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Wound Healing Activity of *Butea Monosperma* in Wistar Rats

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ABSTRACT

Wound is a physical injury that causes breakdown in the protective function of the skin and the loss of continuity of epithelium with or without loss of underlying connective tissues. Proper healing of wound is essential for the restoration of normalcy in disrupted anatomical continuity and altered functional status of the skin. Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers. It can be divided into 3 distinct phases: an immediate haemostatic phase, an early phase of re-epithelialization, a later phase of dermal repair and remodelling. The present investigation was undertaken to determine the efficacy of *Butea monosperma* leaves aqueous extract, isolated acidic ingredient and its newly synthesized amide analogue along with silver sulphadiazine as control on burn wound in *Wistar albino rats*. The histopathological examinations confirmed that the treated animals showed improved rate of epithelialization and wound contraction. On comparison among the impact of test compounds; it was further revealed that, the *Butea monosperma* leaves aqueous extract and synthesized amide analogue showed significant wound healing activity than isolated acidic ingredient.

Keywords: *Butea monosperma*, isolated ingredient, amide analogue, burn wound.

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INTRODUCTION

The physical injury that results breakdown in the protective function of the skin and the loss of continuity of epithelium with or without loss of underlying connective tissue is called wound. Proper healing of wound is essential for the restoration of disrupted anatomical continuity and also altered functional status of the skin. Wound healing is a complex and fundamental activity of all damaged body structures. The same principle underlines the healing of cuts, abrasions, ulcers and areas damaged by chemical attack, invasion by microorganisms or immune reactions. Healing of the skin damaged by a physical assault may be divided into following three aspects¹,

- i. An immediate haemostatic phase,
- ii. An early phase of re-epithelialization,
- iii. A later phase of dermal repair and remodelling.

Research on wound healing agents is one of the developing areas in modern biomedical sciences. Recently there has been a shift in universal trend from synthetic to herbal medicine, which we can say 'Return to Nature'. Medicinal plants have been known for 'millennia' and are highly esteemed all over the world as a rich source of therapeutic agents for treating wounds, cuts and burns. The herbal drugs are popular for wound healing among rural and urban communities of India. The one reason for the popularity and acceptability is the belief that all natural products are safe. The demand for plant-based medicines, health products, pharmaceuticals, food supplements, cosmetics, etc., are increasing in both developing and developed countries due to the growing recognition that the natural products are nontoxic, have less side effects and are easily available at affordable prices².

In the literature, *B. monosperma* is ascribed to have many medicinal properties. The flowers and leaves are reported to possess astringent, diuretic, depurative, aphrodisiac and tonic properties. They are also effective in leprosy, leucorrhoea and gout. The leaves are also used to cure boils, pimples and tumorous hemorrhoids and are internally given in flatulent colic, worms and piles. The leaves are reported to contain alkaloids, protein, fibre and minerals. The bark is useful in tumors, bleeding piles and ulcers³. Topical administration of an alcoholic bark extract was found to improve the different phases of wound repair, including collagen synthesis and maturation, wound contraction and epithelialization⁴.

The present study was undertaken to explore the efficacy of aqueous extract of *Butea monosperma* leaves, isolated acidic ingredient and newly synthesized amide analogue along with silver sulphadiazine as control on burn wound healing in *Wistar albino rats*.

MATERIALS AND METHODS

Plant material:

The leaves of *Butea monosperma* plant were collected seasonally from the Melghat region of Amravati, District of Maharashtra, India. It was authenticated by the taxonomist Dr. S. P. Rothe with Voucher specimen (ML – 101) and was deposited in the herbarium of Department of Botany, Shri Shivaji College, Akola.

Extraction and Isolation:

The leaves of *Butea monosperma* plant were shade dried at room temperature and ground in a manual mill to get coarse powder. The powder was kept in the airtight polythene bag and stored at dry place. This powder was extracted with water as a solvent by using soxhlet apparatus. The extract was concentrated at 40 °C using rotary evaporator. Finally it was dried, crushed and stored in air tight bottle at 4 °C for further study. The aqueous test extract (100g) was dissolved in NaHCO₃ till there was no effervescence then filter and crude acidic ingredients were re-appeared from filtrate using 10% HCl.

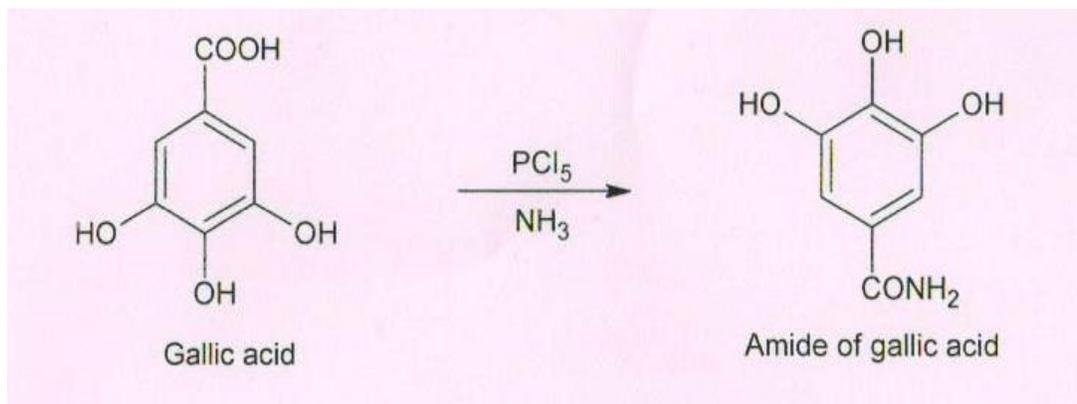
The crude acidic ingredients (35g) adsorbed on to a silica gel (s d fine 60-120 mesh) and subjected to column chromatography over silica gel (100-200 mesh) and eluted with n-hexane followed by petroleum ether-benzene-acetic acid gradient system (20:40:40, 40:20:40, 40:40:20). The fractions were collected in 100ml conical flasks. The eluted fractions were combined to give major fractions by comparing their R_f values, when run on TLC Aluminium sheets Silica Gel 60 F₂₅₄ 0.2 mm thickness with similar solvent systems. The separated components on the silica plate were visualized in UV light and then to Iodine, methyl red, bromophenol blue, vanillin sulphuric acid. After developing TLC profile, similar TLC pattern fractions were mixed together to get major fractions. Total seven fractions were collected (F1-F7). Fraction F-5 was subjected to repeated chromatographic purification over silica gel using elution with petroleum ether-benzene-acetic acid (40:40:20) to give compound 1 (100 mg)^{5&6}.

Characterization of compound 1:

M.P.: 245 °C, **Elemental analysis:** Found C =49.40,

H = 3.52, O = 47.00, Calculated C = 49.42, H = 3.55, O = 47.02 %. **UV spectrum:** λ_{max}283 nm (n → π*). **IR spectrum, ν, cm⁻¹:** 3367.61, 3289.24, 3064.85, 3011.46 (-OH stretching), 3064.85 (Ar-H stretching), 1702.93, 1618.06 (two C=O absorptions), 1600-1450 (aromatic ring), 1300-1000 (C-O). **¹H NMR spectrum (400 MHz):** δ ppm 4.801 (3H, s, aromatic C-OH), 7.087-7.221 (2H, s, Ar-H).

From the characterization it was found that isolated compound 1 was Gallic acid. The major ingredient i.e. Gallic acid (Compound 1) thus separated from test extracts was then used for the preparation of its amide analogue (Compound 2).



Characterization of compound 2: M.P.: 240°C , Elemental analysis for $\text{C}_7\text{H}_8\text{N}_2\text{O}_4$:

Found C = 45.40, H = 4.32, O = 34.73, Calculated C = 45.66, H = 4.38, N = 15.21, O = 34.75 %.

UV spectrum: λ_{max} 283 nm ($n \rightarrow \pi^*$). **IR spectrum ν , cm^{-1} :** 3754 ($-\text{NH}_2$ stretching), 3714 (overtone of 1658), 3273 ($-\text{NH}$ stretching/ $-\text{OH}$ stretching), 2375, 2341 ($-\text{CH}$ stretching) 1658 ($-\text{NH}$ bending, primary amine), 1614 ($\text{C}-\text{O}$ stretching), 1541 (NH bending, secondary amine), 769 ($-\text{NH}$ oop). **^1H NMR spectrum (400 MHz):** δ ppm 1.902 (2H, s, primary amine), 2.007 (1H, s, secondary amine), 4.800 (3H, s, aromatic $\text{C}-\text{OH}$), 7.015 (2H, s, Ar-H).

Animals:

The interdisciplinary part of proposed study was carried out after getting permission from the Institutional Animal Ethical Committee, Pusa (CPCSEA/IAEC/CP_PL/07-2012). The care of laboratory animals was taken according to the guidelines of CPCSEA, Ministry of Forests and Environment, Government of India (registration number 729/02/a/ CPCSEA).

All the experiments were carried out using adult *albino Wistar male rats* weighing about 130-150 gm. The animals had free access to food and water and they were housed in cages in a natural (12 hrs each) light-dark cycle. The animals were acclimatized to the laboratory conditions for at least 5 days before behavioral experiments which were carried out between 0900 h and 1800 h.

Burn wound healing activity⁷:

Dorsal skin of the *Wistar rats* were shaved at full thickness. The animals were anesthetized by ketamine injection and burn wound of approximate 2 cm in diameter were created (circular area) by the brass probe which was immersed in boiling (100°C) water until thermal equilibrium was reached. The probe was then placed on the back of the rats for 20 s without applying pressure. They were then housed individually in separate cages after complete recovery from anaesthesia.

Rats were randomly divided into five groups of ten animals each:

Control group:

Immediately after burning, burn areas were covered with propylene glycol solution once a day for 20 days.

Standard Silver Sulphadiazine group:

Immediately after burning, burn areas were covered with Silver Sulphadiazine cream once a day for 20 days.

Crude extract group:

Immediately after burning, burn areas were covered with crude extract of leaves sample which was prepared in propylene glycol once a day for 20 days.

Isolated acid group:

Immediately after burning, burn areas were covered with isolated acid sample prepared in propylene glycol, once a day for 20 days.

Prepared analogue group:

Immediately after burning, burn areas were covered with the prepared analogue sample also prepared in propylene glycol once a day for 20 days.

The wounds were clinically observed in all groups every day. After 10th and 20th days later, the rats were sacrificed after being anesthetized.

BIOPHYSICAL PARAMETERS

Measurement of wound contraction:

The animals were inspected daily and the observations of percentage wound closure were made on 10th, 15th and 20th post wounding days and the photographs were taken. The wound closure was monitored by planimetric measurement of the wound area which was achieved by tracing the wound on a graph paper. The healed area was calculated by subtracting it from the original wound area (2nd day). The percentage of wound contraction was determined using the following formula:

$$\text{Percent wound contraction} = \frac{\text{Healed area}}{\text{Total wound area}} \times 100$$

To apply this equation, the wound margins were traced and measured to calculate the non-healed area which was then subtracted from the original wound area⁸.

Measurement of thickness of epithelial:

The thickness of granulation tissues (epithelial) was measured by planimetric measurement of center of wound⁹. The thickness of granulation tissue was measured on the isolated burn wound sample of skin at 10th day and at 20th day of sacrifice of respective group of animals. The thickness was measured by planimetric, by simply placing the sample of wound area between the

plane surfaces of two slides. The gap between the two slides after placing the skin sample between them was measured. This was considered as thickness (cm) of granulation tissue.

Histopathological examination:

At the end of 10th day and 20th day's trial, the rats were sacrificed and the burned skin tissue samples were collected for histopathological examination. The tissue samples were fixed in 10% formalin solution and then dehydrated through dehydrated alcohol series, cleared in xylene and embedded in paraffin wax. The series of sections were cut and stained with Hematoxylin and Eosin. The sections were examined under light microscope and the photographs were taken.

Statistical analysis:

The results were expressed as mean \pm SD of 5 rats in each group. One way ANOVA followed by Tukey's test and Newman-keuls multiple comparison test were carried out to determine the significant difference on rate of percentage contraction and thickness of granulation tissue of wound area between the control group and the test samples at different trial periods ($p < 0.05$).

RESULTS AND DISCUSSION:

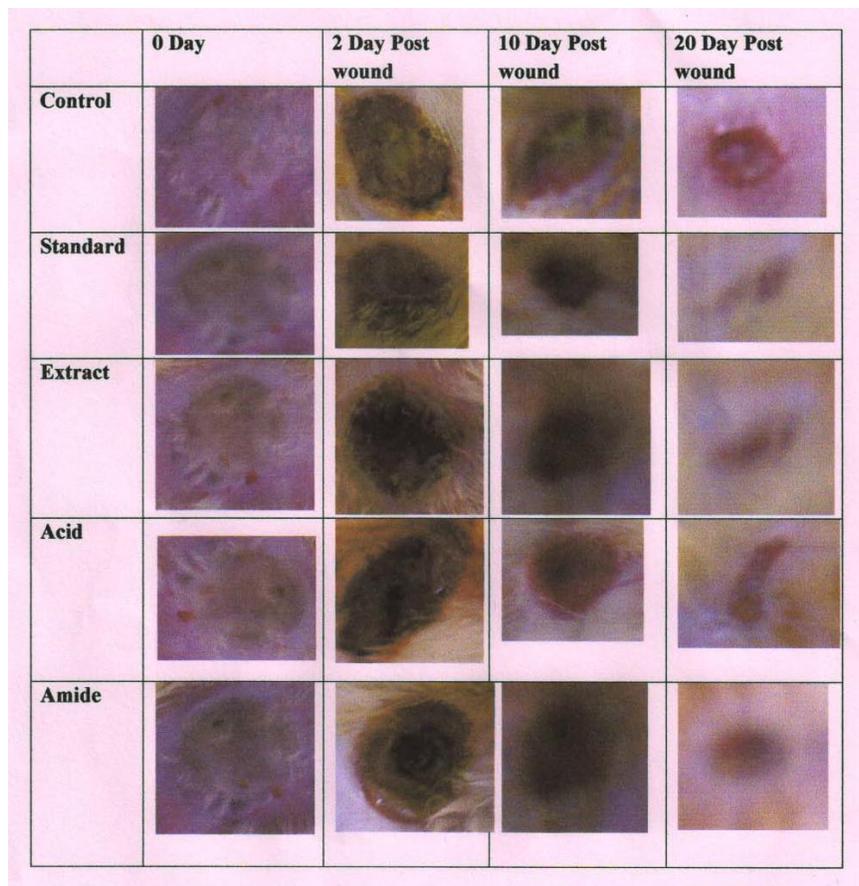


Figure 1: Photographic representation of contraction rate on different days of control and experimental wounds

No mortality was observed in animals during the study period. The wounds in the controlled group of animals displayed a greater degree of inflammation on the basis of three clinical sign of the inflammation process i.e. heat, redness and swelling which appeared to be lessened in wounds treated with test samples. Up to 5 days, the degree of redness was found to remain comparatively same in all animals including treated group of animals. At the end of 10th day of trial period, the redness was still present on the surface of wound in control and isolated acid treated group of animals, while in silver sulphadiazine, crude extract and amide treated group, the degree of redness on the surface of wound appeared to be lessened. On 20th day of trial period there was complete disappearance of redness in all treated groups as shown in figure 1.

Table 1: Percent wise effect of treatments on wound contraction

Treatments	On 2 Day	After 10 Days	After 15 Days	After 20 Days
Control	1.84±0.101 (100)	1.24 ±0.12 (32.6) ^a	0.82±0.103 (55.44) ^b	0.51±0.13 (72.29) ^c
Standard (SSD)	1.88±0.231 (100)	0.887±0.24 (52.8) ^{a, 1}	0.425±0.13 (77.4) ^{b, 1}	0.13±0.047 (92.91) ^{c, 1}
Extract (2%)	1.84±0.102 (100)	0.69±0.058 (62.5) ^{a, 3}	0.36±0.06 (80.44) ^{b, 3}	0.15±0.05 (91.85) ^{c, 3}
Acid (2%)	2.02±0.132 (100)	0.83±0.13 (58.91) ^{a, 2}	0.46±0.04 (77.1) ^{b, 2}	0.24±0.096 (88.25) ^{c, 2}
Amide (2%)	1.825±0.227 (100)	0.725±0.075 (60.28) ^{a, 4}	0.33±0.047 (81.74) ^{b, 4}	0.13±0.047 (92.7) ^{c, 4}

All the readings were in mean of centimeter (N=5) ± S.D. Values in parenthesis were given in percent of wound contraction considering their 2nd day reading as 100 %. ^{abc} The Values in row shown significant different as compared to their 2nd day values; ¹²³⁴ The Values in column indicated significant different as compared to their corresponding control readings (One way ANOVA followed by post hoc Newman-keuls multiple comparison test)

The rate of contraction of control and experimental wounds are represented in Table 1 and Figure 1 & 2. The treated wounds were found to contract much faster. At the end of 20th day after burn wound creation, the control rats showed 72.29 % of wound contraction indicating the natural healing property of skin. The acid demonstrated 88.25%, extract represented 91.85 % while amide derivative indicated 92.7 % wound contraction. These results demonstrated the treatment increased the healing property of burn wound. Statistically, the percentage of wound contraction showed that, there was significant difference between the different groups (p<0.01). The wound healing potential for the aqueous extract and amide analogue demonstrated excellent activity as compared to isolated acid of *Butea monosperma*.

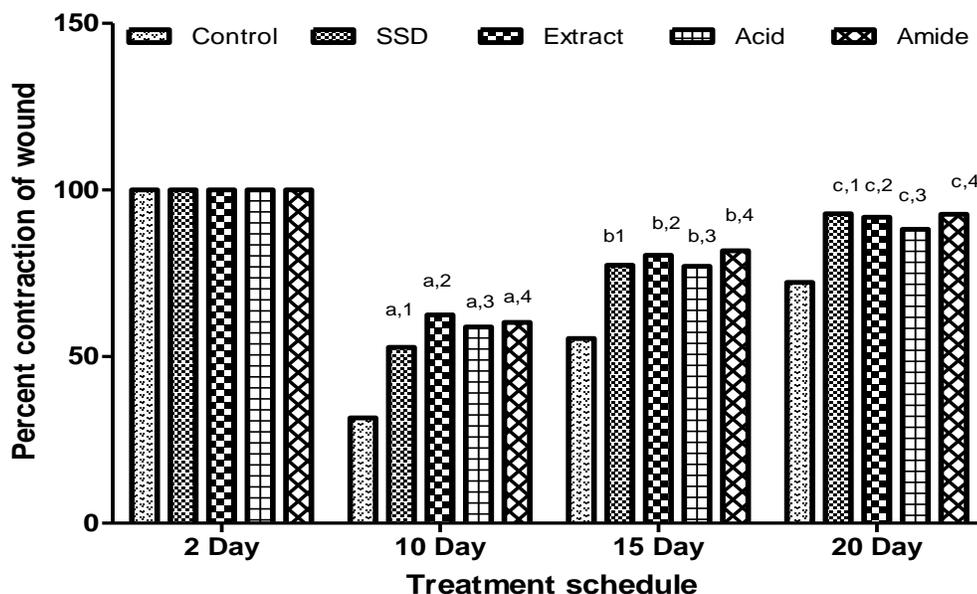


Figure. 2.The periodical rate of contraction in control and treated group rats.

The wound healing activity in treated group animals indicated significant ($p < 0.05$) increase in the thickness of granulation tissue which were measured among the groups at 10th and 20th day. On the 10th day of the trial period, the thickness of the granulation tissue was on higher side in all treated group animals as compared to control group animals. The statistical difference was observed on 20th day of the trial period. The thickness of the granulation tissue was statistically different ($p < 0.05$) among all treated groups except the isolated acid treated group animals. The mean values of thickness of granulation tissues in the center of wound are shown in table 2.

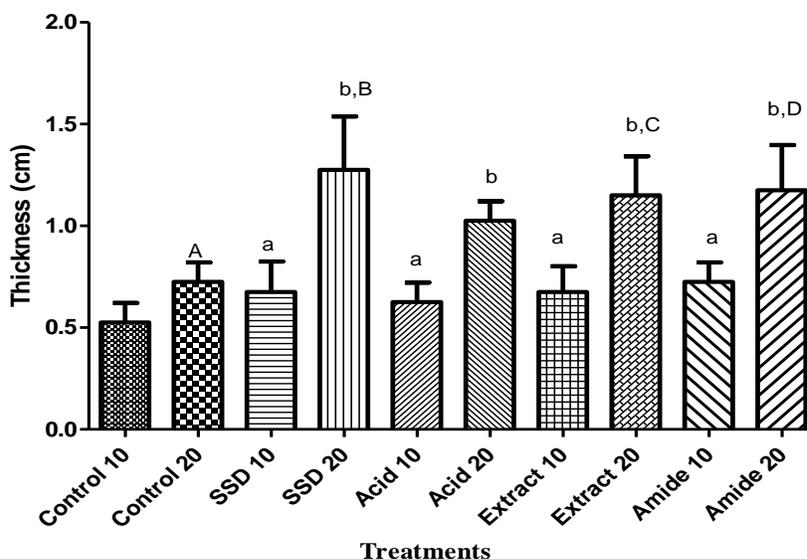
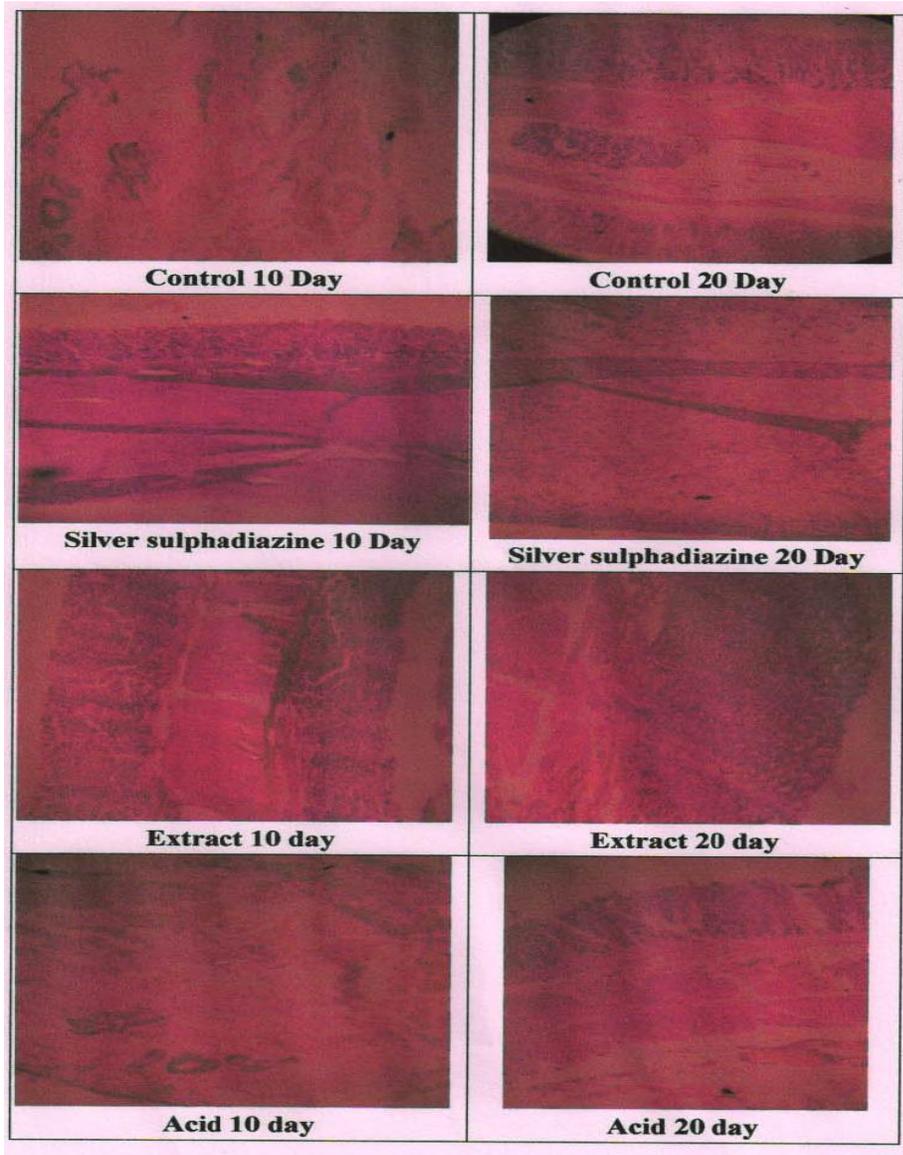


Figure 3:Effect of different treatment on thickness of granulation tissue of wound

Table 2: Thickness of granulation tissues in the center of the wound

Days	Groups	SSD	Extract	Acid	Amide
10 th Days	Control	0.525±0.083	0.725±0.109 ^a	0.625±0.083 ^a	0.725±0.083 ^a
20 th Days	Control	0.77±0.083 ^A	1.27±0.23 ^{bb}	1.02±0.083 ^b	1.175±0.192 ^{bd}

(SSD=Silver Sulphadiazine); All the reading were in mean of centimeter ±S.D (N=5); ^{ab} values in same column were significantly different (p<0.05); ^{ABCD} values in same row were significantly different (p<0.05); One way ANOVA followed by post hoc Tukey's test.



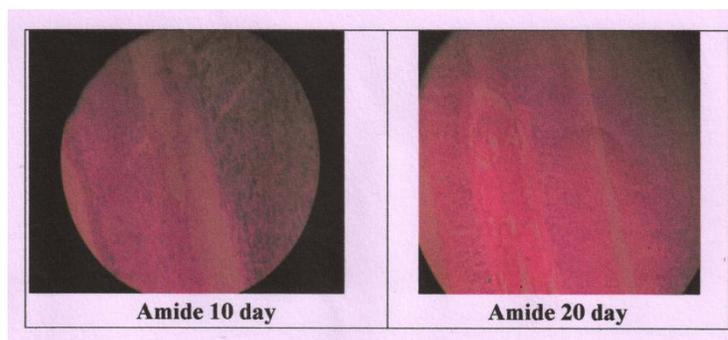


Figure 4: Photographs of Histopathological study

At the end of each 10th and 20th day's trial period, the experiment of histological examination was carried out for treated and untreated wound samples. At 10th day of histological examination in control group animals, the collagen fibers were found loosely packed with irregular arrangement and incomplete epithelialization with less fibrous tissues at wound site. Whereas in treated animals at 10th day the collagen fibres were found orderly packed and keratinocytes were easily differentiated from epidermis. After 20th day of histological examination, there was lessened inflammation in treated group wounds as compared to control group wounds. Granulation tissues in dermis and epithelial layers had better appearance in treated animals as compared to control group animals.

CONCLUSION

On the basis of the results obtained in the present investigations, it can be concluded that the 2% w/w of isolated acid ingredient had significant burn wound healing activity. While the results of extract and synthesized amide analogue (2% w/w) had excellent wound healing activity than the isolated acid treated group. These results were supported by the folklore reflecting the use of plant and its analogues in the management of burns.

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