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Wound healing potential of various successive fractions obtained from *Aegle marmelos* (AM) and *Mucuna pruriens* (MP) in acid burn wound models: An experimental animal study

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ABSTRACT

Traditionally leaves of Aegle marmelos (Linn.) Correa (Rutaceae) and seeds of Mucuna pruriens Linn. (Fabaceae) are applied on cuts and wounds. We report the acid burned wound healing activities of sequential ethyl acetate, methanol and aqueous extracts of AM and MP in acid burn wound models in rats. Forty-eight Wistar-albino rats (220±20g) were divided into eight groups (n=6) and were subjected to acid burn (100 mm^2) on the back of their necks. Animals were divided into control group, standard group and treatment groups. The hydrogel was applied topically once daily to the treatment groups. The parameters observed were epithelialization period, wound contraction % and histopathological analysis as indicative of the process of healing. After the 20^{th} day of treatment, wound closure % was 77.75% in control group, for the standard group it was 100%, for the test group III, IV, V, VI, VII and VIII it was 86.21, 94.81, 93.43, 89.66, 94.63 and 80.74%,respectively.Test groups III,IV,VI and VII showed significant ($p<0.01$)improvement in wound contraction % in comparison to control group. Similarly the period of epithelialization also decreased significantly in test groups. All extracts facilitated the wound healing process as demonstrated by a decrease in the period of epithelialization and faster wound contraction in acid burn wounds. Ethyl acetate and methanolic extract seemed to have the most active component for healing the acid burned wound.

Keywords: Rejuvenate; Bel; Kiwanch; Ameliorated acid burns; Wistar-albino rats

INTRODUCTION

Chemical, thermal, or radiation burns to the skin or other tissues produce a remarkably different healing response due to their effects on the viability of cells and tissues. Animal models of wound repair can provide reliable and reproducible information on the behavior and response of wounds to experimental therapy. Models should strive for reproducibility, clinical relevance, humane treatment and quantitative interpretation. More than 25,000 chemicals commonly used in industry, agriculture, house cleaners etc., have been identified as having the potential to cause burns. This makes chemical burns an important risk in the household and in industrial settings. The criminal use of chemicals to assault others is common. There has been an increase in the use of these chemical agents in aggressions involving domestic violence, mainly to women, spraying them on the face and body. The criminal use of chemicals to assault others is not uncommon [1, 2]. Acid burn causes a nonthermal trauma, with higher prevalence in developing countries. These burns are potentially lethal if it involves a significant proportion of the body surface.

Sulphuric acid is one of the agents most often involved in acid burns [3, 4]. Sulphuric acid burns can occur in work environments and acts of violence in places where the sale is not restricted or banned. In most cases, the non-work related sulphuric acid burns are caused by drain cleaners [5]. Sulphuric acid and its precursor, sulfur trioxide is

strong acids and cause injury by dehydration and by creating excessive heat in the tissue. It produces coagulation and necrotic eschars with thrombus formation in the lesion's microvasculature [6]. The concentration of acid that may produce permanent damage is still very unclear. Most research till date has been performed on animal models [7, 8].Chemical burns continue to pose a variety of dilemmas to the clinician managing such cases.

Medicinal plants have been used since time immemorial for the treatment of various ailments of the skin and dermatological disorders especially cuts, wounds and burns [9].Traditionally *Aegle marmelos*(Linn.) Correa (Rutaceae) leaves [10, 11] and *Mucuna pruriens* Linn. (Fabaceae) seeds [12, 13] are applied on cuts and wounds for healing. AM is commonly known as bael (or bel), is a moderate-sized, slender and aromatic tree. It is indigenous to India and is abundantly found in the Himalayan tract, Bengal, Central and South India [14]. A number of coumarins (including xanthotoxol and alloimperatorin methyl ether), flavonoids (including rutin and marmesin), alkaloids (including α-fagarine), sterols and essential oils have been isolated from plant parts of AM [15]. Two new cytotoxic furoquinoline alkaloids were isolated from the leaves of *Aegle marmelos* (Linn.) Correa; one from the total alkaloidal fraction (acid/base shake-out method) of the $CHCl_3$ extract and identified as 7, 8-dihydroxy-4-hydrofuroquinoline and named trivially as Aegelbine-A. The other new alkaloid isolated from the petroleum ether extract and identified as 4-hydro-7-hydroxy-8-prenyloxyfuroquinoline and named trivially as Aegelbine-B, together with a known alkaloid; aegeline and a known phenolic acid; ρ-hydroxybenzoic acid [16]. MP is commonly known as cowhage plant or kapikacho or kevach in Hindi, is the most popular drug in the Ayurvedic system of medicine [17]. The seeds of MP contain the alkaloids, mucunine, mucunadine, mucunadinine, prurieninine, pruriendine and nicotine, besides β-sitosterol, gluthione, lecithin, vernolic and gallic acids. Apart from alkaloids MP also contains tryptamine, alkylamines, steroids, flavonoids, coumarins and cardenolides. L-DOPA is present in the seed, stem, leaves and roots [18]. AM and MP leaf paste are applied topically for skin diseases and to reduce pain [19].

Identifying the beneficial effects of herbal plants on wound healing and tissue repair may offer tremendous opportunities to enhance the quality of life of acid burn patients. 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 acid burn wound model in rats.

MATERIALS AND METHODS

Materials

Animals

Wistar-albino rats (220±20g) were procured from animal house of VNS Group of Institutions, Faculty of Pharmacy, Bhopal MP and maintained under constant conditions (temperature 25±2 $^{\circ}$C, humidity 40-60%, 12h light/12h 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 MP (VNISP/IAEC/2011/6695/A).

Anesthesia

The rats were anesthetized with single intramuscular injections of 6 mg/kg xylazine hydrochloride (Indian Immunologicals Ltd.) and 85 mg/kg ketamine hydrochloride (Neon Laboratories Ltd).

Chemicals

All the chemicals used in the study were of analytical grade. Hydroheal^{TM} AM (Dr. Reddy's) was used as reference standard which is an amorphous hydrogel wound dressing with colloidal silver.

Collection of plant material

The seeds of *Mucuna pruriens* were purchased in the month of November from Bhopal, Madhya Pradesh (India) local market and leaves of *Aegle marmelos* were collected in the month of September from the medicinal garden of VNS Group of Institutions, Bhopal, Madhya Pradesh (India). The seeds and leaves were washed, shade dried, powdered moderately and stored in well closed container.

Extraction and preparation of topical herbal formulations (Hydrogel)

The leaves of AM and seeds of MP were authenticated by Dr. S. N. Dwivedi (HOD), voucher specimens (*Aegle marmelos* (JC/B/263), *Mucuna pruriens* (JC/B/264)) as herbarium were deposited in the Department of Botany, Janata PG College, A.P.S. University, Rewa (M.P.). The leaves of AM (400 gm) and powdered seeds (500 gm) of

MP were extracted in soxhlet assembly for 36 h with petroleum ether 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 keeping the temperature below 50 $^{\circ}$C to yield semisolid mass and were stored in the well-closed container for further studies.

The gel base formulation hydrogel of different extracts of AM and MP was prepared using the method described by Khan et al., 2013 [20]. Hydrogel was prepared by properly mixing the hydrogel base (97.5%) and extract (2.5%) with continuous stirring to obtain the required consistency [21].

Methods

Wistar-albino rats (220±20g) of either sex were divided into eight groups of 6 rats each. Each rat was kept in a separate cage with free access to standard laboratory diet and water.

1. Group I animals were considered as control group;
2. Group II animals served as the reference standard and were treated with hydroheal;
3. Group III animals were treated with AM ethyl acetate extract formulation (AMEA);
4. Group IV animals were treated with AM methanol extract formulation (AMM);
5. Group V animals were treated with AM aqueous extract formulation (AMA);
6. Group VI animals were treated with MP ethyl acetate extract formulation (MPEA);
7. Group VII animals were treated with MP methanol extract formulation (MPM) and
8. Group VIII animals were treated with MP aqueous extract formulation (MPA).

The rats were anesthetized, their backs were shaved and acid burn wounds were induced on the back of their necks of about 100mm^2 by pouring two drops of concentrated sulphuric acid with the help of 1ml pipette. Then rats were maintained to recover from anesthesia and immediately after recovery, the wound was rinsed with the normal saline solution. The test substances (hydrogel of extracts) were applied topically once daily, until complete healing.

The acid burnt area was measured immediately and thereafter on alternate day using millimeter-scale graph paper. The degree of wound healing was calculated as described below.

Gross examination of the acid burns wound lesion

The wound was grossly examined on alternate day after acid burn injury. The lesion of the wounds was examined using the following criteria: wound bed, color, exudates, swelling of the wound surface, and the consistency of tissues surrounding the wound.

Assessment of the degree of healing of burn wounds

Wound contraction which mainly contributes to wound closure was studied by tracing the raw wound area on transparent paper every alternate day till wounds were completely covered with epithelium. These wound tracings were retraced on a millimeter scale graph paper, to determine the wound area. Wound contraction (WC) was calculated as a percentage change in the initial wound size i.e., WC (%) = initial wound size-specific wound size/ initial wound size x 100 [22].

Epithelialization period was monitored by noting the number of days required for eschars to fall away, leaving no raw wound behind. Falling of scab leaving no raw wound behind was taken as the end-point of complete epithelialization and the days required for this were taken as the period of epithelialization [23].

Histopathological examination of acid burn wounds

In order to collect samples of the healed skin for the ascertaining epithelialization process, animals were anesthetized with ketamine i.p. (85 mg/kg). A specimen of the skin (0.5 × 0.5cm) was taken from the middle of the acid burnt area. The collected samples were fixed in 10% buffered formalin for at least 24 h, progressively dehydrated, embedded in paraffin under vacuum, sectioned at 5µm thickness, deparaffinized and sections were stained with hematoxylin and eosin dyes, sections were examined using a light microscope (Labomed CXR3-Labo America, Inc.) [23].

Statistical analysis

Results are presented as mean ± standard error of mean (SEM). Differences among experimental groups were compared by one-way analysis of variance (ANOVA), followed by Dunnett's *t*-test using the software Graph Pad Instat (Graph Pad Software, Inc., USA).

RESULTS AND DISCUSSION

There was no change in food intake, water consumption and body weight of the animals subjected to burn injury. The wound lesions showed swelling immediately after the acid burn injury. Subsequently the lesions became necrotic and started covering crust from day 6. The acid burn sizes differ between day 1 and day 6. The wounds size decreased from day 8 onwards. Complete and healthy healing was observed in group III, IV, VI, VII and VIII. The group treated with AM aqueous extract formulation (AMA) showed signs of edema, the presence of koilocytes noticed in the re-epithelialized areas. This was further substantiated from the gross examination of the pictures of wound contraction in acid burned wound lesion (Figure 1).

The wound areas were measured at alternate day after acid burn injury. The results are listed in Table 1 and are expressed as mean ± standard error of mean (SEM). No important change in contraction of the wounds was observed in the first three days of treatment (as these are the days when inflammatory processes take place). The cellular proliferation was observed after day 3, and the significant reduction in the wound areas was achieved at days 4, 6, 8 and 10. It was noticed that the treatment with the hydrogel of different extracts of AM and MP shows favorable and even effects from the 12th day of the experiment and all results were found to be significant ($p<0.01$) when compared with the control group. After 20 days of treatment, wound closure% for control group was 24.6±0.98 (77.75%), for standard group it was 0±0 (100%), for the test group III, IV, V, VI, VII and VIII it was 13.8±1.10 (86.21%), 5.3±0.66 (94.81%), 6.6±0.84 (93.43%), 10.6±0.66 (89.66%), 5.6±0.95 (94.63%) and 20.6±0.98 (80.74%).

The mean period of epithelialization in the control group was 24.0±0.51 days. This was significantly shorter in the test groups when compared with control group. The mean period of epithelialization was significantly ($p<0.01$) reduced in group III, IV, VI and VII (18.3±0.61, 17.6±0.33, 20.6±0.66 and 20.5±0.42 days) when compared with control group (Table 2).

The histopathological examination of the skin samples prelevated from the affected areas that were treated with the hydrogel of AM and MP revealed the presence of newly formed well-structured capillary vessels and tissue regeneration (Figure 2a-h). In test group V and VII it was observed that there is slow regeneration with few changes in inflammatory reaction showing incomplete healing (Figure 2e &2h).

The histopathological evaluation of the skin samples of the control group showed that there was an intense inflammatory reaction with increased neovascularization and appearance of loose collagen fibers and a few fibroblasts. In test group III there was less intense inflammatory reaction with moderate neovascularization (Figure 2c). In the test group IV there was more intense inflammatory reaction with moderate neovascularization. Regenerative and reparative attempts in the epidermal layer were also observed (Figure 2d). In the test group VI there was an inflammatory reaction which is less intense with newly formed blood vessels (Figure 2f). In the test group VII there was more intense inflammatory reaction with few new blood vessels and increased deposition of fibroblasts (Figure 2g).

AM and MP contain a number of bioactive substances including flavonoids [15, 18]. Studies have shown that constituent like flavonoids are known to promote the wound-healing process mainly due to their antimicrobial properties, which appear to be responsible for wound contraction and increased rate of epithelialization [24].The higher the flavonoids content the stronger the antioxidant activity. Flavonoids can scavenge the reactive oxygen species (super-oxide anions) and free radicals produced. These reactive intermediates are potentially implicated in delayed wound healing [25]. Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis, but also by improving vascularity. Lipid peroxidation is an important process in several types of injuries like burns, inflicted wound and skin ulcers. A drug which inhibits lipid peroxidation is believed to increase the viability of collagen fibrils [26]. Collagen is the main component of fibrous and cartilage tissue which provides structural support and its synthesis is stimulated by various growth factors which in turn hasten wound healing process [27]. Angiogenesis in granulation tissues improves circulation to the wound site, thus providing

oxygen and nutrients essential for the healing process [28] that include re-epithelization. Stimulation of epithelial cell proliferation and angiogenesis are important for wound healing process [29].

Table 1: Effect of *Aegle marmelos* and *Mucuna pruriens* extracts on wound healing % in experimental rats.

Groups Days	I	II	III	IV	V	VI	VII	VIII
0 day	110.6±1.28	104.1±0.30**	100.1±0.70**	102.3±0.80**	100.6±0.66**	102.6±0.66**	104.3±1.20**	107.0±0.85*
2 day	108.3±1.40 (2.07%)	101.6±0.61** (2.40%)	97.3±0.84** (2.79%)	99.6±0.61** (2.63%)	98.3±0.95** (2.28%)	100.5±0.71** (2.04%)	102.0±1.03** (2.20%)	104.0±1.15* (2.80%)
4 day	106.0±1.15 (4.15%)	99.3±0.42** (4.61%)	95.1±0.83** (4.99%)	97.5±0.5** (4.69%)	96.1±0.83** (4.47%)	98.6±0.66** (3.89%)	100.3±1.20** (3.83%)	102.3±0.95* (4.39%)
6 day	103.6±0.95 (6.32%)	97.8±0.54** (6.05%)	93.3±0.84** (6.79%)	95.6±0.61** (6.54%)	94.5±0.88** (6.06%)	96.8±0.74** (5.65%)	98.1±1.10** (5.94%)	100.3±0.95* (6.26%)
8 day	102.0±1.36 (7.77%)	95.67±0.61** (8.09%)	91.5±0.71** (8.59%)	93.8±0.54** (8.30%)	92.3±0.95** (8.25%)	94.8±0.54** (7.60%)	96.3±1.20** (7.67%)	98.0±0.73* (8.41%)
10 day	97.1±0.83 (12.20%)	89.3±0.84** (14.21%)	87.3±0.71** (12.78%)	89.8±0.54** (12.21%)	90.6±1.22** (9.94%)	90.8±0.54** (11.50%)	92.3±1.20** (11.50%)	93.3±0.66* (12.80%)
12 day	95.1±0.83 (14.01%)	79.8±1.16** (23.34%)	85.6±0.61** (14.48%)	82.3±0.61** (19.55%)	88.6±1.22** (11.92%)	88.3±0.61** (13.93%)	84.6±0.98** (18.88%)	90.3±0.61** (15.60%)
14 day	89.0±0.85 (19.52%)	60.3±1.40** (42.07%)	70.6±0.98** (29.47%)	62.3±0.61** (39.10%)	78.6±1.22** (21.86%)	74.1±0.54** (27.77%)	64.6±0.98** (38.06%)	80.6±0.98** (24.67%0
16 day	84.1±0.74 (23.96%)	42.1±1.37** (59.55%)	61.1±2.03** (38.96%)	46.6±1.22** (54.44%)	48.6±1.22** (51.68%)	60.3±0.95** (41.22%)	45.6±0.95** (56.27%)	70.6±0.98** (34.01%)
18 day	54.6±0.98 (50.63%)	13.6±1.40** (86.93%)	22.1±1.10** (77.92%)	15.3±0.66** (85.04%)	28.6±1.22** (71.57%)	20.6±0.66** (79.92%)	15.6±0.95** (85.04%)	30.6±0.98** (71.40%)
20 day	24.6±0.98 (77.75%)	0±0** (100%)	13.8±1.10** (86.21%)	5.3±0.66** (94.81%)	6.6±0.84** (93.43%)	10.6±0.66** (89.66%)	5.6±0.95** (94.63%)	20.6±0.98** (80.74%)

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

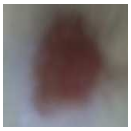
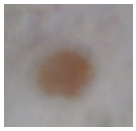
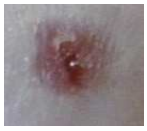
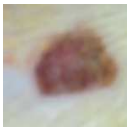
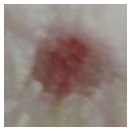
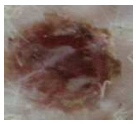
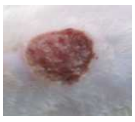
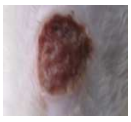
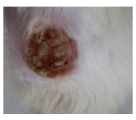
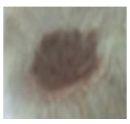
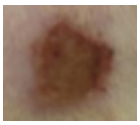
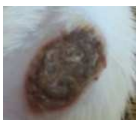
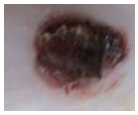
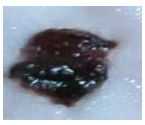
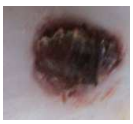
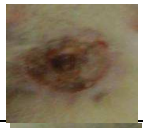
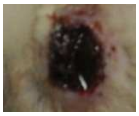
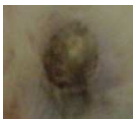
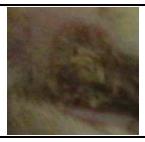
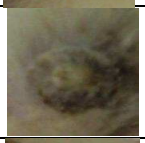
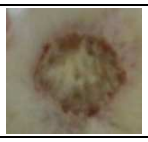
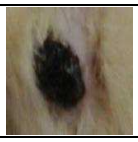
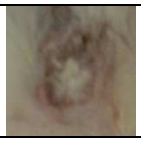
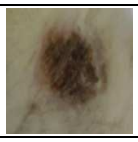
Table 2: Period of epithelization in acid burn wound model

Groups	Period of epithelization (Days)
Group I (Control)	24±0.51
Group II (Standard)	15.8±0.65**
Group III (AMEA)	18.3±0.61**
Group IV (AMM)	17.6±0.33**
Group V (AMA)	21.3±0.66*
Group VI (MPEA)	20.6±0.66**
Group VII (MPM)	20.5±0.42**
Group VIII (MPA)	21.6±0.61*

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

Reports about medicinal plants affecting various phases of the wound healing process, such as coagulation, inflammation, fibroplasia, collagenation, epithelialization and wound contraction are abundant in the scientific literature [30-33]. Approximately one-third of all traditional medicines in use are for the treatment of wounds and skin disorders, compared to only 1-3% of modern drugs [34]. In the past, several studies have been done using natural products for the treatment of burn wound, but these were mainly aimed at controlling infections [35, 36]. The anti-inflammatory activity of certain natural products could also play a part in the healing of burn wound [37]. Burn injuries involve stimulation of intravascular neutrophils and initiate systemic inflammatory reactions by producing toxins such as reactive oxygen species (ROS) almost in every tissue. Antioxidants at wound site provide a favorable environment for tissue healing [38]. Antioxidant and antimicrobial activity of *Aegle marmelos* [39, 40] and *Mucuna pruriens* [41, 42] have been reported. The literature survey reveals the presence of several phytoconstituents like tritepenoids, saponins, alkaloids and flavonoids are known to promote wound healing process due to their antioxidant, antimicrobial activities [43] and astringent property [44]. Various phytochemical constituents have been isolated from the different extracts of the *Aegle marmelos* leaves *i.e.*, alkaloids, cardiac glycosides, terpenoids, saponins, tannins, flavonoids and steroids [45]. Phytochemical screening of *Mucuna pruriens* confirms the presence of flavonoids [46, 47].

Fig. 1: Photographic representation of contraction rate showing percent wound contraction area on different days of control, standard and hydrogel of *Aegle marmelos* and *Mucuna pruriens* extracts treated rats.

GROUPS DAYS	I	II	III	IV	V	VI	VII	VIII
0 day								
2 day								
4 day								
6 day								
8 day								
10 day								
12 day								
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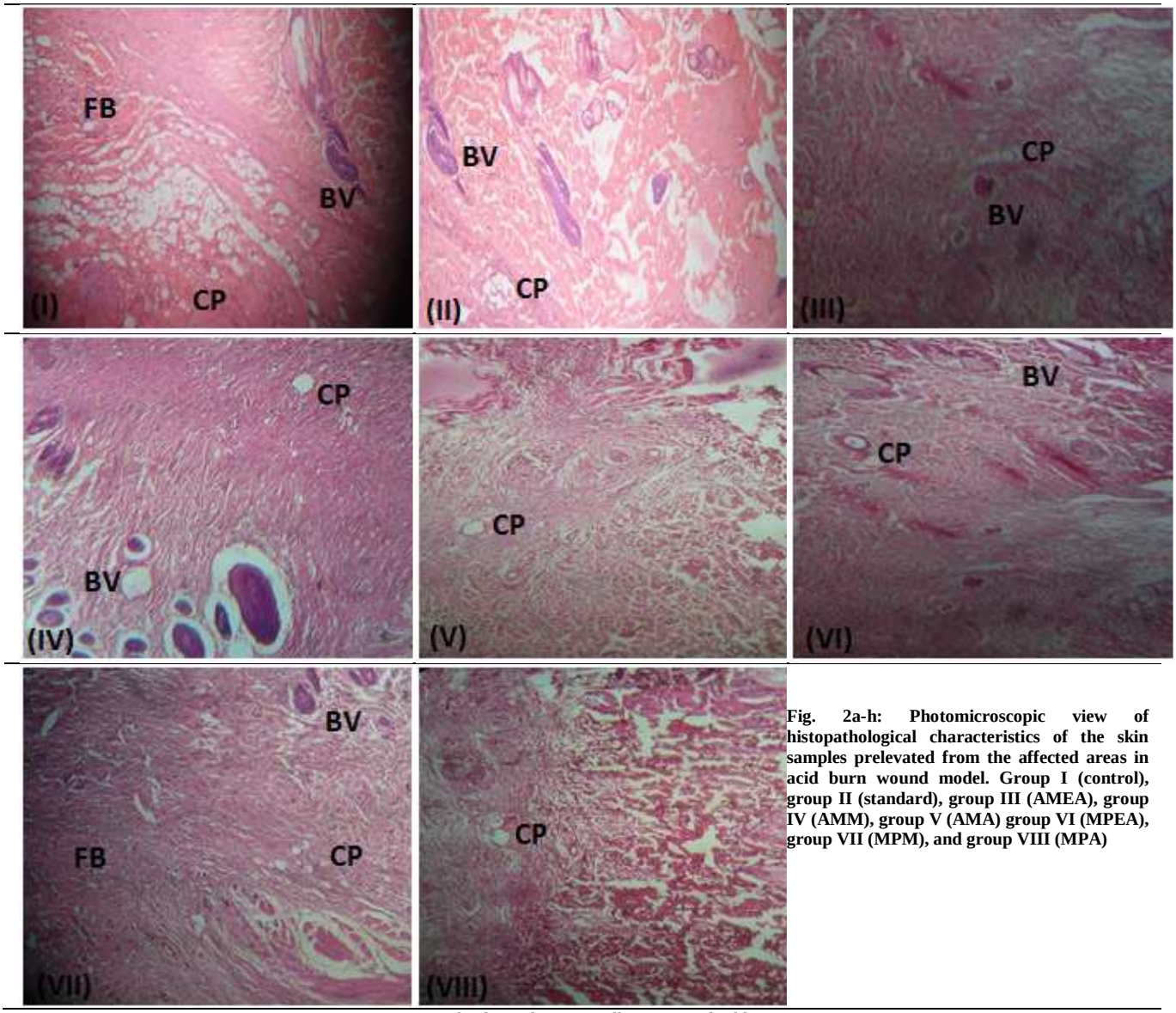
20 day								

Fig. 2a-h: Photomicroscopic view of histopathological characteristics of the skin samples prelevated from the affected areas in acid burn wound model. Group I (control), group II (standard), group III (AMEA), group IV (AMM), group V (AMA) group VI (MPEA), group VII (MPM), and group VIII (MPA)



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**BV:- Blood vessel, CP: Capillary, FB:- Fibroblast*

The antimicrobial activities of methanol extract may be due to the presence of tannins, triterpenoids and flavonoids. Tannins have been known to form irreversible complexes with prolene rich protein resulting in the inhibition of cell wall synthesis [48]. Triterpenoids are known to weaken the membranous tissue, which results in dissolving cell wall of microorganism [49]. Flavonoids, another constituent of methanol extract, have exhibited a large number of biological activities like anti-inflammatory, antioxidant and antimicrobial properties [50]. Although the present study did not explore the exact mechanism of healing by AM and MP, it could be attributed to both anti-inflammatory and antimicrobial properties.

CONCLUSION

Acid burn injury needs special attention and management because of their huge human and economic impact. Although the burned patient has many problems to face during the stages of recovery from a burn injury, the major

and persisting problems for survivors are those associated with the problems of healing and the outcome of healing in terms of scarring. Problems associated with wound management, treatment and healing have always been important challenges for clinicians and investigators. A substantial number of drugs are developed from plants, minerals and animals and are described in the Ayurveda for their wound healing properties. The majority of these involve the isolation of the active ingredient found in a particular medicinal plant and its subsequent modification. In conclusion, plant-based traditional medicine has been used throughout generations; the efficacy of such treatments requires experimental backup and scientific verification. Our study shows that the hydrogel of ethyl acetate and methanolic extract ointment of AM and MP effectively stimulates wound contraction as compared to the control group. These findings justify the inclusion of these plants in the management of wound healing. Hydrogels prepared from AM and MP extracts hydrogel can be used as alternative agents to existing wound healing therapies in the future. However, further studies are certainly needed to shed more light on the healing mechanism of *Aegle marmelos* and *Mucuna pruriens* extracts.

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