Evaluation of Wound Healing Potential of Some Indian Herbal Extracts and it’s Formulation in Acne Vulgaris

S. A. Thube1*, M. J. Patil2
1Department of Pharmacognosy, M.C.E. Society’s Allana College of Pharmacy, Pune, Maharashtra, India,
2Department of Pharmacognosy, M.M’s College of Pharmacy, Tathawade, Pune, Maharashtra, India

ABSTRACT

Objective: Butea monosperma, Barleria prionitis, Casuarina equisetifolia, Dalbergia sissoo, and Lagenaria siceraria are reported to possess anti-acne, anti-inflammatory, anti-ulcer, and wound healing activity. The present work is aimed at evaluating the wound healing potential of these plant materials in acne vulgaris. Methodology: Individual gel formulations containing extracts of the above mentioned drugs and their polyherbal gels were evaluated by excision and incision wound model (in vivo) in Sprague Dawley rats. Results: The individual gel formulations showed significant reduction in wound size when compared with the untreated group. The rates of wound closure after the application of the gels were compared with the untreated wounds. The polyherbal formulation containing all the extracts was found to be more beneficial when compared to remaining treated groups. Healing under scab and formation of normal epithelial cells were better than other individual formulations. As well as vascular changes, cellular infiltration, and necrosis were found less. Conclusion: From the results, it may be concluded that the plants are endowed with significant wound healing activity, thereby justifying its traditional medicinal use in acne vulgaris as a separate entity and in combination. Chemical components such as tannins, flavonoids, saponins, and alkaloids present in the extracts can be responsible for the foresaid activity.

Keywords: Acne, excision model, incision model, wound healing

INTRODUCTION

Wounds are physical injuries that result in opening or break of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. The basic principle of optimal wound healing is to minimize tissue damage and provide adequate tissue perfusion and oxygenation, proper nutrition and moist wound healing environment to restore the anatomical continuity and function of the affected part.1 Inflammation, which constitutes a part of the acute response, results in a coordinated influx of neutrophils at the wound site. These cells, through their characteristic “respiratory burst” activity, produce free radicals.2 Wound related non-phagocytic cells also generate free radicals by involving non-phagocytic nicotinamide adenine dinucleotide phosphate-oxidase mechanism.3 Thus, the wound site is rich in both oxygen and nitrogen centered reactive species along with their derivatives. The presence of these radicals will result in oxidative stress leading to lipid peroxidation, DNA breakage, enzyme inactivation, and free-radical scavenger enzymes. Evidence for the role of oxidants in the pathogenesis of many diseases suggests that antioxidants may be of therapeutic use in these conditions. Topical applications of compounds with free-radical-scavenging properties in patients have shown to improve significantly wound healing and protect tissues from oxidative damage.4 In India, medicines based on herbal origin have been the basis of treatment and cure for various diseases and physiological abnormalities under practice such as ayurveda, siddha and Unani. Moreover, Indian folk medicine comprises numerous prescriptions for therapeutic purposes such as healing of wounds, inflammation, skin infections, leprosy, diarrhea, scabies, venereal disease, ulcers, and snake bite. More than 80% of the world’s population still depends upon traditional medicines for various skin diseases. Herbal medicines in wound management involve disinfection, debridement...
and providing a moist environment to encourage the establishment of a suitable environment for natural healing process. Research on wound healing agents is one of the developing areas in modern biomedical sciences. Several drugs of plant, mineral, and animal origin are described in the traditional texts of Indian systems of medicine like ayurveda for their healing properties under the term “Vranaropaka.” Besides the classical systems of Indian medicine, the folk and the tribal medicine also employ a number of plants and animal products for the treatment of cuts, wounds, and burns. Some of these plants have been screened scientifically for the evaluation of their wound healing activity in different pharmacological models and human subjects, but the potential of most of the plants remain unexplored.6

Inflammation plays a major role in the pathogenesis of acne. As microcomedones form, a lymphocytic infiltrate occur, which triggers inflammation.7 This tends to elicit follicular keratinocytes further to produce more keratin, as well as stimulating increased sebum production and reducing linoleic acid content in the sebum generated by the sebaceous glands. Most Westernized people have experienced the inflammatory nature of acne vulgaris, given the various red, swollen, tender lesions associated with it, particularly papules, pustules, nodules, and cysts.

Herbs that relieve inflammation could therefore also be useful for limiting or resolving acne. Berberine-containing herbs, besides their antimicrobial action have been shown to be inflammation-modulating.8 Besides Oregon grape, Berberis vulgaris (barberry), Captis chinensis (gold thread), Hydrastis canadensis (golden seal), and Xanthorrhiza simplicissima (yellow root) all contain berberine and similar alkaloids. Oregon grape has been shown repeatedly to be helpful in clinical trials for patients with psoriasis, another inflammatory skin condition.9

Wound healing is a complex process that can be roughly divided into three overlapping phases of inflammatory reaction, proliferation, and remodeling. The inflammatory phase involves vascular responses characterized by blood coagulation and hemostasis as well as cellular events, including infiltration of leukocytes with varied functions in antimicrobial and cytokine release, which initiates the proliferative response for wound repair. During the proliferative phase, there is formation of the epithelium to cover the wound surface with a concomitant growth of granulation tissue to fill the wound space. Granulation tissue formation involves proliferation of fibroblasts, deposition of collagens and other extracellular matrices, and development of new blood vessels. Once the new tissue within the wound is formed, the remodeling phase begins to restore tissue structural integrity and functional competence.10

Traditionally, the leaves of Barleria prionitis (Acanthaceae), leaves of Butea monosperma (Papilionaceae), Casuarina equisetifolia bark (Casuarinaceae), Lagenaria siceraria fruit (Cucurbitaceae) and Dalbergia sissoo bark (Papilionaceae) were used for the treatment of acne.11 In our past study on these plants showed a promising result in terms of their ability to reduce Propionibacterium acne and Staphylococcus epidermidis population, the causative organisms for acne and to act as antioxidants.12 The present study has been undertaken to assess the wound healing ability of the individual extracts in a gel form and their polyherbal formulation. The ability of these crude drugs to heal wounds can be thought to be helpful in resolving the clinical symptoms associated with acne.

MATERIALS AND METHODS

Plant materials

The leaves of B. prionitis (Acanthaceae) and B. monosperma (Papilionaceae), C. Equisetifolia bark (Casuarinaceae), L. siceraria fruit (Cucurbitaceae) and D. sissoo bark (Papilionaceae) were collected from Ahmednagar district and authenticated at Botanical survey of India. Voucher specimens of the plants have been deposited at BSI, Pune.

Preparation of extract and preliminary phytochemical screening, the plant materials were dried, coarsely powdered and extracted by continuous hot extraction (soxhlet) method using ethanol (95% v/v). The extracts obtained were concentrated and subjected to phytochemical screening.13

Molisch test

The extracts were combined with a small amount of Molisch’s reagent (α-naphthol dissolved in ethanol) in a test tube. After mixing, a small amount of concentrated sulfuric acid was slowly added down the sides of the sloping test tube, without mixing, to form a layer. A positive reaction is indicated by appearance of a purple ring at the interface between the acid and test layers.

Biuret test

The extracts were treated with an equal volume of 1% strong base, i.e., potassium hydroxide followed by a few drops of aqueous copper (II) sulfate. Formation of a purple color confirmed for the presence of proteins.
Ninhydrin test

Three drops of 1% solution of ninhydrin in ethanol were added to 1 ml of the extract solution, and the solution was heated for 5 min in a boiling water bath. The formation of red, blue, or purple color gives a positive result for the presence of proteins.

Dragendorff’s test

A volume of 2-3 ml of the solution few drops of Dragendorff’s reagent, i.e., potassium bismuth iodide solution was added. An orange-brown precipitate was formed.

Mayer's test

A volume of 2-3 ml of the sample solution few drops of Mayer's reagent, i.e., potassium mercuric iodide solution is added. A white precipitate is formed.

Ferric chloride test

To the extract solution a few drops of 1% FeCl₃ was added. Formation of green or blue color indicated the presence of tannins.

Lead acetate test

To the test solution add 10% lead acetate solution. Formation of the white precipitate of lead tannate indicates the presence of tannins.

Shinoda test

To the test solution few magnesium turnings and concentrated hydrochloric acid was added drop-wise. Pink scarlet to green to blue color appears after min, which confirms for the presence of flavonoids.

Liebermann Burchard’s test

The extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled, and cooled. Concentrated sulfuric acid was added through the sides of test tube. The formation of brown ring at the junction indicated the presence of steroidal saponins.

Foam test

The extracts were diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 min. The formation of 1 cm layer of foam indicates the presence of saponins.

Determination of total phenolic content (TPC)

TPC was analyzed by the Folin–Ciocalteu colorimetric method using gallic acid as standard developed by Ragazzi and Veronese with modification and expressed as mg/g gallic acid equivalent (GAE) on dry weight basis.¹⁴ The 25 mg plant extract was dissolved in 10 ml of 50% MeOH:H₂O (1:1), at room temperature and in its 1.0 ml, 1.0 ml of Folin’s Reagent (1 N) and 2.0 ml of Na₂CO₃ (20%) were added subsequently. The test mixture was mixed properly on cyclomixer, left at room temperature for 30 min and maintained to 25 ml with water. The absorbance of test mixture was measured at 725 nm. The reported TPC were expressed as GAE mg/g.

Development of the formulation

Literature survey showed that carbomers have many advantages as a gelling agent viz., high viscosity at low concentrations, stability to heat, unaffected by aging, do not support microbial growth, non-toxic and nonirritant, etc.¹⁵,¹⁶ Taking into consideration above facts carbomer 940 was taken for the formulation of gel. Eight different gel formulations were prepared containing the extracts at different concentrations.

Wound healing activity

Experimental animals

Wister albino rats (Rattus norvegicus), (male) of 4 weeks, weighing between 100 and 150 g were used for wound healing activity. The animals were housed in standard environmental conditions of temperature (31 ± 1°C), humidity (60 ± 0.2%) and 12 h light and 12 h dark cycle. Rats were fed with standard rodent diet and tap water. The approval was obtained from the Animal Ethical Committee of M.C.E Society’s Allana College of Pharmacy, Pune.

Excision wound model

The experiments were carried out in accordance with the Animal Ethical Committee after obtaining approval. The backs of the animals were shaved and sterilized with 70% ethanol and tincture Iodine. 5 mm × 5 mm excisions were created by a surgical blade from a predetermined shaved area on the back of each animal. The wounds were left undressed to the open environment, and no local or systemic antimicrobial agents were used. This model was used to monitor the rate of wound contraction. The experimental groups were topically applied with the extract twice daily for consecutive 10 days. The group treated with...
Aloe vera gel served as a standard. The wound contractions were measured by tracing paper on the wounded margin and calculated as a percentage reduction in wound area. The actual values were converted into a percentage value taking the size of the wound at time of wounding as 100%. The granulation tissues were monitored on the 4, 8, and 16th post wound days and analyzed for scab formation, necrosis, vascular changes, healing, cellular infiltration, and epithelial cell formation. The animals were divided into 10 groups each having five rats (Figure 1).

Group 1: Served as the untreated group.
Group 2: Served as reference standard treated with Aloe vera gel
Group 3: Served as test group treated with formulation 1 (B. prionitis gel)
Group 4: Served as test group treated with formulation 2 (B. monosperma gel)
Group 5: Served as test group treated with formulation 3 (C. equisetifolia gel)
Group 6: Served as test group treated with formulation 4 (D. sissoo gel)
Group 7: Served as test group treated with formulation 5 (L. siceraria gel)
Group 8: Served as test group treated with polyherbal gel formulation 6 (B. prionitis and C. equisetifolia gel)
Group 9: Served as test group treated with polyherbal gel formulation 7 (C. equisetifolia and D. sissoo gel)
Group 10: Served as test group treated with polyherbal gel formulation 8 (containing all the extracts).

Figure 1: Wound healing activity of the control, standard and the formulations by excision wound model. Scab formation (yellow arrow), necrosis (black arrow), vascular changes (red arrow), healing (green arrow), cellular infiltration (blue arrow), and epithelial cell formation (white arrow). (a) Control ×400, (b) Standard ×400, (c) Formulation 1 ×400, (d) Formulation 2 ×400, (e) Formulation 3 ×400, (f) Formulation 4 ×400, (g) Formulation 5 ×400, (h) Formulation 6 ×400, (i) Formulation 7 ×400, (j) Formulation 8 ×400.
Incision wound model

Longitudinal para-vertebral incisions of 5 cm in length were made through the entire thickness of the skin and cutaneous muscle with the help of a scalpel. After complete homeostasis, the wounds were closed by means of interrupted sutures placed at equidistant points of 1 cm apart. The sutures were removed on the 8th post wound day, and the topical application of gel formulations and oral administration of the extracts continued. The animals were divided into ten groups each having five rats as in excision wound model19 (Figure 2).

Histopathological examinations

Wound tissue specimens from untreated and treated rats were taken at day 14 (after treatment). The tissue specimens were taken on 14th day (after treatment), so that the influence of the extracts and reference drugs on skin cells and its components can be evaluated well-instead of

Figure 2: Wound healing activity of the control, standard and the formulations by incision wound model. Scab formation, (yellow arrow), necrosis (black arrow), vascular changes (red arrow), healing (green arrow), cellular infiltration (blue arrow), and epithelial cell formation (white arrow). (a) Control group ×400, (b) Standard ×400, (c) Formulation 1 ×400, (d) Formulation 2 ×400, (e) Formulation 3 ×400, (f) Formulation 4 ×400, (g) Formulation 5 ×400, (h) Formulation 6 ×400, (i) Formulation 7 ×400, (j) Formulation 8 ×400.
the last day of complete wound closure where there will not much difference in the skin tissues and structures. The cross-sectional full-thickness wound scar of about 5 mm thick sections from each group were collected for the histopathological evaluation. Samples were fixed in 10% buffered formalin for 24 h and dehydrated with a solution of the sequence of ethanol-xylene series, processed followed by blocked with paraffin at 40-60°C, and sectioned into 5-6 μm thick sections. The sections were stained with hematoxylin and eosin stain. Collagen deposition was identified by staining with the sections with Van Gieson’s stain. Mast cells were stained with toluidine blue.

Measurement of shrinkage of wounds

The measurements of the wound areas of the excision wound model were taken following the initial wound using transparent paper and a permanent marker. The recorded wound areas were measured with graph paper. Progressive decrease in the wound size was monitored periodically.

Measurement of tensile strength

Tensile strength is a measure of the restored tissues resistance to breaking under tension and shows the strength of the healed tissue. The tensile strength was measured after 10th day after wounding. The newly formed tissue including scar was excised, and the tensile strength was measured using a tensiometer (Kruss, GmbH, Germany). The wound breaking strength was measured as the weight of water at the time of wound breaking per area of the specimen.

High performance thin layer chromatography (HPTLC) chemoprofiling of the extracts and the optimized formulation

The HPTLC chemoprofiling of the extracts was done to confirm for the presence of the chemical constituents in the extract as well as the optimized polyherbal formulation. About 100 mg of dry powdered herbal extract was mixed with little quantity of methanol by sonication and then the volume was made up to 10 ml with methanol. Pre-coated silica gel G,F, aluminum plates were used. 10 μl of the

Figure 3: HPTLC chemoprofiling showed the presence of tannins in the individual extracts as well as the optimized polyherbal formulation. Chemoprofiling 1 - The HPTLC fingerprinting under visible light, Chemoprofiling 2 - The HPTLC fingerprinting under UV 254 nm, Chemoprofiling 3 - The HPTLC fingerprinting under UV 366 nm, Chemoprofiling 4 - The HPTLC fingerprinting for the confirmation of tannins after spraying with Ferric Chloride reagent and visualized under visible light, Chemoprofiling 5 - The HPTLC fingerprinting for the confirmation of alkaloids after spraying with Dragendorff’s reagent and visualized under visible light. A=Barleria prionitis, B=Butea monosperma, C=Casuarina equisetifolia, D=Dalbergia sissoo, E=Lagenaria siceraria and F=Formulation F8.
individual ethanolic extracts and the extract of the optimized polyherbal formulation was applied by using HPTLC applicator. Toluene:ethyl acetate:formic acid (5:4:1) was used as the mobile phase. The TLC plates were developed in the developing chamber to a sufficient distance. The detection of spots on TLC plates was carried out by using the spray reagents as 5% ferric chloride and Dragendorff’s reagent (Figure 3).

Statistical analysis

For all statistical analyses GraphPad Prism Version 5.0 for Windows (GraphPad Software, San Diego, CA, USA) were used. Data are represented as the mean ± standard error of the mean (N = 5) and analyzed by one-way analysis of variance followed by Dunnett’s multiple comparison test. *P < 0.05, **P < 0.01 and ***P < 0.001 were considered as statistically significant in all analyses. The graphs were plotted using Sigma Plot for Windows Version 11.0 (Systat Software Inc., Germany).

RESULTS

The preliminary phytochemical screening revealed the presence of tannins, flavonoids, steroids, alkaloids and saponins (Tables 1-7).

DISCUSSION AND CONCLUSION

A number of secondary metabolite compounds isolated from plants have been demonstrated in animal models (in vivo) as active principles responsible for facilitating healing of wounds. Some of the most important ones include tannins,25 alpha-bisabolol and alpha-terpineol,26 asiaticoside, isolated from Centella asiatica.23 Ethanol extract of the leaves of Embelia ribes Burm. (Myrsinaceae) and its
isolated quinone compound embelin\textsuperscript{27} acylated iridoid glycosides from \textit{Scrophularia nodosa}\textsuperscript{28} have been reported.

The plant materials taken for screening have reported anti-inflammatory as well as anti-microbial activity, which supports the wound healing process\textsuperscript{29-32}. The plant materials have shown for the presence of flavonoid compounds\textsuperscript{33,34}. Flavonoids have a good free radical scavenging property as well as lipid peroxidation activity, which also help in the wound healing process.

The antioxidant activities of the individual extracts had been screened in our previous paper and found to be promising\textsuperscript{35}. It is likely that the antioxidant property of the extracts could be linked to its wound healing acceleration\textsuperscript{36,37}. Topical applications of compounds with antioxidant properties significantly improve wound healing and protect tissues from oxidative damage\textsuperscript{38}. Tannins are known to possess antioxidant activity\textsuperscript{39}. It could be conceivable that the extracts as a separate entity and in a polyherbal formulation exert their wound healing activity through the tannins since tannins are reported to improve wound healing and protect tissues from oxidative damage\textsuperscript{14}. The extracts also exert a significant anti-microbial activity which may be contributed to promote wound healing. In conclusion, the current study revealed that wounds treated with individual gel formulations and their polyherbal formulation extracts significantly accelerate the wound healing process. This wound healing process attributed probably due to the tannins can be considered as a very important factor in reducing acne. Further work is in the process for the isolation of active compounds and their screening for the anti-acne activity.
Table 7: Effect of the formulations on wound contraction incision wound model in rats

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<th>Vascular changes</th>
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0: No abnormality detected, +: Damage/active changes up to<25%, ++: Damage/active changes up to<50%, +++: Damage/active changes up to<75%, ++++: Damage/active changes up to more than 75%

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REFERENCES


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