed to study the complex cellular and biochemical processes of wound repair and to evaluate the efficacy and safety of potential therapeutic agents. Plants and their extracts have immense potential for the management and treatment of wounds since these natural agents induce healing and regeneration of the tissue by multiple mechanisms. However, there is need for scientific validation, standardization and safety evaluation of plants of traditional medicine so that they can be recommended for healing of wounds.[1, 2]

The present experimental study evaluates the wound healing action of two Ayurvedic antimicrobial research formulations (RFNA and RFUL) using excision and incision methods in rats and compares its efficacy with standard drug. The Research formulation RFNA contains equal amounts of stem bark of Neem (*Azadirachta indica* A. Juss.) and Ashoka (*Saraca asoca* Roxb.) while RFUL contains stem barks of Udumber (*Ficus glomerata* Roxb. and Lodhra (*Symplocos racemosa* Roxb.). The aqueous extract of each research formulation was used in two different concentrations (5% & 10%) for preparation of the therapeutic ointment used for external application in different wound healing models. These new herbal formulations were not evaluated till now for their wound healing properties although they are likely to exhibit significant antimicrobial action due to the synergetic effect of the phenolic and flavonoid compounds present in these research drugs and the pharmacological properties of the constituent herbs.[3-5]

The research formulation RFNA contains Neem (*Azadirachta indica* A. Juss.) belonging to the Meliaceae family which is found in abundance in tropical and semi-tropical regions like India, Bangladesh, Pakistan and Nepal. It is a fast-growing tree which is 20–23 m tall and having 4-5 ft diameter straight trunk. The leaves are compound and imparipinnate, each comprising of 5–15 leaflets. The main chemical compounds present in its leaves and stem bark include azadirachtin, nimbolinin, nimbin, nimbidin, nimbidol, sodium nimbinate, gedunin, salannin and quercetin. The stem bark and leaves are used for treatment of different types of skin diseases like ringworm, eczema and itching, blood purification, liver diseases, wounds and fever. Its anti-inflammatory, anti-hyperglycemic, anticancer, antipyretic, antioxidant, antidiabetic and hepato-protective medicinal properties have been reported.[6, 7]

The plant known as a *Saraca asoca* Roxb. or Ashoka belongs to the Leguminosae family. The chemical compounds found in its stem bark include glycosides, flavonoids, tannins and saponins. The stem bark of this plant has been used in the Indian system of medicine for its bitter, astringent, sweet, refrigerant, anthelmintic, styptic, stomachic, febrifuge and demulcent properties. It has a

stimulating effect upon the endometrium and ovarian tissues. It is useful in the treatment of dyspepsia, fever, biliousness, burning sensation, colic dysentery, internal bleeding, haemorrhoids, ulcers, menorrhagia, uterine fibroids and leucorrhoea. The pharmacological activities of its stem bark include uterogenic, antibacterial, oxytocic, antitumour, anticancer, antiprogestational, antitumor, anti-inflammatory and antiarthritic properties.[8, 9]

The research formulation RFUL contains the plant *Ficus glomerata* Roxb. or Cluster Fig which belongs to the Moraceae family. The stem bark, fruits, leaves and latex of this plant have been used since ancient times as mentioned in the Ayurvedic text book for treatment of dysentery, diarrhoea, toothache, stomach-ache, vaginal disorders, menorrhagia, haemoptysis, diabetes, piles and glandular swelling, etc. The phytochemical compounds isolated from the stem bark are leucocyanidin-3-o-B-glucopyranoside, leucopelarogonidin 3-O-a-L-rhamnopyranoside, B- sitosterol, stigmasterol, tetracyclic triterpene- gluanol acetate and tiglic acid. The reported pharmacological properties of the different plant parts are hypoglycaemic, antiulcer, antioxidant, wound-healing, anti-inflammatory, anti-diarrhoeal, antibacterial, antifungal, antipyretic and antidiuretic. [6, 7, 10]

Symplocos Racemosa Roxb. known as Lodhra belonging to the Symplocaceae family is found distributed throughout North Eastern India up to 2,500 ft. elevation. It is a small evergreen tree with stem up to 6 m in height and 15 cm in diameter. Its stem bark is useful in bowel complaints such as diarrhea & dysentery, in dropsy, eye disease, liver complaints, wound healing, excessive vaginal discharge, menstrual problems, fevers, ulcers, scorpion-string, etc. The alcohol extract of stem bark indicated the presence of carbohydrates, glycosides, saponins, terpenoids & alkaloids while its ether extract indicated the presence of glycosides, phytosterol, steroids and two phenolic glycosides of salirepin series namely symplocuronic acid and sympocemoside. The prominent pharmacological activities of its stem bark are antibacterial, anti-inflammatory, antiulcer, anti-tumor, antimicrobial and antioxidant. [9, 11, 12]

MATERIALS & METHODS

Plant collection

The stem barks of Lodhra (*Symplocos racemosa* Roxb.), Udumber (*Ficus glomerata* Roxb.), Neem (*Azadirachta indica* A. Juss.) and Ashoka (*Saraca asoka* Roxb.) were collected from crude drug supplier of pharmacy department of the Institute and plant samples were authenticated by the Botanical Survey of India, Howrah, India. Authenticated specimens bearing numbers IPGAE&R/Dravyaguna/M.Gupta/07, 08, 09 & 10 were deposited in the herbarium museum of the department of Dravyaguna at I.P.G.A.E. & R., Kolkata for future reference.

Procurement of chemicals

Chemical reagents such as Toluene, Formic acid, Acetonitrile, Gallic acid, Acetic acid, Vanillin, and HPLC grade water were procured from M/s Merck Specialities Pvt. Ltd and Petroleum ether, acetone, Chloroform, Ethyl Acetate, Ascorbic acid, Acetyl Salicylic acid, Catechol, Ellagic acid and Benzoic acid were purchased from M/s Nice Chemicals Pvt. Ltd. The pharmacognostical and chemical analysis of the research formulation has been done following the protocols of drug standardization mentioned in the Ayurvedic Pharmacopoeia of India.[7]

Pharmacognostical study

The physical parameters such as moisture content, pH value, Ash value and extractive values were determined in the various research formulations with the help of Soxhlet apparatus using different nonpolar to polar solvent systems. Two types of research formulations were prepared for this study - RFNA consisting of equal amounts of stem barks of Neem (*Azadirachta indica* A. Juss.) and Ashoka (*Saraca asoca* Roxb.), and RFUL consisting of equal amounts of stem barks of Udumbar (*Ficus glomerata* Roxb.) and Lodhra (*Symplocos racemosa* Roxb.).[13]

Continuous extraction of research formulation

The stem barks of all the plant samples were washed, air-dried and pre-heated in oven before being powdered in a grinding machine to 40# mesh particle size and stored in an airtight container. Powdered dried barks of these plants were mixed in equal ratio and this coarse powder was sequentially extracted with petroleum ether (60°C – 80°C), chloroform, acetone, ethanol and water using Soxhlet apparatus. These extracts were filtered using a Buckner funnel and Whatman No. 1 filter paper at room temperature and concentrated at reduced temperature and pressure using rotary evaporator. All obtained extracts were stored in refrigerator below 10°C for subsequent experiments. The aqueous extract of each research formulation was used for preparation of ointment used during the study.[14]

Phytochemical screening

The research extracts were subjected to preliminary phytochemical testing to detect the presence of different chemical group of compounds such as saponins, tannins, alkaloids, flavonoids, glycosides, carbohydrates, oils and fats, proteins and amino acids following the standard Ayurvedic pharmacopeia standardization methods.[15, 16]

Determination of total phenol and total flavonoid content

The Total phenol content (TPC) was determined using the Folin-Ciocalteu reagent. To 0.5 ml aliquot of dried aqueous extract, 2.5 ml of 10 % Folin- Ciocalteu's reagent and 2 ml of 7.5% sodium carbonate were added. The absorbance was read after 30 min incubation period at room temperature at 760 nm colorimetrically. A standard calibration plot was generated at 760 nm using known concentrations of Gallic acid (100, 200, 300, 400, and 500 µg/ml). The concentration of phenol in the test samples was calculated from the calibration plot and expressed as mg Gallic Acid Equivalents (GAE) per gm sample extract.

Similarly, the Total flavonoid content was determined using the spectrophotometric method. An aliquot of 0.5 ml of sample (1 mg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 1% aluminium chloride and 0.1 ml of potassium acetate solution (1 M). In the mixture, 2.8 ml of distilled water was added to bring up the total volume to 5 ml. The test solution was shaken vigorously and absorbance at 415 nm was recorded after 30 minutes of incubation. A standard calibration plot was generated at 415 nm using known concentrations of Quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg Quercetin equivalent/gm of sample. [17, 18]

Preparation of Ointment

The ointments of aqueous extracts of both research formulations were prepared by mixing different concentrations of a polyether compound called Polyethylene Glycol (PEG) which

is chemically stable, irritation free, and miscible with water and mucous secretion. Hydrophilic 5% and 10% Ointments of both research formulations were developed by using Poly Ethylene glycol 400. Different ratios of low and high molecular weight polyethylene glycols were tried for preparation of these ointment bases. The investigated physical characteristics included the drop point, the congealing range and the spreadability properties of bases using standard glass apparatus and instruments. The results of studying these physical parameters indicated that PEG 400: PEG 4000 blend gave very good bases for use in wound healing action of both research formulations.[19]

Animals

Healthy Swiss albino rats of either sex weighing between 160 to 220 gm were procured from Government of West Bengal approved breeder of CPCSEA, M/s Saha Enterprises, Kolkata and housed under standard environmental conditions with fixed 12-hour light/dark cycles and at a temperature of approximately 25°C in the animal house of IPGAE&R. The animals were kept in standard polypropylene cages and provided with food (standard pellet diet) and water. These animals were acclimatized for a period of 14 days prior to performing any experiments. All experimental protocols were approved by the Institutional Animal Ethical Committee (I.A.E.C.) vide their Memo No. SVP/PG/401(A) 2014 dated 27.3.2014.

Experimental protocol

All the healthy experimental animals were randomly allocated to one of the following six groups, each group having six animals:

Group A: The animals in this placebo group were treated only with the ointment base.
Group B: The animals in the standard group were treated with Framycetin Sulphate IP.
Group C: The animals were treated with 5 % ointment of **RFNA** consisting of aqueous extract of equal amounts of stem bark of Neem (*Azadirachta indica* A. Juss.*)* and Ashoka *(Saraca asoca* Roxb.*).*
Group D: The animals were treated with 10 % ointment of **RFNA** consisting of aqueous extract of equal amounts of stem bark of Neem (*Azadirachta indica* A. Juss.*)* and Ashoka *(Saraca asoca* Roxb.*).*
Group E: The animals were treated with 5 % ointment of **RFUL** consisting of aqueous extract of equal amounts of stem bark of Udumbar (*Ficus glomerata* Roxb.) and Lodhra (*Symplocos racemosa* Roxb.).
Group F: The animals were treated with 10 % ointment of **RFUL** consisting of aqueous extract of equal amounts of stem bark of Udumbar (*Ficus glomerata* Roxb.) and Lodhra (*Symplocos racemosa* Roxb.).

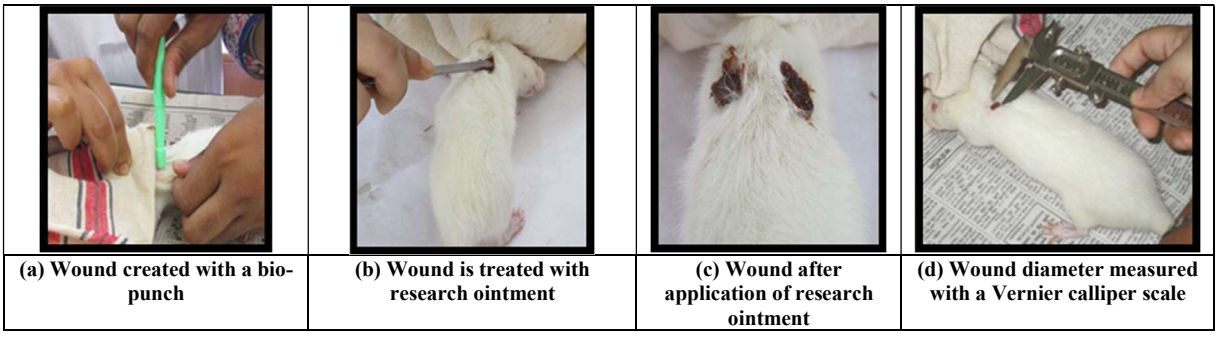
Excision wound model

Excisional wound is one of the most commonly used wound healing models since it is resembling acute clinical wounds, which require healing by second intention, that is the skin edges are not sutured together. These wounds are generated by the surgical removal of all skin layers (epidermis, dermis and subcutaneous fat) from the animal. This model allows the investigation of haemorrhage, inflammation, granulation tissue formation, re-epithelialisation, angiogenesis and remodelling. The wound area can be regularly photographed over time, and wound healing rate or wound closure is calculated based on the wound size relative to its original dimensions. For performing histological analysis, animals can either be euthanized (e.g. mice, rats) or locally anesthetized (e.g. rabbit ear model) and biopsies collected, processed and examined for both the epithelial gap (the quantifiable distance between the epithelial wound margins), granulation bed characteristics (recruited cell populations, vascularity and matrix alterations) and collagen organization.[20]

The lower dorsum of every animal was shaved and cleaned with 70 % liquor. Two round injuries were then made on each side of the lower lumbar spine utilizing aseptic strategies of 6 mm on left side and 8 mm on right side of vertebral column using punch biopsy. The punch biopsy (and going with surgical blade sharp edge extraction) evacuated the whole epidermis, dermis and shallow sash (subcutaneous fat, connective tissue and cutaneous trunci muscle) over the dorsal gluteal muscle district. All animals were treated with the prescribed ointment (placebo, standard or 5% / 10% research formulation) once every day till complete healing of the wounds up to maximum 14 days as shown in figure 1. The progressive changes in the wounded areas, wound width and physical appearance of wounds were monitored on Days 4, 7 and 14. All experimental animals were euthanized utilizing the CO_2 strategy and all injuries were analysed for proof of contamination or neighbourhood/foundational prejudice to treatment.[3, 21, 22] The wound contraction was calculated as a percentage of the reduction in wounded area. A specimen sample tissue was isolated from the healed skin of each group of mice for histopathological examination.

$$\% \text{ Wound contraction} = \left[\frac{\text{Healed area}}{\text{Total wound area}}\right] * 100$$

where Healed area = original healed area – present wound area



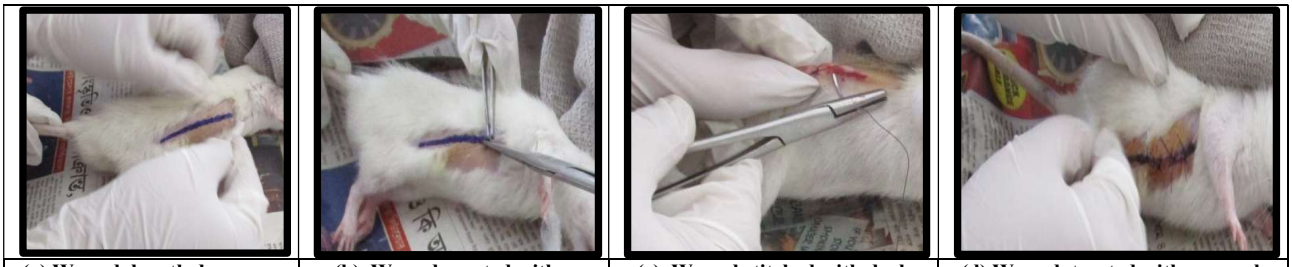
(a) Wound created with a bio-punch	(b) Wound is treated with research ointment	(c) Wound after application of research ointment	(d) Wound diameter measured with a Vernier calliper scale

Figure 1: Different stages of wound during excision model study

Incision wound model

Incisional wounds can be classified as primary or secondary closure, and sutured immediately after wound infliction or not respectively. Primary closure or first intention is an excellent model for biomechanical analysis of wound strength and less suited for histologic assessment of healing, or evaluation of wound tissue biochemistry or epithelialization, due to the limited volume of wound healing activity.[20]

The hair on the back of anesthetized rats was removed using a shaving machine. A longitudinal paravertebral cut of 6cm length was made through the full thickness of the skin 1.5 cm away from the midline on each side of the vertebral column with the assistance of a sharp surgical tool. After complete haemostasis, the injury was sewed utilizing dark silk careful thread (number 000) and a bent needle (number 11) making stitches around 1cm apart. All the sutures were non-absorbable, braided, non-capillary and siliconized. After sewing, the injury was left uncovered and all animals were treated with the prescribed ointment of their group once every day for the next 10 days. On the 10th day, all rodents were sacrificed using the overdose of ether in the laboratory, stitches were removed, and elasticity of restored wound skin was estimated utilizing a tensiometer as shown in figure 2. A sample of tissue taken from the healed wound area was fixed in 10% formalin and embedded in paraffin wax. Serial sections (5 μm thickness) of paraffin-embedded tissues were cut using the microtome instrument which were stained with haematoxylin and eosin, and examined by light microscope (Olympus BX51). Ulceration, necrosis and epithelisation were evaluated in the skin tissues. Congestion, oedema, PNL, mononuclear cells, fibroblasts and vascularization were also qualitatively evaluated as −, +, ++ and +++.[5]



(a) Wound length drawn on rodent	(b) Wound created with a surgical blade	(c) Wound stitched with dark silk careful thread and a bended needle	(d) Wound treated with research ointment

Figure 2: Different stages of wound during incision model study

Statistical Analysis of data

The data obtained regarding percentage of wound healing was statistically analysed using one-way analysis of variance (ANOVA). The values of $p \leq 0.01$ were considered as statistically significant. The histopathologic data was considered non-parametric and hence no statistical tests were performed.

Table 1: Results of physiochemical analysis

Parameter	RFNA	RFUL
Total Ash value (in % w/w)	9.43	8.83
Acid insoluble Ash (in % w/w)	1.89	0.77
Water soluble Ash (in % w/w)	7.65	8.16
pH value	4.62	5.29
Moisture Content (in % w/w)	7.60	8.20

Table 2: Extractive values (in % w/w) of research formulations

Research formulation	Petroleum-Ether	Ethyl acetate	Chloroform	Acetone	Alcohol	Aqueous
RFNA	0.532	0.857	3.201	1.751	3.748	2.91
RFUL	0.846	0.926	0.165	1.25	2.70	2.64

Table 3: Estimation of Total Flavonoid Content and Total Phenol Content

Sl. No.	Parameter	Aqueous Extract of RFNA	Aqueous Extract of RFUL
1.	Flavonoid content (µg Quercetin equivalent / mg of extract) following the standard curve ($R^2 = 0.999$)	54.76	59.14
2.	Phenol content (µg Gallic acid equivalent / mg of extract) following the standard curve ($R^2 = 0.997$)	205.00	225.67

Table 4: Physical Evaluation of parameters in Excision Model

Day	Group A	Group B	Group C	Group D	Group E	Group F
0 Day	Wound colour light pink	Wound colour light pink	Wound colour light pink	Wound colour light pink	Wound colour light pink	Wound colour light pink
4th Day	Slight dark pink with pus on the edges (++++) 80	Slight dark pink no pus	Slight dark pink with pus on the edges (++) 40	Slight dark pink with pus on the edges (+) 20	Slight dark pink with pus on the edges (++) 45	Slight dark pink with pus on the edges (+) 22
7th Day	Brown colour with pus (++++) 82	Dry brown skin no pus	Dry brown skin no pus	Dry brown skin no pus	Dry brown skin no pus	Dry brown skin no pus
14th Day	Dark Brown with pus (++++) 85	Healed skin	Almost healed brownish skin	Healed skin	Almost healed brownish skin	Healed skin

Table 5: Mean wound diameter & percentage inhibition of left wound on 4th, 7th and 14th day

Group	Initial Left Wound size (mm)	Day 4th		Day 7th		Day 14th	
		Wound Diameter (mm)	Percentage Inhibition	Wound Diameter (mm)	Percentage Inhibition	Wound Diameter (mm)	Percentage Inhibition
Group A	6	5.76 ±0.049	4.0	5.004 ±0.078	16.6	4.08 ±0.060	32.0
Group B	6	5.04 ±0.07	16.0	2.10 ±0.076	65.0	0 ±0	100
Group C	6	4.20 ±0.08	30.0	2.26 ±0.073	62.33	0.25 ±0.022	95.8
Group D	6	4.20 ±0.06	30.0	2.10 ±0.089	65.0	0.24 ±0.037	96.0
Group E	6	4.26 ±0.14	29.0	2.40 ±0.269	60.0	0.34 ±0.051	94.3
Group F	6	4.150 ±0.07	30.83	2.26 ±0.249	62.33	0.22 ±0.030	96.2

Table 6: Mean wound diameter & percentage inhibition of right wound on 4th, 7th & 14th day

Group	Initial right Wound Size (mm)	Day 4th		Day 7th		Day 14th	
		Wound Diameter (mm)	Percentage Inhibition	Wound Diameter (mm)	Percentage Inhibition	Wound Diameter (mm)	Percentage Inhibition
Group A	8	7.747 ±0.045	3.16	6.72 ±0.131	16.0	5.60 ±0.196	30.0
Group B	8	6.08 ±0.04	24.0	3.20 ±0.252	60.0	0 ±0	100
Group C	8	6.0136 ±0.04	24.83	3.60 ±0.260	55.0	0.24 ±0.041	97.0
Group D	8	5.84 ±0.04	27.0	3.36 ±0.241	58.0	0.06 ±0.020	99.25
Group E	8	6.08 ±0.03	24.0	3.76 ±0.305	53.0	0.21 ±0.0.45	97.37
Group F	8	5.856 ±0.03	26.8	3.416 ±0.208	57.3	0.16 ±0.024	98.0

Table 7: Physical Evaluation of parameters in Incision Model

Day	Group A	Group B	Group C	Group D	Group E	Group F
0 Day	Wound colour light pink	Wound colour light pink	Wound colour light pink	Wound colour light pink	Wound colour light pink	Wound colour light pink
4th Day	Slight dark pink with pus on the edges (++++) 90%	Slight dark pink no pus	Slight dark pink with pus on the edges (++) 35%	Slight dark pink with pus on the edges (+) 19%	Slight dark pink with pus on the edges (++) 40%	Slight dark pink with pus on the edges (++) 20%
7th Day	Brown colour with pus (+++) 82%	Dry brown skin no pus	Dry brown skin no pus	Dry brown skin no pus	Dry brown skin no pus	Dry brown skin no pus
14th Day	Dark Brown with pus (++) 78%	Healed skin	Almost healed brownish skin	Healed skin	Almost healed brownish skin	Healed skin

Table 8: Mean wound size & percentage inhibition on 4th, 7th & 14th day during Incision wound model study

Groups	Initial wound length (cm)	4th Day	7th Day	14th Day
		Wound Size	Wound Size	Wound Size
Group A	6	5.88 ±0.020	5.28 ±0.088	4.92 ±0.055
Group B	6	4.44 ±0.102	1.32 ±0.107	0 ±0mm
Group C	6	4.68 ±0.076	2.82 ±0.085	0.3 ±0.049
Group D	6	4.32 ±0.060	2.34 ±0.111	0.12 ±0.017
Group E	6	4.62 ±0.070	2.86 ±0.076	0.37 ±0.042
Group F	6	4.48 ±0.047	2.44 ±0.100	0.19 ±0.027

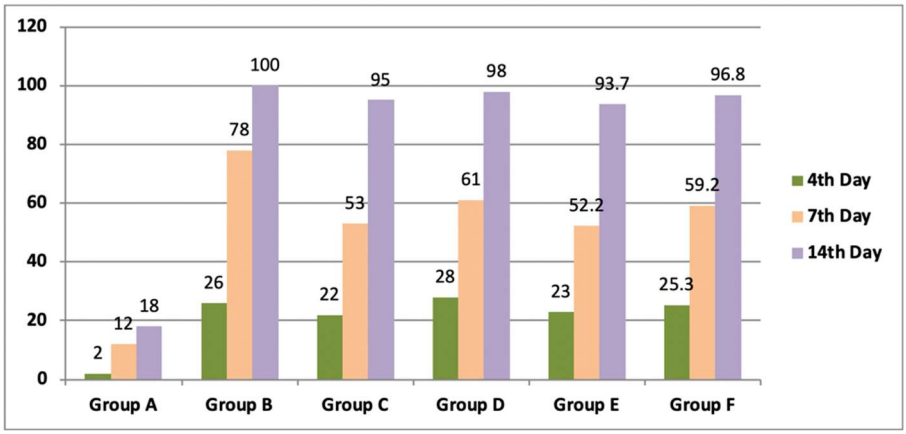


Figure 3: Percentage contraction of wound on 4th, 7th and 14th day during incision wound model study

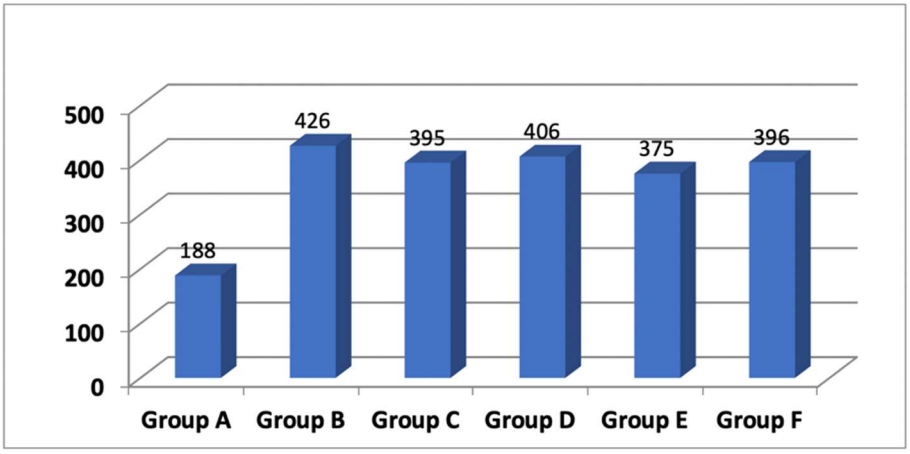
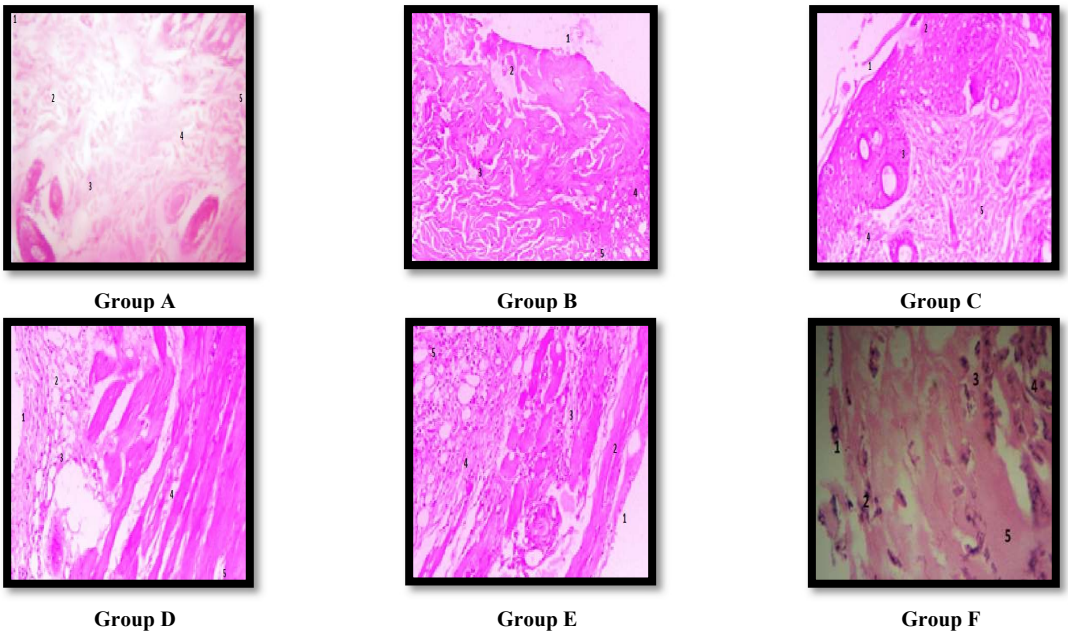


Figure 4: Tensile strength of restored skin in various treatment groups



1.Epithelization; 2. Polymorphonuclear leukocytes (PMNL); 3. Fibroblast; 4. Vessels; 5. Collagen

Figure 5: Histopathological study of the skin using microscope after 14 days in the incision model

RESULTS

Pharmacognostical studies

The results obtained after the physiochemical analysis are given in Table 1 while the extractive values using different solvents are detailed in Table 2.

Phytochemical screening

The preliminary phytochemical analysis highlighted the presence of alkaloids, flavonoids, tannins and carbohydrates in both the aqueous and alcoholic extracts while saponins were found only in the aqueous extract. Fixed oil and fats were found to be present in the petroleum ether, ethyl acetate and chloroform extracts.

Determination of total phenol content and total flavonoid content

The total flavonoid content (TFC) and the total phenol content (TPC) were calculated from the absorbance calibration curves generated using different concentrations of Quercetin and Gallic acid respectively and the obtained data are shown in Table 3.

Excision wound model

During this study, physical parameters such as appearance, colour and amount of pus formation in the wound and its surrounding areas was assessed as shown in Table 4 using an arbitrary scoring scale where the symbols indicate (+) →up to 25%, (+ +) →up to 50%, (+ ++) →up to 75%, and (++++) →up to 100% severity.

The size of wounds created on both the left and right side were systematically studied for all the treatment groups during the study period. The mean contracted wound diameter size and the percentage inhibition observed with reference to the initial values of the left and right wound are shown in tables 5 and 6 respectively.

Incision wound model

During this study, physical parameters such as appearance, colour and amount of pus formation in the wound and its surrounding areas was evaluated using the arbitrary scoring scale mentioned above and the results are detailed in Table 7.

The progressive reduction in wound length for different groups is shown in table 8 and their percentage contraction is depicted in figure 3.

The elasticity of the restored skin was assessed by measuring its tensile strength which is shown in figure 4.

The histopathological study of the wound area was done after 14 days and the results obtained for the various groups are shown in figure 5.

DISCUSSION

Both research formulations RFNA and RFUL had good ash values (9.43% and 8.83%), which was mostly water soluble, and pH values of 4.62 and 5.29 respectively. During the process of preparation of extracts, both formulations showed good extractive values with the commonly used solvents. Phytochemical screening indicated the presence of alkaloids, flavonoids, tannins and carbohydrates in both the aqueous and alcoholic extracts. The total flavonoid content (TFC) of RFNA and RFUL was estimated to be 54.76 and 59.14 µg Quercetin equivalent / mg, while the total phenol content (TPC) was found to be 205.00 and 225.67 µg Gallic acid equivalent / mg respectively.

During the excision wound model study, the assessment of physical condition on 4th day indicated that while Group A had 80 % pus formation, there was no pus formation in Group B, and Groups C, D, E and F showed 40%, 20%, 45% and 22% pus formation respectively. However, on the 7th day only group A had 82% pus while there was no pus formation in any other group. At the end of the observation period on 14th day, the wound had completely healed in Groups B, D and F, and almost healed in Groups C and E, while Group A still had substantial pus formation in the wound. The percentage of wound contraction in Groups B, C, D, E and F on the 14th day was noticed to be 100, 95.8, 96.0, 94.3 and 96.2 in the left wound, and 100, 97.0, 99.25, 97.37 and 98.0 in the right wound respectively as compared to very low values in case of Group A. Hence, the highest therapeutic efficacy among the research formulation groups was noticed in groups D and F which is almost same as the standard group (B), and slightly lower efficacy was seen in groups C and E which had 5% concentration of research drugs. Almost similar trends were noticed on the 4th and 7th day also.

In case of the incision wound, the visual inspection of wound condition on the 4th day indicated that while Group A had 90 % pus formation, there was no pus formation in Group B, and Groups C, D, E and F showed 35%, 19%, 40% and 20% pus formation respectively. However, on the 7th day only group A had 82% pus while there was no pus formation in any other group. At the end of the observation period on 14th day, the wound had completely healed in Groups B, D and F, and almost healed with brownish skin in Groups C and E, while Group A still had substantial pus formation. During the incision wound model study, the percent reduction in the length of wound on the 14th day was found to be 100, 95, 98, 93.7 and 96.8 in Groups B, C, D, E and F respectively while it was 78, 53, 61, 52.2 and 59.2 on the 7th day in these groups. Hence, the best treatment efficacy among the research formulation groups is noticed in Group D followed by Group F, both of which are almost similar to the standard group (B), especially after 14 days. Groups C and E are also highly effective although a little lower in efficacy as compared to groups D and F. These findings are also corroborated by the tensile strength values obtained in respect of the restored skin which are 426.66, 406.66, 396.33, 395.83 and 375.50 gm/mm^2 in case of Groups B, D, F, C and E respectively.

During the incision wound model study, the histopathological study of the skin was also done and the results of the microscopic examination for the various groups reveal the differences in the various healing stages. Physiological wound healing is schematically divided into three partially overlapping phases: inflammation, proliferation and remodelling, that are determined by interacting events on a molecular, cellular and extracellular matrix level that ends with wound closure within days or weeks. An incisional wound is initially held together with suture material, but it must gain enough inherent strength to maintain closure. As the healing process continues, the fibres further organize by forming a dense three-dimensional matrix whose formation is mediated by interactions between the collagen fibres and the surrounding extracellular matrix components.[1, 2, 4] The wound's strength grows rapidly as these fibres cross-link and aggregate into larger fibres which improves the tensile strength of incisional wounds because the fibres bind to cell-membrane proteins across the wound interface. Remodelling of the incised wounds happens when collagen is aligned along tension lines and water is reabsorbed so that the collagen fibres can lie closer together and cross-link, which reduces scar thickness and also makes the skin area of the wound stronger and increases its tensile

strength.[20, 21] Overall much higher progress in all these stages was observed in groups B, D and F followed by groups C and E as revealed by the microscopic examination.

CONCLUSION

During this study, the higher concentration (10%) of both types of research formulations (RFNA and RFUL) exhibited therapeutic efficacy which was almost similar to that of the standard drug. The wound healing properties of the lower concentration (5%) of RFNA and RFUL were also highly significant but a little lower than the standard drug. The results of the experimental studies in both the excision model as well as the incision model indicate that 10% concentration of RFNA provides the best and highly significant wound healing effect closely followed by 10% RFUL group, and a little lower therapeutic efficacy is shown by the 5% RFNA and then the 5% RFUL group although these two groups also provide very high results as compared to the control group. These observed results could be attributed to the high concentration of phenolic and flavonoidic compounds in the research formulations since these phytochemical constituents are well known for their anti-microbial and anti-inflammatory actions and primarily responsible for inhibition of cytokine in the wound surface area thus promoting reepithelization and remodelling of wound skin.

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