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In-Vivo Healing Potential of *Punica Granatum* and *Glycyrrhiza Glabra* on Excisional Dermal Wounds

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ABSTRACT

The methanolic extract of *Punica granatum* (PUG), *Glycyrrhiza glabra* (GLA) and their combination (PUG-GLA) were evaluated for their wound healing activity using excision wound model in rats. Rats treated with PUG-GLA showed higher rate of wound contraction, significant decrease in epithelization period and significant increase in hydroxyproline content of granulation tissue when compared with the controls.

The histological examination of treated wounds showed that the original tissue regeneration was much greater in PUG-GLA, with increase in the restoration of collagen fibers, fibroblasts, blood vessel formation and hair follicle regeneration.

Keywords: *Punica granatum; Glycyrrhiza glabra;* Wound healing; Excision model; Hydroxyproline

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INTRODUCTION

A wound is defined as bodily injury caused by physical means with disruption of the normal continuity of the structure[1]. Healing of wounds is an intricate, well-orchestrated physiological event, which includes formation of fibrin clot, inflammatory response, tissue granulation (re-epithelization and angiogenesis), matrix formation and remodeling[2].

Different phases of wound healing have been recognized, although the process is continuous, with each phase overlapping the next.

Punica granatum L. (Pomegranate) member of family Punicaceae is a large shrub native to Iran and Himalayas of north India and used medicinally in Indo- China, Europe, North Africa and South Africa. Pomegranate peel extract, rich in ellagitannins has proved their efficacy as antioxidant and antineoplastic agents, particularly against breast and colon cancer[3]. Traditionally this folkloric herb is believed to have benefits like antiulcer, hepatoprotective, antihelmintic and used in snakebite and dysentery[4]. The plant is also rich in antioxidants[5], has antibacterial properties[6], has been found useful in treating dental[7] and dermatologic conditions and improves cardiovascular health.

Glycyrrhiza glabra L. (Licorice) member of family Fabaceae is native to Mediterranean region and central and southwest Asia. It was found to be useful from the ancient Roman Empire and also in Chinese herbals[8]. The anti-inflammatory activity of glycyrrhizin and glycyrrhetinic acid has been used in the preparation of skin cosmetics and in treatment of dermatoses and pruritis. The main anti-inflammatory constituent of the plant, Glycyrrhizin, inhibits both cortisone degradation in the liver and generation of reactive oxygen species (O_2^-, H_2O_2,$^{\cdot}OH$) by neutrophils. Since there is no effect on reactive oxygen species generated by cell free system, glycyrrhizin does not act as a scavenger for these entities but decrease their generation by inhibiting neutrophil metabolism[9].

There is no previous report on wound healing activity of PUG-GLA to the best of our knowledge and considering the wide traditional use of both the herbs and lack of their individual adverse effects, its wound healing efficacy was studied.

MATERIALS AND METHODS

Plant materials

G. glabra roots (Fabaceae) and *P. granatum* pericarp (Punicaceae) were collected locally in August 2012 were identified by Dr. H.M. Pandit, Department of Botany, Guru Nanak Khalsa College, Mumbai, India. Voucher specimens [*P. granatum* (3513a) and *G. glabra* (3513b)] were

deposited in the institute.

Extraction and drug formulation

The plants sun dried and powdered (100 g) extracted with Methanol in Soxhlet apparatus gave residue 18.5 g of GLA and 17 g of PUG under reduced pressure with rotary evaporator. A hydrogel containing Carbopol 1% was prepared incorporating 0.25 g of each extract in 50 g of the gel. For the vehicle control group, gel was prepared without incorporating extracts.

Animals

Sprague Dawley rats of either sex, weighing 200-250 g, were individually housed in standard environmental conditions and fed with animal diet and water *ad libitum.* Standard rat pellet food was procured from Pranav Agro Industries Ltd. The study was approved by the Institutional Animal Ethics Committee (IAEC) for animal experimentation (Approval No: IAEC/PR/2012/02) at Bharati Vidyapeeth's College of Pharmacy, Navi Mumbai, India.

Wound healing activity

Excision wound model was used to evaluate wound healing activity of both the herbs. The parameters of efficacy such as wound closure and period of epithelization were studied. Animals were anesthetized and a full thickness skin was excised of area 300 mm^2, 2mm in depth, on the shaved ethanol sterilized dorsal thoracic region of the rats[10]. The wounds were left undressed to the open environment.

Animals were divided into six groups of 6 each and treated topically, till complete epithelization, as follows: Group I was treated with placebo gel; Group II served as wounded control; Group III was treated with 1% gel formulation [PUG (0.5%) + GLA (0.5%)]; Group IV was treated with 0.5% gel formulation [PUG (0.5%)]; Group V was treated with 0.5% gel formulation [GLA (0.5%)] and Group VI served as reference standard control and were treated with Framycetine Sulphate 1% w/w cream.

The area of the wounds were measured by tracing the wounds on to a graph paper on the day of wounding and subsequently on days 4, 8, 12 and 16 post wounding. The number of days needed for falling of the scar without any residual wound, gave the period of epithelization[11].

On the 10^{th} day, granulation tissue from animals of the groups were collected, washed with cold saline and lyophilized for hydroxyproline estimation[12]. Hydroxyproline was measured using Bergman & Loxley method[13]. The granulation tissue was dried in oven at 60ºC for 12 h and weighed. The dried tissue was added to 2 ml of 6 N HCl and kept at 110ºC for 24 h. The neutralized acid hydrolyzed fraction was used for determination of hydroxyproline.

Statistical Analysis

All results, expressed as mean ± SE were analyzed by one-way ANOVA, followed by Dunnet's *post-hoc* test in which all groups were compared with wounded control group. *P*-value < 0.01 was considered statistically significant.

Histopathological study

Samples of the skin tissues from the healed wounds of each group were taken for the histopathological examination[14]. Skin tissue from healed wound was excised from animal of each group and preserved in 10% buffered formalin. Thin sections were stained with Hematoxilin and Eosin solution and photographed under 40X magnification. The histological evaluation was done to study arrangement of collagen tissues, hair follicle regeneration, blood vessel formation, epithelization and fibroblast proliferation[15, 16].

RESULTS AND DISCUSSION

Table 1 shows wound healing in various groups. On day 12, significant increase in wound closure ($P < 0.01$) was observed in animals treated with PUG-GLA combination than other groups. However, the vehicle control group did not show any significant increase, when compared to the wounded control. Thus, the combination of PUG and GLA was significantly more effective than that of each alone.

It was also observed that the epithelization period of the treatment and standard groups were less in comparison with the controls. On the 17th day, the wound treated with the combination gel had healed completely while the wound treated with standard was almost completely healed. On the 18th day, the standard treated group healed completely. Period of epithelization was 17 days for final formulation treated group, whereas it was 27 days for wounded control group. Statistically significant increase in the hydroxyproline content ($P < 0.001$) was observed in PUG-GLA and standard treated group as compared with the controls. (Figure 1)

Microscopic changes in histopathological study of tissue are showed in Figure 2. The histological examination showed that the original tissue regeneration was much greater in the wounds treated with PUG-GLA with increase in restoration of collagen bundles, fibroblasts, blood vessel formation and hair follicle regeneration. Wounded and vehicle control groups showed irregular arrangement of collagen fibers, and wounds were only moderately cellular with fibroblast cells. Mono nuclear cells (MNC) infiltration was seen in standard treated group, which is indication of acute inflammation.

Table 1: Results of % wound contraction and epithelization period in various groups.

Groups	% Wound Contraction				Period of Epithelization (Days)	Hydroxy Proline (µg/100 mg)
	4th day	8th day	12th day	16th day		
Wounded control	6.1± 0.228	27.7± 1.23	52.8±1.19	79.4 ± 2.28	27	9.22±0.24
Vehicle control	11.0 ± 2.46	38.0 ± 2.8 [b]	61.7 ± 1.06	86.2 ±2.76[a]	24	11.03±0.37
P. granatum gel (0.5%)	13.0 ±0.92[b]	35.7± 0.98[a]	67.2 ± 1.7 [b]	90.0 ±2.30[b]	22	12.84±0.75[a]
G. glabra gel (0.5%)	13.6 ±1.45[b]	39.2± 1.43[c]	73.5 ±3.54[b]	95.2 ±0.58[c]	20	13.15±0.72[b]
Combination gel (1%)	15.8 ±0.53[c]	45.9± 1.14[c]	80.0 ±2.25[b]	97.5 ±0.50[b]	17	14.63±0.92[c]
Framycetine Sulphate cream (1% w/w)	14.3 ±0.89[c]	51.3± 2.72[c]	79.1 ±3.45[b]	96.4 ±0.58[b]	18	14.47±0.91[c]

Values are mean ± SEM. N=6 , [a] $P < 0.05$, [b] $P < 0.01$, [c] P value < 0.001 vs. control group.

All wound are measurements were expressed as a percentage of the initial wound size.

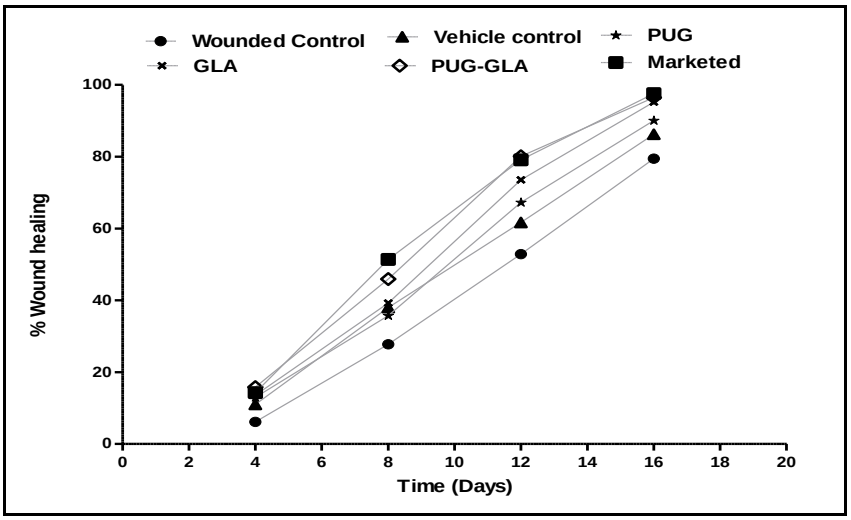

Figure 1: Comparative % wound recovery graph of treatment groups.

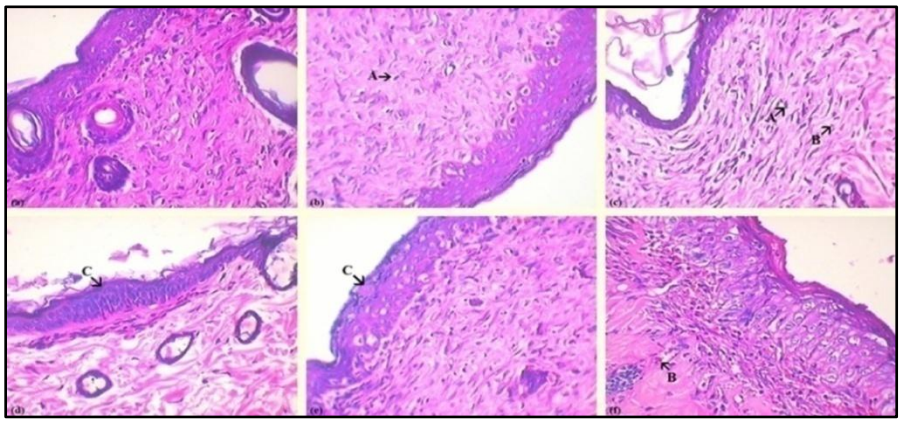

Figure 2: Photomicrographs of sections stained with Haemotoxylin and Eosin of healed wounds. A: connective tissue (collagen), B: Fibroblast, C: Epithelium. (a) Vehicle control, (b) Wounded control, (c) PUG-GLA treated wounds, (d) PUG treated wounds, (e) GLA treated wounds and (f) Standard treated wounds. (Magnification: 40 X)

CONCLUSION

There is no drug in clinical use that may directly act as wound healing drug, though antibiotics, steroids and non-steroidal anti-inflammatory drugs are used for the treatment of wounds, however they have several drawbacks. The search for wound healing natural remedies has drawn attention to herbals[1].The immense literature on wound treatment is mainly focused on skin, which is the most vulnerable part of the body that interacts with the environment and, therefore receives constant insult and injury[17]. Despite rapid advancement in pharmaceuticals the availability of drugs capable of stimulating wound repair still remains ambiguous. The increasing popularity of traditional herbal medicines, easy availability of raw materials, cost-effectiveness and less reported adverse reactions, prompted us to formulate this topical preparation and assess its wound healing ability. Any one of the phytochemical constituents present in the *P. granatum* and *G. glabra* extract may be responsible for the wound healing activity. Recent studies on other plant extracts have shown that phytochemical constituents like flavonoids[18] and triterpenoids[19] are known to promote the process of wound repair due to their antibacterial and astringent properties.The outcome of study showed that, the topical application of *P. granatum* and *G. glabra* extract in the form of gel enhance the rate of wound contraction and level of hydroxyproline, decreased epithelization period and displayed superior result in histopathological study. Further pharmacodynamic research is required to identify active fractions reliable for excision wound healing activity.

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