ORIGINAL ARTICLE

Appraisal on the wound healing activity of different extracts obtained from *Aegle marmelos* and *Mucuna pruriens* by *in vivo* experimental models

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Abstract

Aim: The use of a simple and reproducible model is inevitable for an objective statement of the effects of external factors on wound healing. Hence, the present study was conducted to evaluate wound healing activities of sequential different extracts of *Aegle marmelos* leaves (AM) and *Mucuna pruriens* seeds (MP) by *in vivo* experimental models.

Materials and Methods: Wistar albino rats were subjected to excision, incision and dead space wounds measuring approximately 250 mm^2, 3 cm and implanting sterilized polyvinyl chloride tube on the back of each rat near either side of the vertebral column respectively. The experimental animals were randomized into eight groups (n = 6), control, standard and treatment groups. Hydrogel of different extracts were applied topically once daily. The parameters observed were percentage of wound contraction, epithelization period, tensile strength, hydroxyproline content of the granulation tissue, and histological changes during wound healing.

Results: The statistical study revealed that in excision, incision, and dead space wound models all formulations have significant (P < 0.01) wound healing potential. However, methanolic extract formulation was found to be superior to all other treatments as evidenced by rapid wound contraction, lesser number of days required for complete epithelization, increased tensile strength and significant increase in hydroxyproline content.

Conclusions: As compared to the reference standard treated group the wound healing process of the experimental groups was decelerated. All extracts obtained from AM and MP facilitated the wound healing process in all experimental models.

Key words: *Aegle marmelos*, dead space wound, excision wound, incision wound, *Mucuna pruriens*, wound healing

Date of Acceptance: 02-Dec-2015

Introduction

A wound is an injury to a part of the body, especially one in which a break is made in the skin. There are various types of wounds, including an incised wound, lacerated wound, abrasion, contusion, ulcer, and burn wound.[1] Wound healing, or wound repair, is an intricate process in which the skin (or another organ-tissue) repairs itself after injury.[2] The process of wound healing occurs in different phases such as coagulation, epithelization, granulation, collagenation, and tissue remodeling. The healing cascade is activated when platelets come into contact with exposed

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Access this article online

Quick Response Code:

Website: www.njcponline.com

DOI: 10.4103/1119-3077.181364

How to cite this article: Toppo FA, Pawar RS. Appraisal on the wound healing activity of different extracts obtained from Aegle *marmelos* and *Mucuna pruriens* by *in vivo* experimental models. Niger J Clin Pract 2016;19:753-60.

collagen leading to platelet aggregation and the release of clotting factors resulting in the deposition of a fibrin clot at the site of injury. The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing.[3] Inflammatory cells also arrive along with the platelets at the injury site providing key signals known as growth factors.[4] The fibroblast is the connective tissue cell responsible for collagen deposition required to repair the tissue injury.[5] Collagen accounts for 30% of the total protein in the human body.[6] In normal tissues, collagen provides strength, integrity, and structure. When tissues are disrupted following injury, collagen is required to repair and restore normal structure and function.

In India, there has been interesting in the potential of the medicinal plant for the development of drugs with wound healing properties as taught in a popular form of Indian medicine known as Ayurveda.[7] Recent studies with other plant extracts have shown that phytochemical constituents such as flavonoids[8] and triterpenoids[9] are known to promote the wound healing process mainly due to their astringent and antimicrobial properties, which appear to be responsible for wound contraction and increased rate of epithelization. Based on the information furnished in the literature, the main effects of the active constituents of the plant extracts toward wound healing are phytochemical constituents contributing to antimicrobial activity, phytochemical constituents working as antioxidants, active components having enhanced mitogenic activity (contributing to increased cell proliferation), angiogenesis, enhanced collagen production, and increased DNA synthesis.

Several drugs obtained from plant sources are known to increase healing in different types of wounds. Traditionally, leaves of *Aegle marmelos* (AM) (Linn.) Correa commonly known as bael (or bel), belonging to the family *Rutaceae*[10,11] and seeds of *Mucuna pruriens* (MP) Linn. commonly known as cowhage plant or kevach, belonging to the family *Fabaceae*[12,13] are applied on cuts and wounds. AM is indigenous to India and is abundantly found in the Himalayan tract, Bengal, Central and South India.[14] The leaves are having astringent, laxative, and expectorant.[15] Antimicrobial activity[16] and antioxidant activity[17] have been reported. MP is the most popular drug in the Ayurvedic system of medicine.[18] Antioxidant activity[19] and antimicrobial activity[20] have been reported.

Therefore, the aim of treating a wound is to either shorten the time required for healing or to minimize the undesired consequences.[21] Attention should be directed toward discovering an agent, which will accelerate wound healing either when it is progressing normally.[22] Thus, the purpose of this study was to evaluate wound healing activities of sequential ethyl acetate, methanol, and aqueous extracts of AM and MP in excision, incision, and dead space wound models in rats.

Materials and Methods

Materials

All the chemicals used in the study were of analytical grade. Hydroheal™ AM (Dr. Reddy's) was used as reference standard that is an amorphous hydrogel wound dressing with colloidal silver. Hydrogel without extract was taken as control and treatment groups received hydrogel formulations (HFs) of different extracts of AM leaves and MP seeds.

Identification and collection of plant material

The seeds of MP were purchased from Bhopal local market, and leaves of AM were collected in the month of September from the medicinal garden of VNS Group of Institutions, Bhopal, Madhya Pradesh. These were identified and authenticated by Dr. S. N. Dwivedi (HOD) and voucher specimens (AM: JC/B/263; MP: JC/B/264) were deposited in the herbarium of the Department of Botany, Janata PG College, APS University, Rewa, Madhya Pradesh. The seeds and leaves were washed, shade dried, powdered moderately, and stored in a well-closed container.

Extraction of crude drugs

The leaves of AM (400 g) and powdered seeds (500 g) of MP were extracted with petroleum ether (36 h) for defatting. The defatted plant materials were dried and then exhaustively extracted with ethyl acetate, methanol, and water in soxhlet apparatus successively. The completion of extraction was confirmed by evaporating a few drops of the extract on the watch glass and ensuring that no residue remained after evaporating the solvent. The extracts were concentrated under reduced pressure using a water bath keeping the temperature below 50°C to yield semisolid mass and stored in a well-closed container for further studies.

Animals

Wistar albino rats (220 ± 20 g) were procured from animal house of VNS Group of Institutions, Faculty of Pharmacy, Bhopal MP and maintained under constant conditions (temperature 25 ± 2°C, humidity 40–60%, 12 h light/12 h dark cycle). During maintenance, the animals received a diet of food pellet supplied from the animal house and water *ad libitum*. These experiments were approved by the Institutional Animal Ethics Committee, VNSFP, Bhopal, Madhya Pradesh (VNISP/IAEC/2011/6695/A).

Methods

Preparation of topical herbal formulations (hydrogel)

Gel base formulations were prepared by dispersing 1 g of carbopol 934 in 50 ml of distilled water with continuous

stirring and kept overnight to get a smooth gel. Two milliliters of distilled water was taken, and the required quantity of sodium metabisulfite was dissolved by heating on water bath. Five milliliters of distilled water was taken, and required quantity of methyl paraben and propyl paraben were dissolved by heating on water bath. The solution was then cooled to add sodium metabisulfite solution. Finally, fully mixed ingredients were mixed properly with the carbopol gel with continuous stirring. Then, the required amount of extract (2.5%) was mixed in the above mixture and its volume was increased to 100 ml by adding distilled water and triethanolamine was added dropwise to the formulation for adjusting the required skin pH (pH: 6.5–7.0) and to obtain required consistency[23,24] [Table 1].

Evaluation of topical herbal formulations

Physical appearance

All formulations were tested for their appearance, phase separation, and presence of any aggregates.[24,25]

Skin irritation study

Wistar albino rats of either sex weighing 220 ± 20 g were used for this test. The hairs were removed from the rats 3 days before the experiment. Hydrogel of different extracts were applied on test animals up to 7 days and finally the treated skin was examined visually for erythema and edema.[25,26]

Evaluation of wound healing activity

Excision wound model

The wound site was prepared following the excision wound model. Excision wounds were made as described by Morton and Malone.[27] Surgical interventions were carried out under sterile conditions using diethyl ether. A circular wound of about 250 mm^2 was made on depilated ethanol-sterilized dorsal thoracic region of rats. Animals were closely observed for any infection and those which showed signs of infection were separated and excluded from the study and replaced. Wistar albino rats (220 ± 20 g) of either sex were divided into eight groups of six rats each. Group I animals were considered as control group; Group II animals were served as reference standard and were treated with Hydroheal; Group III animals were treated with HF-1; Group IV animals were treated with HF-2; Group V animals were treated with HF-3; Group VI animals were treated with HF-4; Group VII animals were treated with HF-5 and Group VIII animals were treated with HF-6. Hydrogel of different extracts were applied topically once daily.

An excision wound margin was traced after wound creation by using transparent paper and area measured by graph paper. Wound contraction was measured in each 2 days interval and epithelialization time was measured from the initial day.[28,29]

Incision and dead space wound model

The effect of the test drug on the incision and dead space wound was evaluated by noting effect on the formation of granulation tissue in subcutaneously implanted polyvinyl chloride (PVC) tube. Selected animals were randomly divided into eight groups of six each as mentioned in excision wound model. A midline incision of 3 cm was made and a tunnel was created subcutaneously in which sterilized PVC tube (2 cm length and 1 mm diameter) was inserted and the incision was closed with the help of two interrupted sutures as described by Ehrlich and Hunt.[30] Hydrogel was filled in the PVC tube before implantation and also applied locally over the incision wound daily for 12 consecutive days. The sutures were removed on the 8th postwound day and skin breaking strength was measured on the 12th day by the method described by Lee.[31] The anesthetized animal was secured to the table and a line was drawn on either side of the wound 3 mm away from the suture was gripped with forceps was supported firmly, whereas the other was connected to a freely suspended lightweight measuring jar. Water was slowly added continuously until the wound began to gap. As soon as wound gapping appeared the addition of water was stopped. The volume of water was determined and noted as a measure of breaking strength in grams. The granulation tissue harvested on the implanted tube was carefully dissected out along the tube. Tubular granulation tissue was cut lengthwise to obtain a sheet of granulation tissue, dried at 60°C for 24 h to get a constant weight and weighed. The tissue was then used for the determination of hydroxyproline.[32] Along this, the tissue of the incision wound was also preserved for histopathological study.

Histopathological studies

Wound tissue specimens were fixed in formalin and processed for paraffin embedding. Sections of 5 μ thickness were stained[8] and qualitatively assessed under the light microscope (Labomed, CXR3, Labo America, Inc., CA, USA).[33,34]

Statistical analysis

The experimental results were expressed as the mean ± standard error of mean and the statistical significance was evaluated by one-way analysis of variance followed by Dunnett's *t*-test using the software GraphPad InStat (GraphPad Software, Inc., CA, USA).

Results

The percentage yield of powdered leaves of AM and powdered seeds of MP extracts viz., ethyl acetate, methanol, and aqueous extract were found to be 3.54% w/w, 12.44% w/w, 6.10% w/w, 2.80% w/w, 4.90% w/w and 13.08% w/w [Table 2]. The physicochemical properties

Table 1: Formula of the composition of medicated topical herbal formulations (hydrogel)

Compositions → / Formulations ↓	Carbopol 934 2%	Methyl paraben 0.02%	Propyl paraben 0.002%	Sodium metabisulphite 0.2%	Triethanolamine QS	Purified water QS	Extracts 2.5%
HF-1	✓	✓	✓	✓	✓	✓	AM-EA*
HF-2	✓	✓	✓	✓	✓	✓	AM-MeOH*
HF-3	✓	✓	✓	✓	✓	✓	AM-AQ*
HF-4	✓	✓	✓	✓	✓	✓	MP-EA*
HF-5	✓	✓	✓	✓	✓	✓	MP-MeOH*
HF-6	✓	✓	✓	✓	✓	✓	MP-AQ*

*AM-EA=Aegle marmelos ethyl acetate extract; * AM-MeOH=Aegle marmelos methanolic extract; * AM-AQ=Aegle marmelos aqueous extract; *MP-EA=Mucuna pruriens ethyl acetate extract; * MP-MeOH=Mucuna pruriens methanolic extract; * MP-AQ=Mucuna pruriens aqueous extract

Table 2: Percentage yield of *Aegle marmelos* and *Mucuna pruriens*

Extracts	Yield (%)	
	Aegle marmelos	*Mucuna pruriens*
Ethyl acetate	3.54% w/w	2.80% w/w
Methanol	12.44% w/w	4.90% w/w
Aqueous	6.10% w/w	13.08% w/w

Table 3: Evaluation parameters of prepared topical herbal formulations (hydrogel)

Formulations	Appearance	Homogeneity	Consistency	Phase separation
HF-1	Green	Good	Excellent	None
HF-2	Green	Good	Excellent	None
HF-3	Green	Good	Excellent	None
HF-4	Brown	Good	Excellent	None
HF-5	Brown	Good	Excellent	None
HF-6	Brown	Good	Excellent	None

of topical herbal formulations are shown in Table 3. The physical appearances of topical herbal formulations of AM and MP extracts were found to be green and brown in color, respectively. From the results, it is evident that all topical herbal formulations showed uniform homogeneity and consistency. The results of skin irritancy studies indicated that the topical herbal formulations were free from dermatological reactions. All the formulations did not produce any skin irritation, i.e., erythema and edema for about 72 h when applied over the skin.

Excision wound model

The wound healing contracting ability of hydrogel was found to be significantly greater than that of the control. The animals treated with hydrogel showed an increase in the percentage of wound contraction when compared with control animals, all results were found significant ($P < 0.01$). The HF-2, HF-4, and HF-5 treated groups showed significant wound healing from the 4th day onwards, which was comparable to that of the standard group. The wound area was lesser, as well as the percentage of wound contraction was much more with the HF-2, and HF-5 treated groups (6.6 ± 0.71 [97.46%] and 8.5 ± 0.42 [96.67%]) as compared to control group (99.0 ± 1.29 [62.57%]) on 18th day [Table 4].

The mean period of epithelialization in the control group was 27.5 ± 0.42 days. This was significantly ($P < 0.01$) shorter in the standard group and test groups when compared with control group. The mean period of epithelialization was significantly ($P < 0.01$) reduced in animals of groups treated with HF-2 and HF-5 (18.8 ± 0.47 and 19.3 ± 0.33 days) when compared with control group [Table 5].

Incision and dead space wound model

The tensile strength [Table 6] of Group IV treated with HF-2 and the Group II treated with reference standard drug was comparable to each other. The Groups VI and VII treated with HF-4 and HF-5 showed a lesser, but significant increase in the tensile strength compared to the control group. Methanolic extract of AM and MP showed a significant ($P < 0.01$) increase in tensile strength in the 12-day-old wound.

In dead space wound model, there was significant ($P < 0.01$) increase in hydroxyproline content (mg/g tissue). This indicates improved collagen maturation by increased cross-linking. While an increase in hydroxyproline content in test groups indicates increased collagen content, since hydroxyproline is the direct estimate of collagen synthesis it supports the wound healing activity of AM and MP.

Histopathological evaluation

Histopathological examination of the wound sections in excision and incision wound model the main activity observed was the regeneration of cells, re-epithelialization,

Table 4: Effect of reference standard drug and different topical herbal formulations (hydrogel) on wound contraction in excision wound model

Post- wounding (days)	Wound area (mm^2) (mean±S.E.M.) and percentage wound contraction							
	Group I (Control)	Group II (Standard)	Group III (HF-1)	Group IV (HF-2)	Group V (HF-3)	Group VI (HF-4)	Group VII (HF-5)	Group VIII (HF-6)
0	264.5±0.56	257.6±0.76**	260.1±0.79**	260.6±0.49**	259.8±0.70**	260.8±0.54**	255.5±0.66**	258.1±0.54**
2	224.5±0.61 (15.12%)	207.3±0.88 (19.52%)**	213.1±0.60 (18.06%)**	211.1±0.47 (18.99%)**	219.6±0.61 (15.47%)**	213.5±0.50 (18.13%)**	213.6±0.42 (16.39%)**	220.0±0.73 (14.76%)**
4	195.8±0.47 (25.97%)	151.6±0.76 (41.14%)**	172.6±1.85 (33.64%)**	164.5±0.61 (36.87%)**	186.1±0.79 (28.36%)**	165.3±0.71 (36.61%)**	158.6±0.71 (37.92%)**	176.8±0.94 (31.49%)**
6	158.0±0.51 (41.85%)	117.6±0.49 (54.29%)**	144.3±0.66 (44.52%)**	123.0±0.57 (52.80%)**	146.5±0.61 (43.61%)**	128.0±0.57 (50.92%)**	123.3±0.66 (51.74%)**	145.5±0.61 (43.62%)**
8	153.8±0.60 (41.85%)	88.1±0.60 (65.79%)**	113.5±0.61 (56.36%)**	96.8±0.30 (62.85%)**	143.5±0.76 (44.76%)**	109.0±0.44 (58.20%)**	100.6±0.66 (60.62%)**	124.0±0.57 (51.95%)**
10	144.0±0.57 (45.55%)	53.8±0.47 (79.11%)**	84.3±0.76 (67.58%)**	59.5±0.71 (77.16%)**	104.1±0.30 (59.93%)**	63.8±0.60 (75.53%)**	69.6±0.55 (72.75%)**	100.1±0.70 (61.21%)**
12	133.6±0.88 (49.48%)	31.8±0.47 (87.65%)**	62.3±0.66 (76.04%)**	40.5±0.76 (84.45%)**	84.6±0.55 (67.43%)**	46.5±0.56 (82.17%)**	44.3±0.76 (82.66%)**	66.8±0.60 (74.11%)**
14	121.6±0.88 (54.02%)	16.1±0.60 (93.75%)**	40.6±1.05 (84.39%)**	21.5±0.76 (91.74%)**	70.8±0.90 (72.74%)**	36.3±0.76 (86.08%)**	29.8±0.87 (88.33%)**	41.5±1.11 (83.92%)**
16	110.6±1.28 (58.18%)	4.8±0.60 (98.13%)**	26.5±0.76 (89.81%)**	12.5±0.99 (95.20%)**	54.0±1.23 (79.21%)**	17.6±0.66 (93.25%)**	15.6±1.14 (93.89%)**	27.6±0.88 (89.30%)**
18	99.0±1.29 (62.57%)	00±00 (100%)**	18.1±0.94 (93.04%)**	6.6±0.71 (97.46%)**	34.0±0.73 (86.91%)**	12.1±0.60 (95.36%)**	8.5±0.42 (96.67%)**	18.0±0.96 (93.02%)**

Values are presented as mean±S.E.M; $n=6$; $^{**}P<0.01$

Table 5: Epithelization periods in excision wound model

Groups	Period of epithelization (days)
Group I (control)	27.5±0.42
Group II (Standard)	15.6±0.33**
Group III (HF-1)	22.3±0.33**
Group IV (HF-2)	18.8±0.47**
Group V (HF-3)	23.8±0.30**
Group VI (HF-4)	22.5±0.22**
Group VII (HF-5)	19.3±0.33**
Group VIII (HF-6)	21.8±0.30**

Values are presented as mean±S.E.M; $n=6$; $^{**}P<0.01$

Table 6: Effect of reference standard drug and different topical herbal formulations (hydrogel) in incision wound and dead space model in rats

Groups	Incision model	Dead space model
	Tensile strength (g)	Hydroxyproline content (mg/g tissue)
Group I (control)	235.8±9.32	6.5±1.25
Group II (standard)	367.6±9.80**	56.8±1.35**
Group III (HF-1)	281.8±5.99**	15.1±0.65**
Group IV (HF-2)	354.5±9.62**	20.1±0.94**
Group V (HF-3)	266.1±4.32*	14.8±1.49**
Group VI (HF-4)	319.1±5.38**	43.3±1.14**
Group VII (HF-5)	338.3±9.62**	47.8±1.13**
Group VIII (HF-6)	269.1±6.34*	37.3±1.05**

Values are presented as mean±S.E.M; $n=6$; $^{**}P<0.01$; $^{*}P<0.05$

and proliferation of fibroblasts. The histopathological data exhibited that no topical herbal formulations induced necrosis on the wound areas. In excision wound model, re-epithelialization capacity of HF-2 and HF-5 was highest as compared to control group. In incision wound model, the control group showed poorly formed granulation tissue and sparse distribution of collagen fibers and fibroblasts. The treated animals also showed a denser distribution and better organization of blood vessels and regenerations of cells within the tissue. These changes were more prominent in groups that were treated with HF-2 and HF-5. This histopathological observation provided additional evidence for the experimental wound healing studies based on the contraction value of wound areas and the measurement of tensile strength [Figures 1 and 2].

Discussion

Plants have traditionally been used as a source of medicine in India by indigenous people of different ethnic groups inhabiting various terrains for the control of various ailments afflicting human and their domestic animals.[35,36] The results of this study revealed that methanolic extract of AM and MP has significant wound healing activity in both excision as well as incision

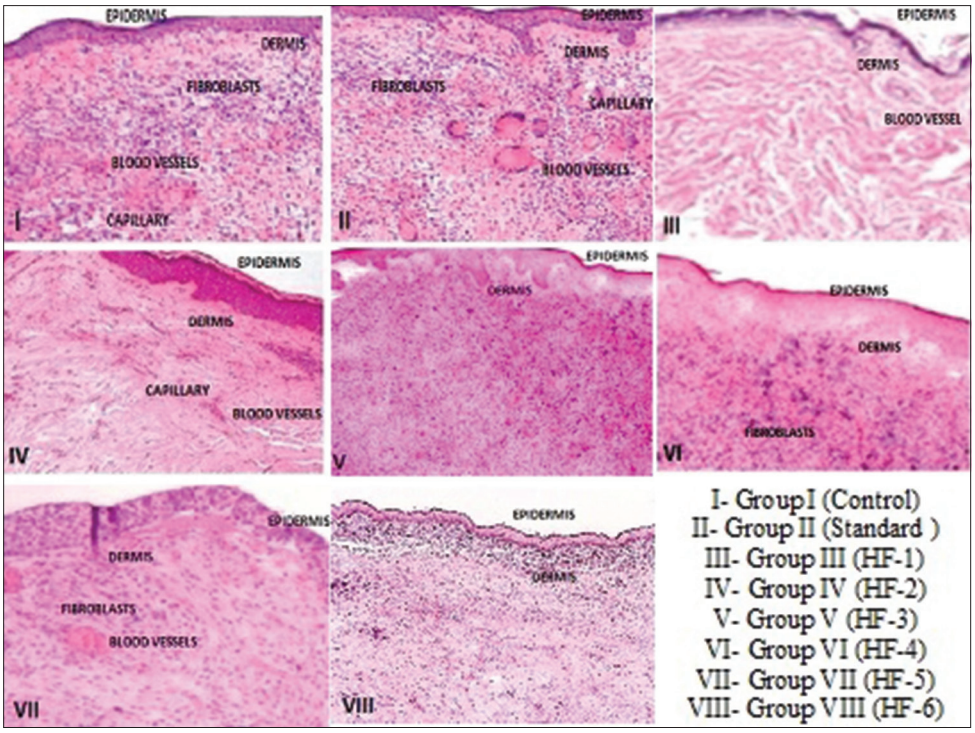

Figure 1: Histopathology of skin in excision wound rats. In excision wound model, when compared to other topical herbal formulations (hydrogel) and the control group re-epithelialization capacity of hydrogel formulations-2 and hydrogel formulations-5 was highest

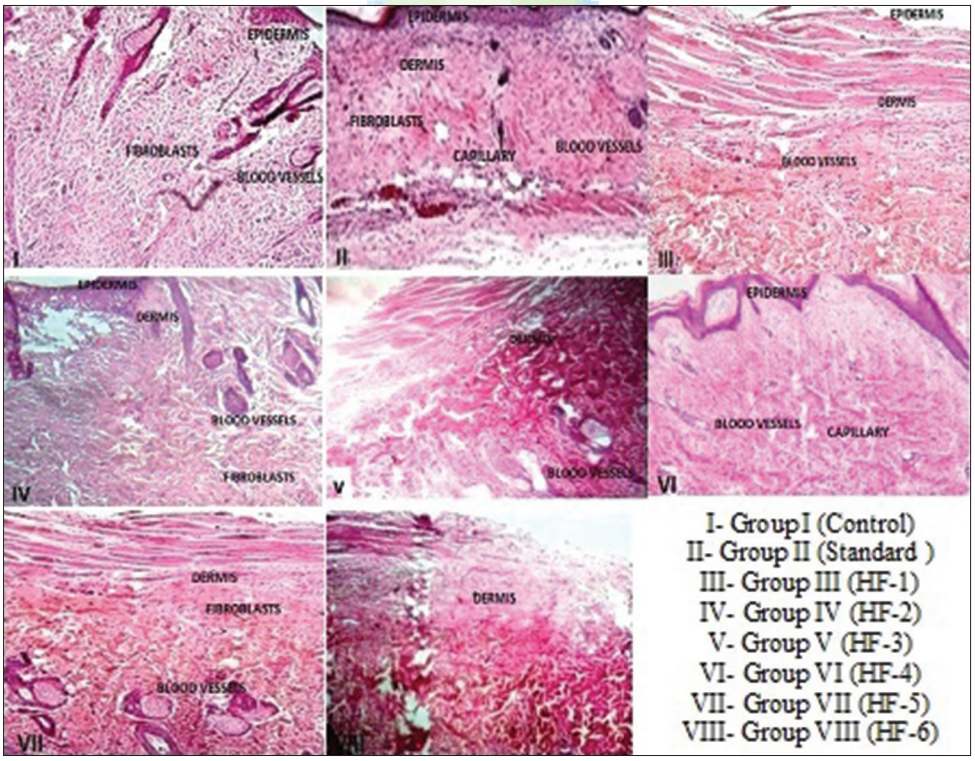

Figure 2: Histopathology of skin at day 12 in incision wound rats. The treated animals showed a denser distribution and better organization of blood vessels and regenerations of cells within the tissue. These changes were more prominent in groups that were treated with hydrogel formulations-2 and hydrogel formulations-5

wound models. In dead space wound model increased hydroxyproline content indicates increased collagen synthesis, which in turn leads to enhanced wound healing. A number of coumarins (including xanthotoxol

and alloimperatorin methyl ether), flavonoids (including rutin and marmesin), alkaloids (including alpha-fagarine), sterols, and essential oils have been isolated from plant parts of AM.[37] The seeds of MP contain the alkaloids, mucunine, mucunadine, mucunadinine, prurieninine, pruriendine and nicotine, besides beta-sitosterol, gluthione, lecithin, vernolic, and gallic acids. They contain a number of bioactive substances including tryptamine, alkylamines, steroids, flavonoids, coumarins, and cardenolides. L-3,4-dihydroxyphenylalanine is present in the seed as well as in the stem, leaves and roots.[38] It can be inferred from this study that the wound healing activity of AM and MP may probably be due to the phytoconstituents present in the plant or could be a function of either the individual or the additive effects of the phytoconstituents, which seems to be responsible for wound contraction, increase rate of epithelization, tensile strength and hydroxyproline content. No single method is adequate to represent the various components of wound healing process.[39] Wound contraction indicates the rate of reduction of the unhealed area during the healing process. Thus, the fast rate of wound closure indicates the better efficacy of the medication. The progressive reduction in wound area of different groups of animals by hydrogel of different extracts of AM and MP is presented in Table 4. The breaking strength is the strength of the healing wound measured by the amount of force required to disrupt it. At the beginning of healing process, a wound has little-breaking strength, but as it heals the breaking strength increases rapidly due to synthesize of collagen and formation of stable intra-and inter-molecular cross-linking.[40] Hence, results obtained from data conclude that methanolic extract hydrogel of AM and MP is capable of promoting wound healing activities in excision, incision and dead space wound model as compared to control.

Conclusion

Since ancient times, people have used plants and preparations thereof to accelerate the wound healing process. Herbal medicines in wound management involve disinfection, debridement and the provision of a suitable environment for the natural healing process. In fact, alternative medicine is of less toxicity and with fewer side effects compared with conventional medicine, and hence it is important to introduce a scientific validation for the medicinal effect of plants used in traditional medicine. To offer scientific proof and justification for their traditional use as wound healing agents, plant based topical remedies should exhibit multiple effects on these wound healing related physiological processes. The current study provided ample evidence of wound healing potential of hydrogel obtained from AM leaves, and MP seeds extract in terms of rapid wound contraction, a lesser number of days required for complete epithelization, increased tensile strength and a significant increase in hydroxyproline content.

Acknowledgment

I would like to thank VNS Group of Institutions, Faculty of Pharmacy, Bhopal, Madhya Pradesh, India for providing all the necessary facilities during this work and especially I also extend my thanks to Indian Council of Medical Research, Government of India for supporting this work with fellowship (ICMR-SRF).

Financial support and sponsorship

ICMR, New Delhi, India.

Conflicts of interest

There are no conflicts of interest.

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