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Systematic investigation of ethanolic extract from *Leea macrophylla*: Implications in wound healing

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ABSTRACT

Ethnopharmacological relevance: *Leea macrophylla* Roxb. ex Hornem. (Leeaceae) commonly known as Hastikarnapalasa is mainly distributed throughout the tropical parts of India. Traditionally, the plant is found to be effective against guinea worm, ringworm and is applied to sores and wounds.
Aim of the study: The present study aims to validate traditional wound healing claim of *Leea macrophylla* scientifically.
Material and methods: Box–Behnken design (BBD) was used to optimize the extraction process. The optimized root tuber extract of *Leea macrophylla* was standardized with chlorogenic acid by HPLC for the first time. Both oral and topical routes were selected as administrative means for the wound healing study using excision and incision wound model. For topical treatment bioadhesive gel was formulated and characterized for mechanical and physical characteristics by texture profile analysis (TPA) and scanning electron microscopy (SEM). The effect on wound healing was also assessed by evaluating antioxidant enzymes viz. glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT), free radicals lipid peroxidation (LPO) and nitric oxide (NO), inflammatory marker myeloperoxidase (MPO), collagen markers hydroxyproline, hexosamine and hexuronic acid along with the histopathological examination. Furthermore, the effect on the level of the proinflammatory cytokines interleukin-1β (IL-1β), interleukin -6 (IL-6), tumor necrosis factor-α (TNF-α) and growth factor, vascular endothelial growth factor (VEGF) were determined. The expression of cell proliferation nuclear marker Ki-67 was also analyzed by Western blot analysis.
Results: With mesh openings Sieve no. 20, semi polar nature of solvent (92.5:7.5 ethanol-water blend) and extraction time of 18 h, substantially greater extraction efficiency (29%) and phenolic yield (181.54 mg/g) were obtained. The content of chlorogenic acid in ethanol extracts of *Leea macrophylla* was obtained as 9.01% w/w. In incision model, oral treatment with 500 mg/kg ethanolic extract increased wound breaking strength by 23.41% while bioadhesive gel (5% w/v) showed a higher increase of 44.68%. Topical application produced complete wound contraction in 20 days against 22 days taken by oral treatment. Topical treatment also produced a significant ($p < 0.05$) increase in antioxidants glutathione, superoxide dismutase and catalase whereas the level of enzymes lipid peroxidation and nitric oxide and inflammatory markers myeloperoxidase were reduced. Further advantageous effects were reflected by significantly ($p < 0.05$) increased levels of hydroxyproline, hexosamine and hexuronic acid. Favorable effects on the level of proinflammatory cytokines interleukin-1β, interleukin-6, tumor necrosis factor – α and growth factor, vascular endothelial growth factor were also observed. The wound healing potential of *Leea macrophylla* was further supported by its ability to promote cell proliferation during wound healing as demonstrated by Western blot analysis of proliferation marker Ki-67.
Conclusion: The study justified traditional use of *Leea macrophylla* in wound healing and demonstrated that the bioadhesive gel of ethanolic extract produced faster and more significant healing as compared to oral treatment.

Abbreviations: BBD, Box–Behnken design; ELM, Ethanolic extract of *Leea macrophylla*; HPLC, High Performance Liquid Chromatography; CGA, Chlorogenic acid; CMC, Carboxymethylcellulose; SCMC, Sodium carboxy methyl cellulose; PVP, Polyvinyl pyrrolidone; PC, Polycarbophil; ELMO, Oral treatment with ethanolic extract of *Leea macrophylla*; ELMT, Topical treatment with bioadhesive gel of ethanolic extract of *Leea macrophylla*; WBS, Wound breaking strength; HA, Healed area; TWA, Total wound area; CAT, Catalase; SOD, Superoxide dismutase; GSH, Reduced glutathione; NO, Nitric oxide; LPO, Lipid peroxidase; HTAB, Hexadecyl trimethyl ammonium bromide; MPO, Myeloperoxidase; IL-1β, Interleukin-1β; IL-6, Interleukin-6; TNF-α, Tumor necrosis factor-α; VEGF, Vascular endothelial growth factor; ELISA, Enzyme linked immunosorbent assay; ANOVA, Analysis of variance; TPA, Texture profile analysis; SEM, Scanning Electron Microscopy; ROS, Reactive oxygen species; FRSE, Free radical scavenging enzymes

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1. Introduction

Compromise in congruity of any tissue is classified as a wound (example skin breaks, muscle tears, burns or a bone fracture). It may result from a random fall, a bruising accident, surgery, an infection or a covert etiological ailment. For instance, disruption of skin integrity leads to cutaneous wounds (Mallefet and Dweck, 2008), whereas abrupt collision with a moving vehicle may cause visceral, deep lying wounds. The causative factors, signaling intermediaries, pre-existent diseases, and type of injury all cumulatively determine whether the healing process will be acute or chronic (Schreml et al., 2010).

Wound healing is a dynamic, complex mechanism aimed towards re-attainment of tissue integrity and homeostasis (Eming et al., 2007) involving inflammation, re-epithelization, granulation tissue formation, neovascularization, wound contraction and remodeling of extracellular matrix (Singer and Clark, 1999). It is coordinated by a complicated signaling system involving various growth factors, cytokines and chemokines. Cell proliferation is an imperative step in tissue repair and regeneration in wound healing process (Xing et al., 2015).

Constrained healing hampers the life of plenty, and consequently, more efforts are being directed towards investigating cost efficient and accessible therapeutic approaches. Plants and their phytoconstituents have been long-established to treat wounds (Wang et al., 2011). *Leea macrophylla* Roxb. ex Hornem. (Leeaceae), commonly known as Hastikarnapalasa is a wild edible plant with high nutritive value in terms of minerals and vitamins content (B1, B2, C and B12) (Jadhao et al., 2009). The dried powdered root of *Leea macrophylla* is taken along with clarified butter in the morning as age sustainer (Jadhao and Wadekar, 2010). Traditionally, the plant has been reported to be effective against guinea worm, ringworm and is applied on sores and wounds (Kirtikar and Basu, 1975; Bhavamishra, 2010). Roots are applied externally to allay pain and are alexipharmic (Kirtikar and Basu, 1975). In Uttar Pradesh (India) its local name is Bado hanshia where the local tribes use the root tubers orally and locally for treating wound (Anonymous, 1999). Pharmacologically, the plant has been reported to possess anti- urolithiasis (Nizami et al., 2012) and anti-inflammatory activities (Dewanjee et al., 2013). Recently, we have successfully evaluated the potential in vitro antioxidant and antibacterial activity of ethanolic extract of *Leea macrophylla* root tubers (ELM), which is mainly attributed to the presence of phenolic, tannins, flavonoid, steroids and alkaloid (Joshi et al., 2016). However, the root tubers have not been scientifically validated for their wound healing activity.

The present study intends to investigate wound healing potential of *Leea macrophylla* root tuber extract to scientifically validate its traditional claims. Apart from employing statistical descriptors to optimize the extractive process, the current manuscript also entails the development and thorough mechanical characterization of bioadhesive hydrogel for topical application of obtained extract and comparison of its wound healing effect with its oral formulation, to discern the most feasible route of administration.

2. Material and methods

2.1. Plant material and extraction

The root tubers of *Leea macrophylla* were collected from a medicinal plant garden of Department of Dravyaguna, Banaras Hindu University in the month of September – October 2013 and were authenticated by Prof. V.K. Joshi, Department of Dravyaguna, Banaras Hindu University and submitted to Department of Pharmaceutics, Indian Institute of Technology, Banaras Hindu University, Varanasi (No. COG/LM/01). The acquired root tubers were weighed (200 g), and shade dried followed by a cycle of powdering before extraction. The process of Soxhlet extraction was optimized using Box–Behnken design (BBD) where three discerning factors influencing the extraction process were analyzed including mesh size, extraction solvent and extraction time. Design Expert (Version 6.0.0 Trial, Stat-Ease Inc., MN) was used to determine the boundary of the experiment, for evaluating response and for finally optimizing the process. Each specific design factor was constrained at three levels: low, medium and high level.

Following the design, the powdered drug was passed through the sieve (Sieve no. 10, 20 and 40) and then subjected to extraction in Soxhlet apparatus using ethanol-water blend at different ratios (85:15, 92.7:7.5 and 100:0) for a particular time (12 h, 18 h and 24 h) at 70 °C. The solvent was removed by concentrating the extract in a rotary evaporator (IKA) at 40 °C under reduced pressure. The residue left behind was reddish brown in colour and was stored in a desiccator.

2.2. Working with BBD towards response surface

The efficiency of Soxhlet extraction depends on factors such as the degree of sample homogenization, nature of the solvent used for extraction and length of time invested in the completion of extraction process (Gullberg et al., 2004). These critical factors were identified by preliminary experimentation and data mining as being capable of influencing extraction. Please refer to Supplementary information for detailed optimization of soxhlet extraction process.

2.3. Confirmation of chlorogenic acid in ethanolic extract by High Performance Liquid Chromatography (HPLC)

A validated HPLC method (Yuan et al., 2005) was followed for standardizing ethanolic extract of *Leea macrophylla.* Commercially procured chlorogenic acid (CGA, Sigma-Aldrich [purity: 95%]) was employed as standard. HPLC system (Waters) equipped with gradient pumps was used for analysis. A Cosmosil C18 column (150 mm × 4.6 mm, 5 μm particle) was used for chromatographic separation of the sample. The injection volume of sample was 10 μL. An aqueous phase consisting of 0.4% acetic acid and 4.5% tetrahydrofuran in triple distilled water was modified with acetonitrile in a variable pattern to form the mobile phase flowing at 1 ml/min. Gradient elution began with an aqueous phase to acetonitrile phase ratio of 5:95 and changed up to 25:75 during the initial 15 min. Further, the ratio of the mobile phase was altered from 25:75 to 60:40 for next 35 min. An equilibration period of 10 min was allowed using the initial mobile phase composition before injecting the next sample. The entire operation was carried out in ambient conditions. The peak area of extracted data was calculated at 326 nm using class VP series software. The identity of the peak was affirmed by cross checking retention time of the standard chlorogenic acid sample.

2.4. Experimental animals

Young albino rats (Charles Foster) of either sex weighing from 150 to 200 g bred in the Institutional animal facility, were utilized for animal studies. Rats were granted free access to standard feed and water. Temperature and relative humidity were maintained at 25 °C and 50% respectively. The animals were afforded with sufficient time for acclimatization before experiment initiation. Care and non-experimental handling of animals were performed by dedicated animal house staff. All experimental protocols were conducted after approval from Central Animal Ethical Committee