Original Article

Experimental and histopathological observation scoring methods for evaluation of wound healing properties of *Jatyadi Ghrita*

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Abstract

Introduction: *Jatyadi ghrita* is a classical Ayurvedic formulation indicated in the treatment of various types of ulcers. **Aim:** The study was designed to explore the wound healing properties of *Jatyadi Ghrita* in diabetes - induced rats. **Materials and Methods:** In the present study, diabetes mellitus was induced to 6 to 8-week-old male Wistar rats by injecting streptozotocin cut 65 mg/kg body weight intravenously by 15 min prior to the administration of Nicotinamide at 230 mg/kg body weight intraperitoneally. Animals having diabetes were used for grouping namely, diabetic control (DC), *Ghrita* control (GC), positive control (PC), i.e., mupirocin HCl, *Jatyadi Ghrita* treatment and one group of non-DC. Full-thickness excision wound was created and diameter was recorded. Daily clinical observations were recorded. A wound scoring method was developed. Wound diameter and score were recorded on days 1, 2, 3, 5, 7, 9, 12, 14 and 15. Photographs were taken at the same time interval points. Body weight and feed consumption were recorded weekly. Animals were sacrificed at regular intervals to collect the wound area tissue for histopathology analysis. Obtained data was analyzed statistically. **Results and Observation:** It was observed that there was no significant difference in diameter and percent change in wound healing as compared to any control. However, clinical score and histopathological changes in *Jatyadi Ghrita* group were improved from the second day of the study as compared to control. **Conclusion:** This indicates that the drug has similar wound healing activity as compared to the modern drug mupirocin HCl.

Keywords: Ayurveda, experimental wound scoring, *Jatyadi Ghrita*, rat

Introduction

Loss in the integrity of normal anatomical structure of skin is called wound. Hence, healing means restoration of normal anatomical structure and function.[1] Healing is a sequence of events, i.e., movement of specialized cells, signals for influx of connective tissue cells and restoration of blood flow. Certain time-dependent changes occur during healing process, i.e., platelets appear immediately, neutrophils appear within 24 h and macrophages appear within 48 h.[2] These cells occupy the wound bed and release healing signals.[3] These signals are called cytokines. Platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) participate in healing process.[4,5] Proteins such as fibroblast and collagen help in the restructuring of the lost integrity of tissues.

Jatyadi Ghrita is a classical Ayurvedic formulation[6] indicated in ulcers in vital points, oozing/weeping ulcers, deep-rooted ulcers, painful ulcer, bleeding ulcer and non healing ulcer. A study of burn wound healing activity of *Jatyadi Ghrita* and *Jatyadi Taila* was performed by Dhande *et al.* (2012).[7] The study concludes that *Jatyadi Ghrita* and *Jatyadi Taila* help in reducing the period of epithelialization as compared to silver sulfadiazine. Wound healing activity of only *Jatyadi Taila* was

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indicated. *Jatyadi Taila* caused reduction in wound area as compared to the application of modern topical antimicrobial agent neosporin.[8]

Jatyadi Ghrita contains parts of plants namely, *Myristica fragrans* Houtt.[9] *Azadirachta indica* A Juss.[10] *Trichosanthes dioica* Roxb. [11] *Terminalia Chebula* Retz.[12] and *Hemidesmus indicus* R. Br.[13] [Table 1] which are already reported for their wound healing and anti-inflammatory activities. The review of literatures indicated the following basic arenas of exploration for this study:

1. Whether *Jatyadi Ghrita* helps in wound healing?
2. How to evaluate the clinical signs of wound healing in a comparable manner?

Hence, the study was designed to explore the wound healing properties of *Jatyadi Ghrita* in diabetes-induced rats.

Materials and Methods

Ethical clearance

This study was performed as per the animal ethical guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals Guidelines for Laboratory Animal Facility after obtaining approval from the Institutional Animal Ethics Committee (IAEC) of National Research Institute of Ayurvedic Drug Development (NRIADD), Kolkata, with ethical approval number IAEC/2014/01 dated 24.04.2014.

Temperature and relative humidity was maintained at 25 ± 1°C and 40%–70%, respectively and illumination was controlled to give approximately a sequence of 12 h light and 12 h dark cycle. Animals were acclimatized for 7 days before initiation of the study. The health examination of the animals was performed by a veterinarian during acclimatization period.

Drug details

Jatyadi Ghrita was obtained from the Arya Vaidya Sala, Kottakkal. The drug contained ingredients as given in classical text as shown in Table 1.[14]

Table 1: Ingredients of *Jatyadi Ghrita*

Aushadhi Yog	value
Purana Ghritam	8.242 ml
Puranakera Tailam	2.747 ml
Jatipatra (*Myristica fragrans* Houtt.)	10.989 g
Nimba Patra (*Azadirachta indica* A. Juss.)	10.989 g
Patola Patra (*Trichosanthes dioica* Roxb. leaf)	10.989 g
Katuki (*Picrorhiza kurroa* Root ex. Benth [Rz.])	10.989 g
Darvi (*Berberis aristata* DC [stem])	0.086 g
Nisha (*Curcuma longa* Linn. [Rz])	0.086 g
Sariva (*Hemidesmus indicus* R. Br. [Rt])	0.086 g
Manjishtha (*Rubia cordifolia* Linn. [Rt])	0.086 g
Abhaya (*Terminalia chebula* Retz. [Rt.])	0.086 g
Tutha (blue vitriol)	0.086 g
Madhuka (*Glycyrrhiza glabra* Linn.)	0.086 g
Naktahvabija (*Pongamia pinnata* Pierre [Sd.])	0.086 g
Sikta (Beeswax)	0.086 g

Study design

Eight-week-old Wistar rats were selected based on the body weight and were randomly distributed into four groups in such a way that the means of groups are same and body weight variation is ± 20% of the mean body weight. For gross evaluation group, randomization was done keeping minimum variation in the mean of body weight between the groups before induction of diabetes. Each gross evaluation group contained six male rats and histopathological evaluation group contained seven male rats which were sacrificed at the given time points at days 1, 3, 5, 7, 9, 11 and 15 after wound creation. The five groups for gross and histopathological evaluation were as follows, i.e., negative control (NC), diabetic control (DC), vehicle control (*Ghrita*) (GC), positive control (mupirocin HCl, trade name-T-Bact, Mfg.-Sanofi) (PC), and *Jatyadi Ghrita* group (JG). A total of 65 male Wistar rats were obtained from in-house breeding facility of the NRIADD, Kolkata, i.e., 30 for gross evaluation group of which 24 were diabetic and 6 were nondiabetic and for histopathological evaluation group, 35 male Wistar rats were obtained of which 28 were diabetic and 7 were nondiabetic.

Diabetes induction

Diabetes was induced by administering Nicotinamide (Mfg. Himedia Laboratories Pvt., Ltd.) at 230 mg/kg body weight intraperitoneally followed by streptozotocin (Mfg. Himedia Laboratories Pvt., Ltd.) at 65 mg/kg body weight intravenously after 15 min to 8-week-old Wistar rats.[15] Blood glucose levels were evaluated with the help of blood biochemistry analyzer (Robonik Inc.), within the 1st week, after 21 days, before wound creation and at the termination of the study, i.e. before necropsy. Blood glucose levels were evaluated by strip method only to check reversibility of the diabetes whenever desired. The rats showing hyperglycemia (glucose level above 250 mg/dl) were further placed in the respective groups.

Blood was collected through retro-orbital plexus of rats. For glucose estimation, blood collection tubes without anticoagulants were used. The serum was separated and processed.

Wound creation and scoring

Rats were given ether anesthesia for depilation. For wound creation, rats were first tranquilized with Sequil (triflupromazine hydrochloride) and later were given local infiltration of lignocaine as local anesthetic. Hairs in the intrascapular region were removed by shaving with the help of a blade. A small round was drawn on the shaved area and wound was created. Full-thickness excision wound was inflicted on the intrascapular region of the rats. Diameter of the wound was recorded. After wound creation, all the rats were maintained on a thermal pad in order to maintain thermoregulation. Rats were given *ad libitum* feed and water. Recovery from tranquilization was observed carefully and rats were shifted to cage with blotting paper over the rice husk (sterilized) so as to avoid infection and irritation.

Wound contraction and scoring

Diameter of the wound was recorded on days 1, 2, 3, 5, 7, 9, 12, 14 and 15 and percentage change in Diameter was calculated by the following formula:

$$\frac{(\text{Initial wound Size} - \text{Current wound size})}{\text{Initial Wound Size}} \times 100$$

Feed consumption was measured daily up to 1st week and thereafter at weekly intervals. The feed consumption was calculated on g/100 g body weight basis.

A unique scoring system based on clinical observations was developed [Table 2].

Animals were weighed and sacrificed using CO_2 euthanasia followed by exsanguinations and were subjected to a detailed gross pathological examination at termination of the study or on the 16th day of wound creation. The observations were recorded as raw data.

Table 2: Wound healing score card

Observation	Score
Dryness	
Fresh/heavy blood or pus on wound surface: Initially, fresh blood oozes out of wound which later stops due to hemostasis. This score will be given only if bleeding does not stop	1
Blood or pus on wound surface (less bleeding than score 1): Hemostasis has taken place and still remnants of clots are visible in wound bed	2
Apparently dry but blood or pus oozes on moping: Pus is formed under a thin layer of crust. Upon moping, this crust is removed and pus or blood is visible (improved as compared to score 2)	3
Little dry or watery discharge: The wound bed is dry, free of infection, but serosanguinous discharge is visible from the borders (improved as compared to score 3)	4
No blood or completely dry surface	5
Borders	
Initially, borders are swollen due to inflammation and hemorrhages. This requires keen observation of borders at the time of wound creation	1
In case of infection of wound, the borders are moist and rose with blood or pus. A scanty crust formation may take place at the borders. Pus formation around the border area is visible (improved as compared to score 1)	2
Proliferation of granulation tissue is seen at the borders. Crust formation is clearly visible. Borders are comparatively dry (improved as compared to score 2)	3
Crust formation has taken over the borders. Hence, borders are uneven and dry. After the crust formation is complete, the borders are sharp. No swelling of borders is visible as it was visible previously (improved as compared to score 4)	4
Dry and sharp borders. No major abnormality visible	5
Crust formation	
Initiated at the borders (primary or secondary crust)	1
Apparently dry surface but occupied by scanty crust formation. A thin layer of dead tissue is visible below which is pus or blood (improved as compared to score 1)	2
Crust with folds/primary crust/crust not so hard is visible (improved as compared to score 2)	3
Hard crust about to fall. This crust may be removed during observations (improved as compared to score 3)	4
Secondary crust formation	5

Histopathological scoring

Wound tissue collected was subjected to detailed histopathological processing. Tissue was fixed in formalin, trimmed and processed, i.e. dehydrated in graded alcohol, cleared in xylene, and embedded at 58°C–60°C in paraffin. These tissue blocks were cut at 4–5 µ in thickness and stained with hematoxylin and eosin and finally mounted with digital picture exchange. Slides were then studied under light microscope and photographs were taken. Histopathological evaluation and scoring was done as per the method described by Tkalcević *et al*. (2009).[16] Granulation tissue was described as follows:

i. Immature granulation tissue: loose granulation tissue (macrophages, fibroblasts) with emerging vessels
ii. Mature granulation tissue: fibroblasts and sparse extracellular matrix proteins forming layers and vessels running perpendicular
iii. Fibrosis: extracellular matrix proteins (mainly collagen) dominating the granulation tissue, fewer fibroblasts, and vessels.

The score for each wound was given in a semi-quantitative manner with grades of 1–3 for each of the three granulation tissue categories.

1. Wound bed partially covered with granulation tissue
2. Thin granulation tissue over the whole wound bed
3. Thick granulation tissue over the whole wound bed.

The data was analyzed statistically as Groups C (*Ghrita* control), D (PC, mupirocin hydrochloride), and E (*Jatyadi Ghrita*) and were statistically compared with Group A (NC) and Group B (DC) in order to find the treatment-related effects. Further, parameters such as feed consumption in g/100 g body weight/day, percentage change in wound area and scores of clinical observations were statistically evaluated as compared to NC and DC.

Results

Gross evaluation group

Diabetes was well established indicating hyperglycemia, asthenia, polyphagia, polydipsia and polyurea in clinical evaluation. No significant difference was observed in body weights of all the groups. No significant alteration in feed consumption was observed in group-wise comparison with controls. On days 2 and 3, there was a significant contraction in NC as compared to DC. On day 2, rats treated with only *Ghrita* showed significant contraction as compared to DC. However, nonsignificant increase in contraction was observed in groups treated with mupirocin HCl and *Jatyadi Ghrita*. This contraction was comparable to controls. *Jatyadi Ghrita*-treated rats showed significantly improved healing as compared to NC and DC on day 7. This indicates that *Jatyadi Ghrita* has a significant effect on clinical signs such as dryness, borders,

thickness and crust formation [Charts 1-3] on day 7 of the wound creation.

Histopathological evaluation group

Contraction and re-epithelialization

No significant difference was noted between histological wound diameter in *Jatyadi Ghrita*-treated rats and other groups at any point of time, implying that wound contraction rate was similar among all groups. However, the re-epithelialization rate from day 5 onward in *Jatyadi Ghrita*-treated rats was significantly higher as compared to other groups; this was seen in the form of epidermal hyperplasia at the wound borders. Re-epithelialization was a continuous process that ended 14 to 15 days after wounding.

Granulation tissue

Immature granulation tissue, consisting of macrophages, lymphocytes and plump fibroblasts accompanied by early vascular sprouting within edematous fluid, was well formed and covered almost the whole wound bed in the majority of the *Jatyadi Ghrita*-treated rats 5 days after the wound creation, whereas in other group of animals, it was just emerging from the wound edges. This implies that initiation of wound healing was faster in *Jatyadi Ghrita*-treated rats.

By 7 to 9 days after wound creation, the whole wound bed was covered with mature granulation tissue containing fibroblasts and sparse extracellular matrix proteins forming layers and characteristic blood vessels running perpendicular to the directions of the fibroblasts in *Jatyadi Ghrita*-treated rats. Transformation of "immature" granulation tissue into "mature" granulation tissue was characterized by elongation of fibroblasts, early deposition of collagen fibers and angiogenesis. Whereas, mature granulation tissue appeared in other groups with a delay of 2 days.

The process was completed 12–14 days after wound creation in *Jatyadi Ghrita*-treated rats with the whole wound bed filled with collagen and scarce inflammatory cells, indicating the appearance of fibrosis [Figures 1-5]. In contrast, in rest of the groups' rats, 12 days after wound creation, the mature granulation tissue dominated in the wound bed; whereas,

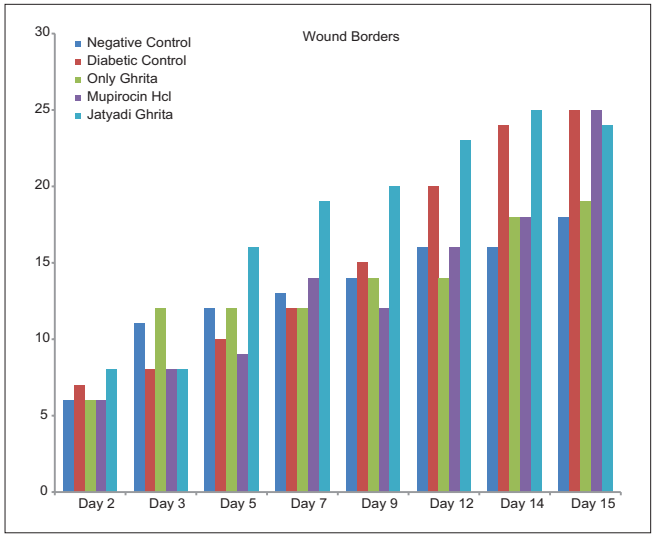

Chart 1: Scores of wound border

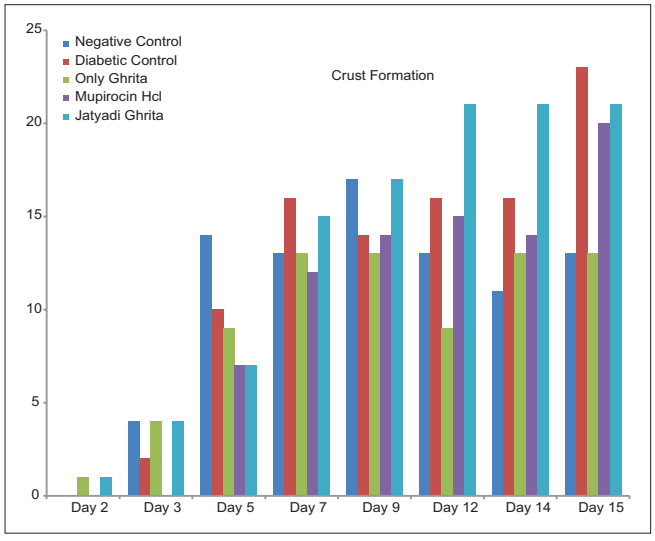

Chart 3: Score of crust formation

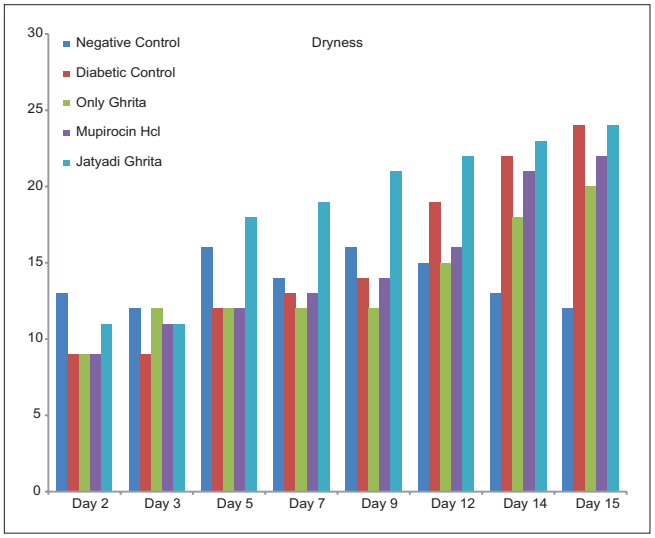

Chart 2: Scores of dryness

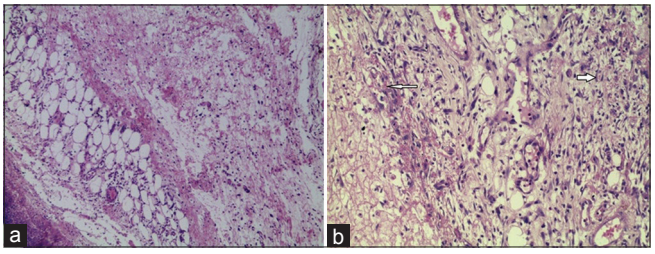

Figure 1: (a) Negative control Day 5: minimal/no immature granulation tissue Deposition as compared to Jatyadi Ghrita group (10 x 10). (b) Jatyadi Ghrita treatment Day 5: Immature granulation tissue with Emerging bloodvessels (angiogenesis) shown in white arrows and fibroblasts and numerous macrophages (10 x 10)

deposition of collagen in the extracellular matrix had just begun at the wound edges.

Inflammation

Acute inflammatory changes including neutrophil infiltration, necrosis, exudate formation, fibrin deposition and scab formation were seen in the first 3 days in all the groups' rats [Table 3]. However, it was comparatively lesser in *Jatyadi Ghrita*-treated rats and mupirocin -treated rats from 5th days onward but present in rest of the groups till 7 days.

Discussion

Alteration in the normal repair process causes delayed wound healing, i.e., chronic wound or ulcer. Pathologic responses leading to fibrosis or chronic ulcers may occur if any part of the healing sequence is disturbed.[2] Delayed wound healing is characterized by excessive infiltration of neutrophils. Neutrophils release a significant amount of enzymes such as collagenase (i.e., matrix metalloproteinase) that is responsible for the destruction of connective tissue matrix.[17,18] Neutrophils also release elastase that is capable of destroying PDGF and TGF-β.[19] This destruction of cytokines delays the healing process.

Wound healing properties of *Jatyadi Ghrita* in diabetes-induced rats were explored in the present study. This study was mainly of exploratory design, hence appropriate controls were used for comparison namely, nondiabetic NC, DC, vehicle control and PC, i.e., mupirocin hydrochloride. Percent are decrease in wound diameter/area, feed consumption, clinical observations and histopathological alterations were mainly studied. The important outcome of the study is that *Jatyadi Ghrita* helps in healing of wound comparable to mupirocin hydrochloride.

The present study reports suitability of streptozotocin–nicotinamide model for any study on diabetes. Food intake was normal in *Jatyadi Ghrita*-treated group which may be due to palliative effect.

Full-thickness excision model was used for the evaluation of *Jatyadi Ghrita*. This model was chosen as it accommodates the broadest assessment of the histomorphological events involved in wound healing, including epithelialization, granulation and angiogenesis.[20] In addition, this model has an advantage of providing large area as medications are can be applied directly to the wound bed.[21,22] This model encompasses all skin components, i.e., epidermis, dermis, hypodermis and blood vessels.[23]

There are two distinct variations in clinical wound in humans and experimental wound in laboratory rats: In rats, healing is by contraction and in humans healing is mainly by re-epithelialization.[24,25] Second, in experimental wound, it is

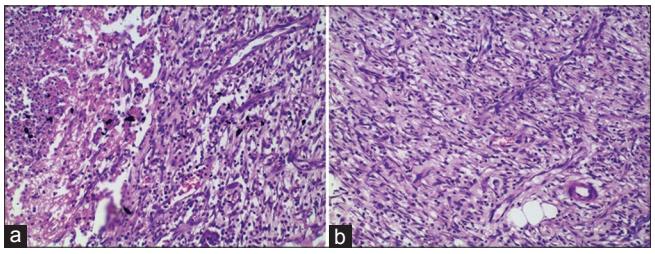

Figure 2: (a) Negative control Day 9: Moderate amount of immature granulation Tissue getting converted to mature (Centre) (20 x 10). (b) *Jatyadi Ghrita* treatment Day 9: Profound amount of mature granulation Tissue with fibroblasts and sparse extra cellular matrix proteins forming layers, Vesselsrunning perpendicular to the fibroblasts direction (white arrow) (20x10)

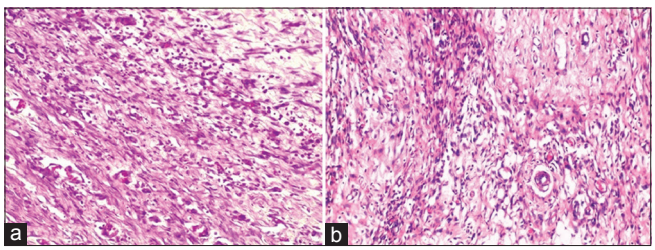

Figure 4: (a) Negative Control Day 15: Fibrosis in progress with moderate amount increased extracellular matrix proteins; however fibroblastsand vessels are still present. Fibrocytes are not properly rearranged and running parallel to each other as compared to *Jatyadi Ghrita* treatment group (20x10). (b) *Jatyadi Ghrita* Day 15: Fibrosis in progress with increased extracellular matrix proteins (mainly collagen) dominating, fewer fibroblasts and vessels. Fibrocytes are rearranged and running parallel to each other (20x10)

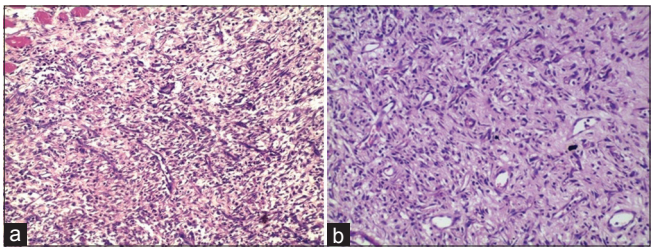

Figure 3: (a) Negative Control Day 11: Profound amount of mature granulation tissue with large number of fibroblasts and scarce extra cellular matrix proteins forming layers as compared to *Jatyadi Ghrita* treated group, vessels running perpendicular to the fibroblasts direction (20x10). (b) *Jatyadi Ghrita* treatment Day 11:Profound amount of mature granulation tissue getting converted to fibrosis with increased extra cellular matrix proteins forming layers, vessels running perpendicular to the fibroblasts direction and presence of fibrocytes (20x10)

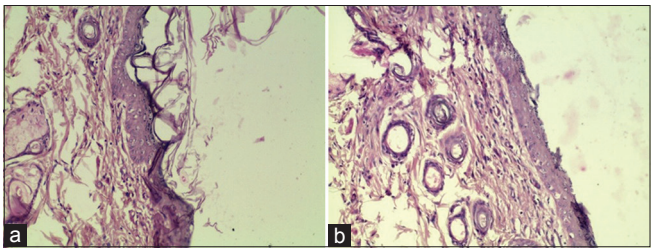

Figure 5: (a) Negative Control Day 15: Re-epithelialization with mild hyperplasia of epidermis and moderate hyperkeratosis (20x10). (b) *Jatyadi Ghrita* Day 15: Re-epithelialization with mild hyperplasia of epidermis (20x10)

Table 3: Histopathological grading score for various histomorphological changes

Groups	Immature granulation tissue	Mature granulation tissue	Re-epithelialization	Fibrosis
Day 1				
Negative control group	0	0	0	0
Diabetic control group	0	0	0	0
Ghrita group	0	0	0	0
Mupirocin HCl group	0	0	0	0
Jatyadi Ghrita group	0	0	0	0
Day 3				
Negative control group	0	0	0	0
Diabetic control group	0	0	0	0
Ghrita group	0	0	0	0
Mupirocin HCl group	0	0	0	0
Jatyadi Ghrita group	1	0	0	0
Day 5				
Negative control group	1	0	1	0
Diabetic control group	1	0	1	0
Ghrita group	1	0	1	0
Mupirocin HCl group	1	0	1	0
Jatyadi Ghrita group	3	0	2	0
Day 7				
Negative control group	2	0	1	0
Diabetic control group	2	0	1	0
Ghrita group	3	0	1	0
Mupirocin HCl group	3	1	1	0
Jatyadi Ghrita group	0	3	2	0
Day 9				
Negative control group	1	2	1	0
Diabetic control group	0	2	1	0
Ghrita group	1	1	1	0
Mupirocin HCl group	0	3	1	0
Jatyadi Ghrita group	0	3	2	0
Day 11				
Negative control group	0	3	1	0
Diabetic control group	0	3	1	0
Ghrita group	0	3	1	0
Mupirocin HCl group	0	2	2	1
Jatyadi Ghrita group	0	1	3	3
Day 15				
Negative control group	0	1	3	1
Diabetic control group	0	1	3	1
Ghrita group	0	1	3	1
Mupirocin HCl group	0	1	3	2
Jatyadi Ghrita group	0	0	3	3

possible to note temporal changes in size and shape in all the rats at a time. This helps in deciding the definitive score to each wound and hence to each group. Clinical observations of wound healing were given appropriate numeric scores. The present study indicated that *Jatyadi Ghrita* does not have significant effect on wound contraction but it improves the healing score for dryness of the wound, decreases the inflammation and helps in the enhancement of crust formation. Histopathological observations indicated that re-epithelialization rate from day 5 onward in *Jatyadi Ghrita*-treated rats was significantly higher as compared to other groups. *Jatyadi Ghrita* also indicated faster maturation of granulation tissue, lesser inflammatory cells and early angiogenesis. The present study concludes that *Jatyadi Ghrita* has analogous wound healing property as that of mupirocin hydrochloride.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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हिन्दी सारांश

जात्यादि घृत के व्रणरोपक गुणधर्म के परीक्षण हेतु प्रयोगजन्य व्रण के लिए निर्मित प्रयोगिक तथा ऊतीविकृति निरीक्षण गणना पद्धति

पल्लवी एस. जमदग्नी, श्रीरंग जमदग्नी, कोयल मुखर्जी, सच्चिदानंद उपाध्याय, सुदेश गायधनी, जयराम हजरा

प्रस्तुत अध्ययन में ६-८ सप्ताह के नर विस्टर चूहों में सिरा द्वारा स्ट्रेप्टोज़ोटोसिन ६५ मि.ग्रा/.कि.ग्रा . देने के १५ मिनट पूर्व २३० मि.ग्रा/.कि.ग्रा .निकोटिनामाइड उदरगत त्वचा के नीचे देकर डायबिटीज़ मेलाईटस कि उत्पत्ति कि गई । डायबिटीज़ युक्त चूहों को विभिन्न समूहों में विभक्त किया गया जैसे डायबिटीज़ नियंत्रित, घृत नियंत्रित, डायबिटीज़ चिकित्सा नियंत्रित, म्यूपायरोसिन एच.सी.एल., जात्यादि घृत चिकित्सा तथा डायबिटीज़ निर्माण नहीं किया हुआ समूह। त्वचा की सम्पूर्ण मोटाई तक काट कर घाव तैयार किया गया और उसका व्यास नापा गया। रोजाना चूहों का निरीक्षण किया गया घाव की अवस्था की गणना पद्धति तैयार की गई। घाव का व्यास तथा घाव गणना से प्राप्तांकों को ०, १, २, ५, ७, ९, १२ तथा १५ वें दिन पर अवलोकन किया गया। घाव का ऊती विकृति परीक्षण करने हेतु चूहों का निश्चित समय पर बलिदान दिया गया । सम्पूर्ण एकत्रित अवलोकन का सांख्यिकीय विश्लेषण किया गया । परिणाम स्वरूप यह पाया गया कि किसी भी नियंत्रण समूह की तुलना में घाव का व्यास तथा व्रणरोपण के प्रतिशत में कोई महत्वपूर्ण अंतर नहीं मिला, परन्तु जात्यादि घृत समूह में चिकित्सीय व्रणरोपण प्राप्तांक तथा ऊती विकृति में द्वितीय दिन से ही नियंत्रण समूह कि तुलना में महत्वपूर्ण सकारात्मक अंतर पाया गया। इस तथ्य से यह निष्कर्ष निकलता है कि औषध का व्रणरोपक गुणधर्म डायबिटीज़ नियंत्रक चिकित्सा औषधि म्यूरोपायरोसिन हायड्रोक्लोराइड के तुल्य है।