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Original Article

Studies on Wound Healing Potential of Polyherbal Formulation using *in vitro* and *in vivo* Assays in Swiss Albino Rats

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ABSTRACT

Background and Aim: Individual herbs, according to Ayurveda, are insufficient to provide the intended medicinal effect. When it is adjusted as a multiple herb composition in a certain ratio, it provides a greater medicinal impact with less toxicity. This investigation is an attempt to evaluate wound healing activity of poly herbal formulation along with *in-vitro* and *in-vivo* pharmacological activity on Swiss albino rats. **Experimental Procedure:** Full skin defects were made in the dorsal region of rats in order to examine wound healing. The activity of the polyherbal formulation was carried out by *in-vitro* (hydroxyproline, collagen and hexosamine) and *in-vivo* (excision and burn) assays along with histopathological study. Silver sulfadiazine was used as standard drug for the experimental work of this project while ketamine hydrochloride (20mg/kg intramuscular) was used as anesthize agent. The use of 2 µg/ml and 4 µg/ml formulations resulted in substantial mobilization of keratinocytes and fibroblasts at the injury site, respectively. In case of *in vitro* activity, effect of polyherbal formulation on biochemical measures such as hydroxyproline, collagen and hexosamine turnover were enhanced compared with untreated group. **Results:** Polyherbal formulation containing herbal extract was applied on wound area on day 0, 3rd, 6th, and 9th and was found to be more significant. Burn wound area on last day was observed 26.30 ± 0.5 while surgically produced wound area was 13.666± 0.8. **Conclusion:** Finally, on the basis of above results, we can conclude that polyherbal formulation may be a breakthrough for the treatment of wound healing in future.

Keywords: Wound healing, Epithelization, Hexosamine, Scar formation, Excised wound, Burn wound.

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INTRODUCTION

Wound healing is a coordinated series of interactions between molecules and cells that results in inflammation, re-epithelization, tissue creation, and remodeling to restore the skin barrier.[1] It is one of the most important subgroups in the broader category of "skin and subcutaneous disorders". A wound is a cellular, anatomic and functional integrity disturbance of the living tissue produced by physical, chemical, electrical or microbiological tissue hazards. They are categorized by the fundamental cause of wound formation.[2] The overall vulnerability of skin and subcutaneous illnesses has grown based upon statistics from the Global Burden of Disease (GBD). Over a ten-year period, the population has increased rapidly, with 605,036, 000 in 2015 compared to 492, 883,000 in 2005.[3] Chronic damage is defined by Werdin *et al.* 2008 and is not carried out in an ordered and prompt manner after 3 months in order to produce anatomical and functional integrity.[4] The wound healing process completes in three phase: the inflammatory phase (establishment of homeostasis and inflammation), the restorative phase, and the regenerative phase. The proliferative phase is the second phase of wound healing process, which includes granulation, contraction, and epithelialization. Lastly, the third phase is the restructuring phase, which determines the strength and look of the cured tissue.[5]

Infections, on the other hand, cause wound healing to be delayed. Wound infections are frequent in emergent countries due to inadequate sanitary conditions. Various robust preparations, components and chemical products from traditionally used medicinal plants were demonstrated to be wound healing in research.[6] These herbs accelerate injury cure by encouraging skin cell proliferation or differentiation, for example *Panax ginseng* C.A. Mey.,[7] *Apis mellifera* L.,[8] *Spermadictyon suaveolens* Roxb.,[9] *Camellia pubipetala* Y. Wan and S.Z. Huang,[10] *Glycyrrhiza glabra* L.[11] and *Curcuma longa* L.[12] which has been described for their wound healing activity. Therefore the historically utilized medicinal herbs are being given greater attention in developing efficient wound cure. Phytominerals rich in a variety of secondary

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metabolites have exceptional wound healing skills that can provide innovative, efficient, cheap and safe treatment for infected lesions. According to the World Health Organization, almost 20,000 medicinal plants were found in 91 countries.[13]

Azaderacta indica A. Juss. (primary component of that composition) is a well-known medicinal plant that is historically, it was used to cure wounds with number of reported pharmacological activity such as it showed significant Hepatoprotective Effect,[14] Antidiabetic Activity,[15] Antimicrobial Effect[16] and Anti-inflammatory activity in cotton pellet granuloma assay in rats[17] etc. In the field of Ayurvedic, some native herbal items have been reported for the accelerating treatment of wounds, including e.g. *Curcuma longa* L., *Terminalia arjuna* Roxb. Wight and Arn., *Centella asiatica* L., *Bidens pilosa* L., *Aloe barbadensis* L. and *Rauwolfia serpentina* L.[18] Plants have been used in traditional medicine to treat a variety of disorders.

Although beforehand preparation, antimicrobial prophylaxis and refining of surgical and an aesthetic treatment are still a severe concern postoperatively,[19] surgical injury still remains a major source of surgical incidence, fatality and extended seriously injuring. The development of post-operative wound infection is an important event that cannot always be prevented. Wound healing is the process of reconstruction of the break or discontinuation of skin surface.[20] Process of healing includes various events like introduction of inflammatory responses, regulation of parenchymal cells, and synthesis of matrix, protein, remodeling and collagenition.[21] However, the poly herbal formulation of wound healing action on the rat-model has not been previously described in detail.

MATERIALS AND METHODS

Selection, collection and identification of plants

Aloe Barbadensis miler (KUK/DOB/AZD/20), *Glycyrrhiza glabra L.* (KUK/DOB/AV/21), *Jasmimum officinale f. officinale* (KUK/DOB/BA/22), *Brassica juncea* (L.) *Czern* (KUK/DOB/CL/23) *Apis mellifera* L. (KUK/DOB/GG/24) and *Azaderacta indica* A. Juss. (KUK/DOB/GG/25) were procured and voucher number was deposited in Herbarium of Institute of Pharmaceutical Sciences, Kurukshetra University, Kurukshetra (Haryana). The sample was authenticated through Department of Botany, and Kurukshetra University, Kurukshetra.

Extraction Procedure

Before extraction, the leaves, stems, and bark of the plants were washed and shade dried. Using an electric blender, the dried plant material was crushed to powder. Using the Soxhlet equipment, the plant powders were extracted with water at 80°C to yield aqueous extracts. These extracts were then concentrated in a Rota evaporator (Buchi Instruments R-210) at a constant temperature of 60°C under decreased pressure and dried to powder, and their extractive yields were determined.[22]

Preparation of poly herbal formulation

To get the optimum formulation, the aqueous extracts of all plants were combined in equal proportions in order to enhance the acceptance and adoption of traditional remedies for tissue regeneration. With continuous stirring, 2.5g of Carbopol-940 (Ozone enterprises, Ahmedabad, India) was disseminated in 100 ml of distilled water. The carbopol-940 was allowed to expand overnight in the beaker. A separate water bath was used to heat a combination of 0.2 ml of 0.5 percent methyl paraben and 0.1 ml of 0.2 percent propyl paraben in 5 ml of distilled water. 5 ml of 5 percent propylene glycol-400, 0.2g sodium meta bisulphide, and the appropriate amount of aqueous combination of all plants (2g for 2 percent formulation and 5g for 5 percent formulation) were correctly combined after chilling. The finished liquid was poured onto a premade Carbopol formulation base, and triethanolamine (TEA) was added drop by drop to achieve the desired skin pH. To create the formulation of the desired consistency, enough water was added to the final composition.[23]

Quality control parameters of polyherbal formulation

Quality control of the polyherbal formulation was carried out by various parameters like pH, spreadibility-time (sec.), extrudability, color, odor, smoothness, and grittiness. pH was determined by using digital pH meter. 1g of polyherbal formulation was dissolved in 100 ml of distilled water and stored for 2 hr. The measurement of pH of polyherbal formulation was done in triplicate. The polyherbal was placed in between the slides under the direction of certain load. The spreadibility was expressed in terms of time (sec.) taken by two slides to slip off from polyherbal formulation. The extrudability of polyherbal formulations was determined in terms of weight (g) required to extrude a 0.5 cm ribbon of formulation in 10 sec. External characters of developed polyherbal formulation were also noted such as color, odor, smoothness and grittiness.

In-vivo Pharmacological Activity

Experimental Animals

Healthy Swiss albino rats weighing between 200-250g were obtained for the present study from Chaudhary Charan Singh Agriculture University, Hisar, and Haryana, India. All the experimental procedures and protocols used in the study were authorized by Animal house, Institute of Pharmaceutical Sciences, Kurukshetra University, Kurukshetra (Haryana). The study protocol was approved by the Institutional Animal Ethics Committee (Register Number: 563/02/A/CPCSEA) and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. The animals were acclimatized to the standard laboratory conditions at 22 ± 2°C, relative humidity 60-70% and light and dark cycle of 12:12 hr and fed with standard diet and water *ad libitum* during the study.

Wound healing activity

The wound healing activity of polyherbal formulation was assessed using excision and incision wound healing model.

Excision wound Model

A circular area of 500mm^2 diameter full thickness surgically excised wounds was created in 30 rats along the marking using toothed forceps, surgical blade and pointed scissors[8] were anaesthetized by ketamine hydrochloride (20mg/kg, intramuscular) over the dorsum portion using depilatory cream (Veet) after shaving and through cleaning. The animals were categorized into three groups, each with six animals (n= 6).

Group I: control group treated with normal saline solution

Group II: standard group treated with silver sulfadiazine (1% w/w)

Group III: test group treated with poly herbal formulation applied

For 9 days, polyherbal formulation were given twice a day in an equal volume of 0.1g of body weight of the animal. Daily dressing was done at least two times. Scar feature and time required to complete epithelization were monitored in control and study groups. In the excision wound model, wound area was measured by tracing the wound with the help of digital vernier caliper (least count = 0.001mm) on day 0, 3rd, 6th and 9th for all groups. Wound contraction was measured every day until complete wound healing and represented as percentage healed of wound area. Percentage of wound contraction was calculated taking the initial size of the wound as 100% and using the following formula:

$$\% \text{ wound contraction} = \frac{\text{Initial wound area} - \text{specific day wound area}}{\text{initial wound area}} \times 100$$

Burn wound model

Burn wound was shaped according to the method[24] already described by Srivastava and Durga Prasad, 2008. Animals (n=6) were fasted overnight and given anesthesia ketamine hydrochloride (20mg/kg, intramuscular) before having partial thickness burn lesions inflicted by pouring hot molten wax (80°C) into a 15 mm^2 diameter metal cylinder put on the shaved backs of the rats. The exposure time was 8 min.

Histopathological Study

The histological study was carried out as per standard protocol.[25] The collected tissue samples (wound area of the skin) of each group was dried with alcohol of different concentration for complete drying and later fixed in paraffin wax. The paraffin wax fixed tissue (5 µm) sectioned[26] were made by using microtome and kept for overnight drying in oven for staining (Hematoxylin and eosin). The histological changes were observed in sequential biopsies taken using a light microscope (BX50 Olympus, Japan) on days 3rd, 6th, and 9th in all three groups for both animal model i.e. excised and burn wound.

In vitro wound Healing Activity

Estimation of Hydroxyproline and Collagen

A sample of skin from the healed wound region was taken and analysed for hydroxyproline content, which is the fundamental component of collagen.[27] Tissue was dried in a hot air oven at 60-70°C for 48 hr to achieve a consistent weight before being hydrolyzed in 5 ml 6 N HCl at 110°C for 18-20 hr in a sealed tube. The hydrolysis was neutralized (pH-7.0) and exposed to chloramine-T oxidation for 15-20 min.[28] The reaction was stopped by adding 0.4 M per chloric acid, and the colour was produced at 60°C using (p-dimethylaminobenzaldehyde) Ehrlich reagent. The collagen content can be find out from the hydroxyproline content by the multiplication of factor 7.46 because collagen is the basic constituent of hydroxyproline.[29]

Estimation of Hexosamine

The weighted granulation tissues were hydrolyzed in 6 N HCl for 8 hr at 98°C, neutralized to pH 7 with 4 N NaOH, and diluted with Milli-Q water for hexosamine measurement. The hexosamine content of granulation tissues was measured using techniques modified from.[30] The diluted solution was combined with the acetyl acetone solution and heated for 40 min at 96°C. The liquid was cooled with 96% ethanol and an Ehrlich agent solution. After the solution was properly mixed and maintained at room temperature for 1 hr, the absorbance was measured with a UV/Visible spectrophotometer at 530 nm (Shimadzu). In milligram per gram of dry weight, the amount of hexosamine in the tissue was measured.[31]

Statistical Analysis

One-way ANOVA was used for test comparison with controls (p-values <0.05 were regarded important) and meaning was measured by multiple scope Duncan test with the help of SPSS software. The results were reported as a group mean ± SEM.

RESULTS

Quality Control of Polyherbal Formulation

The pH, spreadibility-time in seconds, rheology, color, odour, smoothness, and grittiness of the Polyherbal formulation all passed all quality control standards. The final product was consistent and easy to apply on the skin.

In vivo Wound Healing Activity

In polyherbal treated groups substantial increases in wound healing compared to control group were found. During the period of 9 days, Table 1 and 2 shows the reduction of the area of wound in different groups. On the 3rd, 6th, and 9th days after surgery, the wound area was measured in all groups. In the case of the excision wound model (Table 1), the mean wound area of the treatment group was 13.66±0.87 mm^2 in the 9th day post-operative and the saline treated normal was 21.41±1.07 mm^2, whereas the mean wound area of the treatment group was 43.17±.1.4 mm^2 in relation to the control group for the treatment of the burn wound model (Table 2) on the 9th day of post-operative surgery. Polyherbal formulation revealed considerable wound cure activity (p<0.05), whereas standard group of silver sulfadiazine showed progressive wound closure, but the full wound closure was seen by the sixth day of the subsequent surgery and on the ninth day in the control group of all treatment groups.

Histopathological Study

The top surgical wound layer was removed and histological investigations were conducted. The histological examinations of the polyherbal formulation of silver sulfadiazine treated hematoxylin and eosin-stained tissue of rats lead to decreased scar formation and enhanced fibroblast proliferation, angiogenesis, keratinization and epithelialisation compared to vehicle or control group treated. The Figures (Figure 1.1 and 1.2) of

Table 1: Effect of poly herbal formulation on excision wound (mm^2) model.

Treatment	Day 0	Day 3	Day 6	Day 9
Control group	25.37±1.7	56.951 ± 2.1	38.484 ± 1.3	21.410 ± 1.07
Standard group	26.01±1.9**	44.750 ± 1.2**	23.912 ± 0.8**	12.544 ± 0.47**
Polyherbal formulation	25.65±2.0**	43.602 ± 3.3**	27.077 ± 1.5**	13.666 ± 0.87**
F- value	7.8	9.1	37.4	32.6

Each value represents as the Mean ± S.E.M (N=6). Statistical analysis was done by one way ANOVA. *p<0.05, **p<0.01 when compared with control

Table 2: Effect of poly herbal formulation on burn wound (mm^2) model.

Treatment	Day 0	Day 3	Day 6	Day 9
Control group	141.2±1.2	133.54 ± 3.7	56.692 ± 1.1	43.170 ± 1.4
Standard group	145.5±1.0**	112.07 ± 1.4 **	50.329 ± 1.0**	24.77 ± 0.5**
Polyherbal formulation	143.9±1.1**	111.30 ± 1.4 **	53.146 ± 1.1**	26.30 ± 0.5**
F- value	8.4	10.3	59.4	115.1

Each value represents as the Mean ± S.E.M (N=6). Statistical analysis was done by one way ANOVA. *p<0.05, **p<0.01 when compared with control

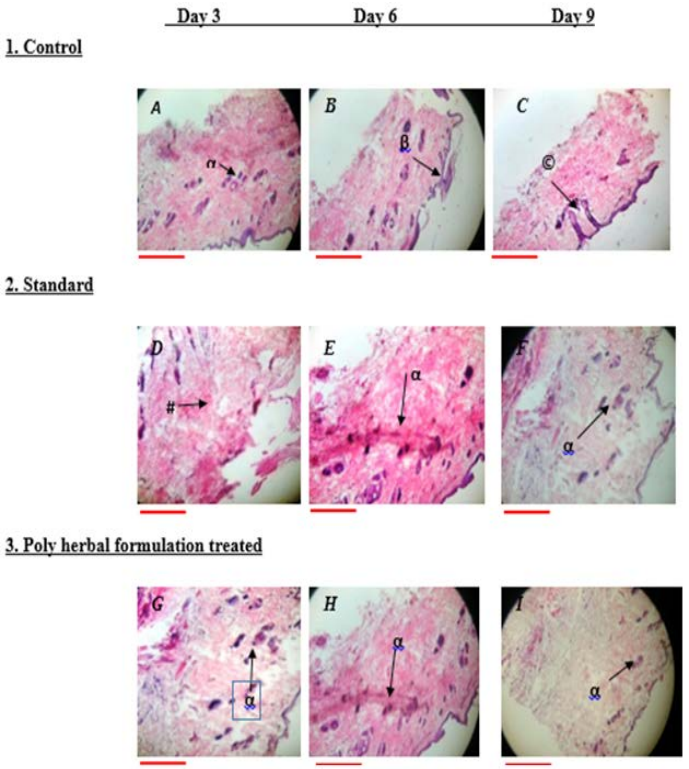

Figure 1.1: Histology photographs of excision wound model collected from different groups of rats on different days. (Magnification 10×) A) Control, B) Standard (framycetin), C) Poly herbal gel (PHG) treated (scale 100.0 pixel) α –sebaceous gland; β- epidermis; ©- granulated tissue; # -collagen.

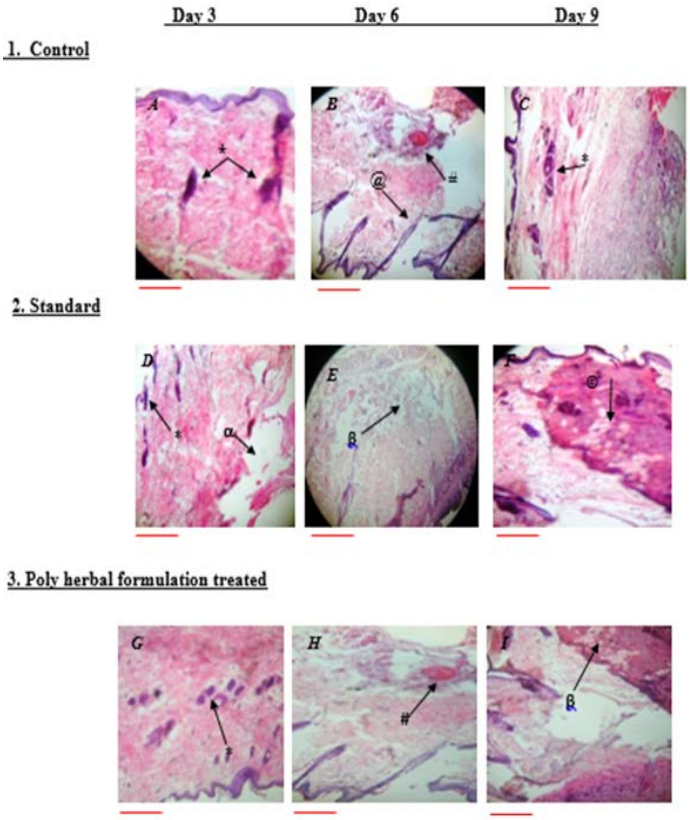

Figure 1.2: Histology photographs of Burn wound model collected from different groups of rats on different days. (Magnification 10×) A) Control, B) Standard (Silver Sulphadiazine), C) Poly herbal gel (PHG) treated (scale 100.0 pixel) # - sebaceous gland;β collagen; * - hair follicle; α-blood vessels; @ - epidermis.

different (3rd, 6th, and 9th) days of histopathology of different group has shown the progress in wound healing. The sample of control rat on day 3rd showed proliferation of epidermis. The dermis shows proliferation of capillaries with the formation of granulation tissue including a significant number of macrophages. The fibers were vertically oriented (Figure 1.1A). Similar changes were observed in standard treated group (Figure 1.1D). However, the skin section from polyherbal treated group showed intense inflammatory responses and epithelization starts earlier. Other histological changes were similar to control group but were more advanced (Figure 1.1G). Histological section (Figure 1.1 D-F) of standard group shows more healing process as compare to control group (Figure 1.1 A-C) owe to deposition of collagen fiber in the tissue. Due to infiltration of lymphocytes in the wound area of the animal shows less healing process. In addition to burn wound model (Figure 1.2), histology of the wound sample of all the groups (treated, standard, control) shows remarkable wound healing process. Comparatively, polyherbal treated group shows advancement in the healing process with respect to standard and control group due to the infiltration of lymphocytes, less inflammatory cells, more collagen fiber etc.

In-vitro wound Healing Activity

Table 3 shows the tissue content of hydroxyproline, collagen and hexosamine on different days after the surgery. Significant increases were seen in the hydroxyproline and hexosamine levels of polyherbal formulation treated groups as control groups. The hydroxyproline level of the standard and polyherbal formulation group was gradually increased in several days but the slow progressive increase till the 9th day was noticed in the control. During cure, a more important extracellular matrix in all treated than control groups has been identified for the hydroxyproline, collagen, and hexosamine content. These are useful indicators for the treatment of wounds. These chemicals mark the wound healing process well.

Table 3: Effect of Poly herbal formulation on biochemical assay of wound healing.

Groups	Hydroxyproline (µg/g)	Collagen (µg/g)	Hexosamine (mg/g)
Control	33.±3.9	244.5±25.9	6.8±2.5
Standard	49.2± 3.3***	367.2±28.9***	22.1±1.5***
Polyherbal formulation	65.3±5.8***	475.6±41.9***	27.9±2.0***

Each Value are expressed as Mean ± SEM (N =6). Statistical analysis was done by one way ANOVA. ***$p < 0.001$ when compared with Control.

DISCUSSION

Polyherbal therapy on injured animals leads to considerable healing activities for wounds. Wound healing is a homeostatic phenomena where the extracellular matrix can be repithelised, granulated and remodelled. The healing process is carried out by the immunological activities of the victim and requires little aid, but different risk factors such as tissue ROS generation, inflammation and microbial infection and the delayed immunity to healing have drawn attention to boost it.[32] Plant-based bio-actives have been shown to help with wound care and treatment by speeding up the process of epithelization and reducing skin scarring.[33]

The treatment of polyherbal produced remarkable healing activity due to the angiogenesis and proliferative capacities, as all the components studied and observed had a major effect on the healing of wounds.

The existing treatments do not have an efficient impact on the process of tissue repair, which is why new drugs need to be developed that have multiple tissue repair approaches. The better alternative would be the biocompatibility, not toxicity and safety of topical wound therapy and plant-derived bio-actives compounds. They enabled quick wound healing with fast shrinkage, shorter epithelization and collagen reshaping. The freshly healed wound was additionally supported by a sufficient tensile power.

The polyherbal formulation exhibited a substantial increase, compared to normal and negative control groups of rats in both the excision wound model and the burn wound model (Figure 1.1, 1.2). On the third and ninth days, the epithelization time was likewise observed to coincide to the wound closure diameter (Figure 1.1). The time of healing is mostly determined by the pace of wound contraction, which promotes and accelerates wound closure by fast re-epithelialization by reducing the nomadic distance travelled by keratinocytes. The extra cells matrix required to retrieve the injured epithelium is also reduced. Epithelization means that the newly produced epithelial cells are migrated and proliferated into damaged wound beds. The sufficient vitality of epithelial cells as compared to normal and negative control might be related to the shorter epithelial period by polyherbal. It had a considerable and effective cure for wound contraction and epithelization compared to normal and adverse group of animals.

All of the indicators observed were considerably altered, indicating that it has a strong pro-healing action. Collagen, a component of developing cells, is synthesized by healing tissue. Collagen concentration is measured by hydroxyproline concentration. The higher the hydroxyproline content, the faster the wound heals. Biochemical study has revealed an increased amount of hydroxyproline that reflects enhanced cell proliferation and an increase in production of collagen. The increased concentration of hexosamine shows the stability of collagen molecules via enhancing electrostatic and ionic interactions.[34] Collagen not only provides strength and integrity to the tissue matrix, but it also plays an important role in homeostatic process and the later stages of healing epithelisation. Improved tissue production and patterning are therefore provided by hydroxyproline and the hexosamine-treated in rats. It has shown a strong wound-healing ability as demonstrated through wound contraction and has thus confirmed the ethnotherapy claim with improved structural integrity and metabolic process in the injured tissue. The healing features were shown more rapidly in burn wound as compare to physically excised wound but there is no significant difference between both of them. From the above study it can be reported that polyherbal has potent action in burn as well as physically excised wound healing.

CONCLUSION

Our results showed that polyherbal formulations produced from the combination of plant extracts of *Azadiracta indica A. Juss, Aloe Barbadensis miler, Glycyrrhiza glabra* L., *Jasmimum officinale* f. *officinale,* and *Apis mellifera* L. accelerate the wound healing effect A major contributory element in the production of collagen, increasing hydroxyproline and hexosamine, wound contraction and the strength of wound break that might be associated with improved healing of the wound might be poly herbal composition. Further studies with advanced therapeutic approaches such as diabetic wound healing and clinical trials are ongoing.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

PHF: Poly herbal formulation; **GBD:** Global Burden of Disease.

Compliance with Ethical Standards

The animal study protocol was approved by the "Kurukshetra University, Kurukshetra Haryana, India Animal Ethics Committee" (Register Number: 563/02/A/CPCSEA) and "CPCSEA, Government of India".

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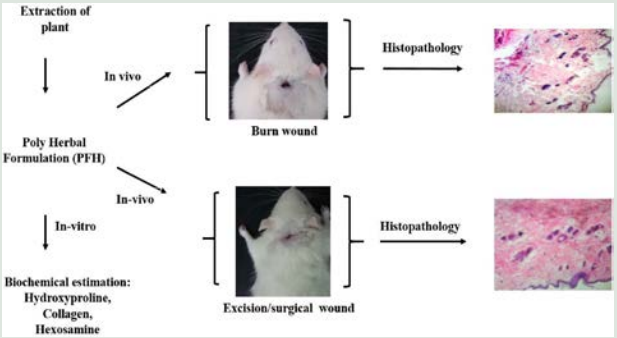
GRAPHICAL ABSTRACT



SUMMARY

- Wound healing activity (excision and burn wound) activities of five herbs and their polyherbal formulation were evaluated.
- *In vitro* assay (Biochemical estimation) of polyherbal formulation was carried out through in hydroxyproline, collagen, and hexosamine.
- Histopathological study of the wound tissue was done on day 3rd, 6th, 9th.
- The polyherbal formulation at a concentration of 2-4 µg/Ml showed significant results against *in vitro* and *in vivo* study.

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