Journal of Ayurveda and Integrative Medicine 8 (2017) 73–81

Contents lists available at ScienceDirect

Journal of Ayurveda and Integrative Medicine

journal homepage: http://elsevier.com/locate/jaim

Original Research Article (Experimental)

Studies on wound healing potential of polyherbal formulation using *in vitro* and *in vivo* assays

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ARTICLE INFO

Article history:
Received 27 May 2016
Received in revised form
21 November 2016
Accepted 26 November 2016
Available online 7 June 2017

Keywords:
Wound healing
Polyherbal formulation
Scratch assay
Fibroblast
Keratinocytes
Angiogenesis
Excision wound

ABSTRACT

Background: The use of herbal plant extracts in wound healing is known through decades, but it is necessary to provide scientific data through reverse pharmacology.
Objective: The aim of the present study is to find the mechanism behind the healing of wounds using *in vitro and in vivo* assays.
Material and methods: The study was designed to determine proliferation and mobilization of fibroblast and keratinocytes at the site of injury, angiogenesis at the site of healing and reduction in oxidative stress while healing. In our earlier studies it was observed that herbal extract of *Vitex negundo* L. (VN), *Emblica officinalis Gaertn* (EO), and *Tridax procumbens* L. (TP) showed rapid regeneration of skin, wound contraction and collagen synthesis at the site of injury in excision wound model. In the present study the cell mobilization was monitored in the scratch assay on L929 fibroblastic cell line and HaCaT keratinocytes cell line under the influence of aqueous plant extracts and its formulation. This formulation was also assessed for its angiogenic potential using CAM assay. Study was carried out to probe synergistic effect of polyherbal formulation using excision model in rat.
Results: The formulation was found to contain high amount of flavonoids, tannins and phenols which facilitate wound healing. At 20 μg/ml concentration of formulation, significant increase in tertiary and quaternary vessels were observed due to angiogenic potential of formulation. Formulation at the concentration of 3 μg/ml and 5 μg/ml showed significant mobilization of keratinocytes and fibroblasts respectively at the site of injury. Polyherbal formulation showed rapid regeneration of skin and wound contraction. Biochemical parameters like hydroxyproline, hexosamine and collagen turnover was increased in test drug treated animals as compared to untreated, whereas antioxidants such as catalase and GSH were increased significantly and decreased amount of tissue MDA was observed.
Conclusion: Polyherbal formulation prepared from the plant extracts accelerates wound healing process by proliferation and mobilization of fibroblast and keratinocytes, and angiogenesis at the site of injury. It also shows fast contraction of wound with its beneficial improvement in tissue biochemical and antioxidant parameters.

1. Introduction

Wound healing is a process of reconstruction of injured skin, coordinated by interaction of various epithelial and mesenchymal cells with cytokines, chemokines and growth factors [1]. Keratinocyte growth factor (KGF) is a paracrine growth factor synthesized by fibroblasts, endothelial cells, smooth muscle cells and dendritic epidermal T-cells [2]. KGF also known to induce mitogen activated protein activation and directly acts as angiogenic factor *in vitro* [3]. Natural plant products play major role in proliferation of fibroblasts and keratinocytes [4]. Plant products were reported to contain growth factors, cell signaling molecules and cell adhesion molecules [5,6]. In our previous studies aqueous extract of *Vitex negundo*

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Peer review under responsibility of Transdisciplinary University, Bangalore.

http://dx.doi.org/10.1016/j.jaim.2016.11.007

L. (VN), *Emblica officinalis Gaertn* (EO) and *Tridax procumbens* L. (TP) showed rapid regeneration of skin along with collagen turnover [7–9].

Leaves of VN are useful in toothache, inflammation, leucoderma, skin-ulcers, rheumatoid arthritis, and the methanolic extract of leaves was studied for wound healing activity [10]. Leaves of *Tridax* have effect on blood pressure, heart rate, immunomodulation, antimicrobial properties, and arrests bleeding from cuts and bruises [11,12].

EO is reported to have antimicrobial, antioxidant, anti-inflammatory, analgesic and antipyretic, adaptogenic, hepatoprotective, antitumor and antiulcerogenic activities [13]. The properties found in VN, EO and TP may be supporting characteristics as wound healing properties which were useful as selection criteria of plants for wound healing activity.

In the present studies we have used *in vitro* assays to evaluate efficacy of polyherbal formulation in terms of mobilization of fibroblasts and keratinocytes to the site of injury and their angiogenic potential as well as its *in vivo* efficacy.

2. Material and methods

2.1. Cell lines, drugs and reagents

L929 mouse skin fibroblasts and HaCaT (Human keratinocytes) cell lines were kindly supplied by Cell Repository of National Centre for Cell Science (NCCS) Pune, India. Cipladine, a standard used for effective wound healing was obtained from Cipla Ltd, Mumbai, India. Day zero fertilized chicken eggs were obtained from Venkateshwara Hatcheries, Pune, India. Media and reagents used in this study were of analytical grade and procured from Sigma-Aldrich, St. Louis, USA.

2.2. Animals

Eighteen Wistar rats of either sex, weight range of 150–200 gms were procured from National Toxicology Centre, Pune. All the animals were provided with water and food *ad libitum*. Rats were housed in standard laboratory condition as per CPCSEA norms. Animals were divided into three groups (control, standard, and polyherbal formulation treated group) of 6 animals each.

2.3. Collection and authentication of plant materials

Leaves of *V. negundo*, bark of *E. officinalis* and whole plant of *T. procumbens* were collected from Kem village, Maharashtra, India, Pune. The herbarium was made and authentication was carried out at Botanical Survey of India (BSI), Pune. No TAY3.BSI/WRC/Tech/2011. One copy of the herbarium specimens were submitted to APT Research Foundation, Pune, India.

2.4. Extraction of the plants

The leaves, stem and bark of respective plants were cleaned and shade dried prior to extraction. The dried plant material was then ground to powder using electric blender. The plant powders obtained were extracted with water at 80° C to obtain aqueous extracts using Soxhlet apparatus. These extracts were then concentrated in a Rota evaporator under reduced pressure and constant temperature at 60° C. and dried to powder and their extractive yields were measured [14].

2.5. Quantification of total flavonoids, phenols and tannins

Total flavonoids [15], phenols [16] and tannins [17] from aqueous extracts were determined using standard protocols.

2.6. Determination of antioxidant potential

The antioxidant effect of the extracts were studied using ABTS (2, 2-azino-bis-3 ethyl benzathiazoline-6-sulphonic acid) radical cations (ABTS+) decolourisation assay according to protocol of Shirwaikar et al., [18]. The concentration equivalent to ascorbic acid was determined from the standard curve of ascorbic acid. The percent inhibition was calculated.

2.7. Preparation of polyherbal formulation

The aqueous extracts of three plants were mixed in equal proportion, to obtain the best formulation in order to increase the acceptability and adoptability of herbal medicine for wound healing [19,20]. Liquid paraffin 20% was added in 30% emulsifying wax and 50% white soft paraffin (oily phase) was kept warm. Warm aqueous phase i.e 30% (emulsifying ointment) 1% chlorocrysol and 69.9% double distilled water were added in warm oily phase and stirred gently until cooled [21,22]. The cream was homogenized using mortar and pestle. It was stored in wide mouth glass bottle and placed in cool place.

2.8. Quality control parameters of formulation

Quality control of the formulation at different concentrations was carried out to evaluate the pH, spreadability, and extrudability. Acute dermal toxicity for individual aqueous extracts of three plants was carried out and was found safe at limit dose (2000 mg/kg). The pH of various formulations was determined by using digital pH meter. One gram of cream was dissolved in 100 ml of distilled water and stored for 2 h. The measurement of pH of each formulation was done in triplicate. The cream was placed in between the slides under the direction of certain load. The spreadability was expressed in terms of time in seconds taken by two slides to slip off from cream. The extrudability of cream formulations was determined in terms of weight in grams required to extrude a 0.5 cm ribbon of cream in 10 s. External characters of developed cream formulation were also noted, such as color, odor, smoothness and grittiness.

2.9. Angiogenesis activity

Angiogenetic potential of polyherbal formulation was determined using chick embryo chorioallantoic membrane assay (CAM) described by Surekha et al. [23]. It is a unique assay to study the blood vessels sprouting in response to angiogenic agent. The surfaces of freshly laid fertilized chicken eggs were wiped with 70% ethanol. On 4th day a small window of one centimeter square was made at the blunt end to puncture the air sac. Windows were sealed with durapore sealing tape and kept horizontally in the incubator till day nine. On 10th day Whatman filter paper rings treated with various concentrations of drug formulations (5 ng, 10 ng, and 20 ng were placed in each embryo). Eggs treated with equal volume of phosphate buffered saline (PBS) served as normal control. After further incubation for 72 h, CAMs were excised from the eggs and fixed in 4% ice cold paraformaldehyde/PBS for 30 min. The membranes were placed on glass slides and image of control and treated CAMs were captured for comparative analysis. Increase