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RESEARCH ARTICLE

Formulation and evaluation of the hydroalcoholic extract of *Caesalpinia Pulcherrima* (Stem bark) on wound healing model in wistar rats

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Abstract

Caesalpinia Pulcherrima is commonly known as a Peacock's pride. Plant is enriched with flavonoids, triterpenoids, tannins and phenolic compounds. Wound healing process is complex, and involves many cellular intermediate pathways. Two models were adopted to evaluate the wound healing activity of *Caesalpinia pulcherrima* (Stem bark): excision and incision wound model in wistar rats. Hydroalcoholic extract of *C. pulcherrima* (stem bark) was incorporated in to the lipophilic base ointment to achieve the 10 and 20% w/w concentration. In excision and incision skin wound models, herbal medicated ointment formulation was applied topically for 21 days and 10 days respectively after the induction of wound injury. In excision wound model, high dose (20% w/w) of ointment was found to be more statistically significant ($p<0.05$) and ($p<0.001$) on wound contraction on 7^{th} and 14^{th}day respectively when compared to the control group. Low dose (10% w/w) group was significant ($p<0.05$) but less significant as compared to high dose group statistically. In incision wound model, high dose group (20% w/w) showed maximum tensile strength (502.50) and was found statistically significant ($p<0.01$) when compared with the control group.

INTRODUCTION

Wound healing is the process of tissue repair involving the tissue response to injury. It is the body's natural process of regenerating dermal and epidermal tissue. It consists of several overlapping phases such as hemostasis, inflammation, proliferation and maturation.[1] When tissues are disrupted following injury, collagen is required to repair and restore normal structure and function.

Caesalpinia pulcherrima is commonly known as peacock flower or "Peacock's pride" in English and as "Ratnagadhi" in Tulu language and belongs to the Leguminosae/Fabaceae family.[2]Phytochemical investigations on the flowers and leaves of *Caesalpinia pulcherrima* have revealed the presence of various phytoactive constituents such as glycosides, retinoids, flavones, chalcones, flavanols and sterols which possess significant anti-inflammatory and antioxidant properties.[3] The preliminary investigations of hydroalcoholic extract of *C. pulcherrima* (stem bark) have shown the marked presence of saponin glycosides, tannins and phenolic compounds, flavonoids and triterpinoids.[4] Wound healing process gets accelerated in the presence of healing agents with antioxidant and anti-inflammatory properties. The present study is taken up to study the wound healing activity of the stem bark of *C. pulcherrima.*

MATERIAL AND METHODS

Plant material and extract preparation

Stem portions of *C.pulcherrima* was collected from Mangalore area in the month of August-September. Stem bark was removed, shade dried and powdered. The powder was loaded into Soxhlet extractor in batches of 200

grams of each and subjected to extraction with distilled water (1 part):95% ethanol (2 part) at 40- 45°C. The percolate was cooled, filtered and concentrated under reduced pressure on a water bath at a temperature below 50 °C till syrup consistency obtained. Then it was dried in a desiccator and stored in refrigerator (2-8°C) temperature for further use.

Ointment preparation

An ointment of hydroalcoholic extract of *C. pulcherrima* stem bark (HAECPS) was prepared and extract was incorporated in to the ointment containing highly lipophilic bases. Ointment was prepared for control group and two different test drug treatment groups (10% and 20% w/w). Ointment was topically applied using sterile cotton buds.

Animals

The study was carried out at Central animal house, KMC, Manipal. Adult albino rats of wistar strain of both sex and weighing 150-200 g was selected for the study. All the animals were housed in polypropylene cage using sterile steel grid floor covering with provided paddy sterile husk for bedding to the cage. Animal were kept at 28±1º C temperature and 50±5% humidity with 12 hours light and dark cycle. Three animals were housed in each cage to prevent overcrowding. The animals were provided with standard rat feed and free access to water ad libitum.All the procedures on experimental rats were carried out in accordance with standards of Institutional Animal Ethics Committee of Manipal University, Manipal and approval (IAEC/KMC/54/2014).

Excision wound model:

A total of 18 wistar rats were divided into 3 main groups. Each group contains 6 rats (n=6). Group I was treated with ointment vehicle base. Group II and III were treated with medicated ointment (10%, 20% w/w of HAECPS ointment respectively) for 21 days or until complete epithelialization, whichever is earlier. The excision wounds were created under ketamine anesthesia (50 mg/kg body weight), given intramuscularly. Lab working area was sterilized by industrial spirits. A shaved rat skin was cleaned with cotton swab soaked in 70% alcohol and round skin area of 500 mm^2 approximately in diameter was impressed on the depilated dorsal thoracic region 5cms away from the ears as described by Morton and Malone.[5] The entire full thickness of skin from the demarcate area was excised. Contraction which mainly contributes for wound closure was studied by tracing the raw wound area on transparent paper every alternate day till wounds were completely covered with epithelium. These wound tracings were retraced on a millimeter scale graph paper, to determine the wound area.

Wound Contraction (WC) was calculated as a percentage change in the initial wound size.[6]

$$WC\ (\%) = \frac{\text{Initial wound size- Specific day wound size}}{\text{Initial wound size}} \times 100$$

Epithelialization period was monitored by noting the number of days required for eschar to fall away, leaving no raw wound behind.

Incision wound model:

The incision wound was created. Two 6 cms long paravertebral straight incision was made, 1cm lateral to the vertebral column on either side through the entire thickness of skin, under ketamine anesthesia (50 mg/kg body weight), given intramuscularly. After mopping the wound dry, intermittent sutured were done with black nylon suture (size 4-0) and needle were placed 1cm apart.[7] On day 7, sutures were removed. On day 10, the animal were sacrificed using excess dose of ketamine intramuscularly and the tensile strength of the wound will be measured by applying tearing force in the form of slowly increasing weight of accumulating water by a continuous water flow technique of Lee,[8] with a slight modification.

A total of 18 wistar rats were divided into 3 main groups. Each group contains 6 rats (n=6). Group I was treated with ointment vehicle base. Group II and III were treated with medicated ointment (10%, 20% w/w of HAECPS ointment respectively) for 7 days.

Measuring of breaking/tensile strength of wound

Tensile strength is the force required to open a healing wound which is used for measuring healing. The instrument used is tensiometer. It is designed on the same principle as the thread tester used in textile industry. It consists of a 6X12 inch board with one post of 4 inch long fixed on each side of the loner ends. The board is placed at the end of the table. A pulley with a bearing was mounted on top of one of the posts. An alligator clamp with 1cm width was tied on the tip of the post without pulley by a piece of fishing line so that clamp could reach the middle of the board. Another alligator clamp was tied on a piece of fishing line with a 1L polyethylene bottle tied on the other end.The excised granuloma tissue was then placed on a stack of paper towels that could be adjusted so that the polyethylene bottle was freely hanging in the air. Water added to the polyethylene bottle was weighed and considered as tensile strength of the wound.

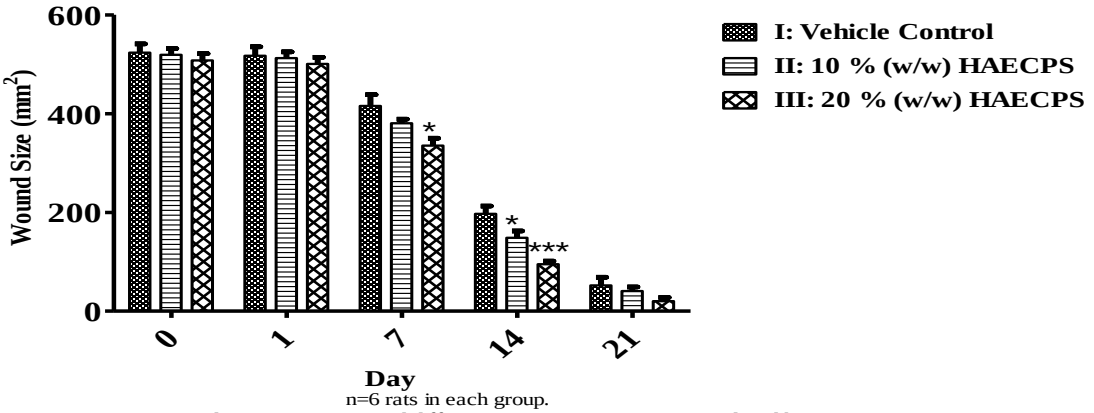
Statistical Analysis

Using Statistical Package for the Social Sciences (SPSS version 16.0;SPSS Inc., Chicago, USA), normally distributed data were expressed as mean ± standard error of mean and analyzed by one way analysis of variance (ANOVA) followed by post hoc Tukey test.

RESULTS

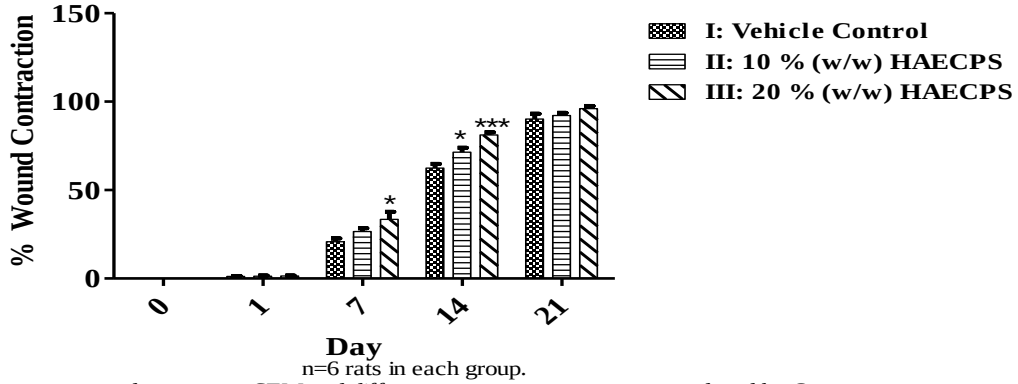
Wound healing activity was observed in both incision and excision models. Wound contracting ability of the ointment (10% topical) was significantly greater (p<0.05) than control on day 14(Figure 1, 2). The period of complete epithelization for excision wound model was found earlier with the 20 % (w/w) of HAECPS ointment treatment group as compared to control and 10 % (w/w) of HAECPS ointment group (Table no.1). The breaking strength of the incised wound which showed significance (p<0.01) compared to control in 20% w/w of HAECPS topical group (Table no. 2).

Fig. 1 Effect of formulated Ointment on Wound Area (mm^2)



n=6 rats in each group.
Data are expressed as mean ± SEM and different treatment groups were analyzed by One way ANOVA followed by post-hoc Tuckey test.
*- p<0.05 as compared to control
**- p<0.01, compared to control
*** - p< 0.001 as compared to control

Fig. 2 Effect of formulated ointment on wound contraction



n=6 rats in each group.
Data are expressed as mean ± SEM and different treatment groups were analyzed by One way ANOVA followed by post-hoc Tuckey test.
*- p<0.05 as compared to control
**- p<0.01 as compared to control
*** - p< 0.001 as compared to control

Table no. 1: Period of epithelization

Group	Period of epithelization(mean ±SEM)
I: Vehicle control	23.83 ± 0.30
II: 10% (w/w) of HAECPS	24.33 ± 0.33
III: 20% (w/w) of HAECPS	22.83 ± 0.30
n=6 rats in each group Data are expressed as mean ± SEM and different treatment groups were analyzed by One way ANOVA followed by post-hoc Tuckey test. Data was not statistically significant as compare to the control	

Table no. 2: Breaking/ tensile strength of the wound:

Groups	Breaking/ tensile strength of wound (mean± SEM)
I: Vehicle control	397.50 ± 17.30
II: 10% (w/w) of HAECPS	446.67 ± 14.06
III: 20% (w/w) of HAECPS	502.50 ± 13.67*
n=6 rats in each group Data are expressed as mean ± SEM and different treatment groups were analyzed by One way ANOVA followed by post-hoc Tuckey test. *- $p<0.01$, compared to control	

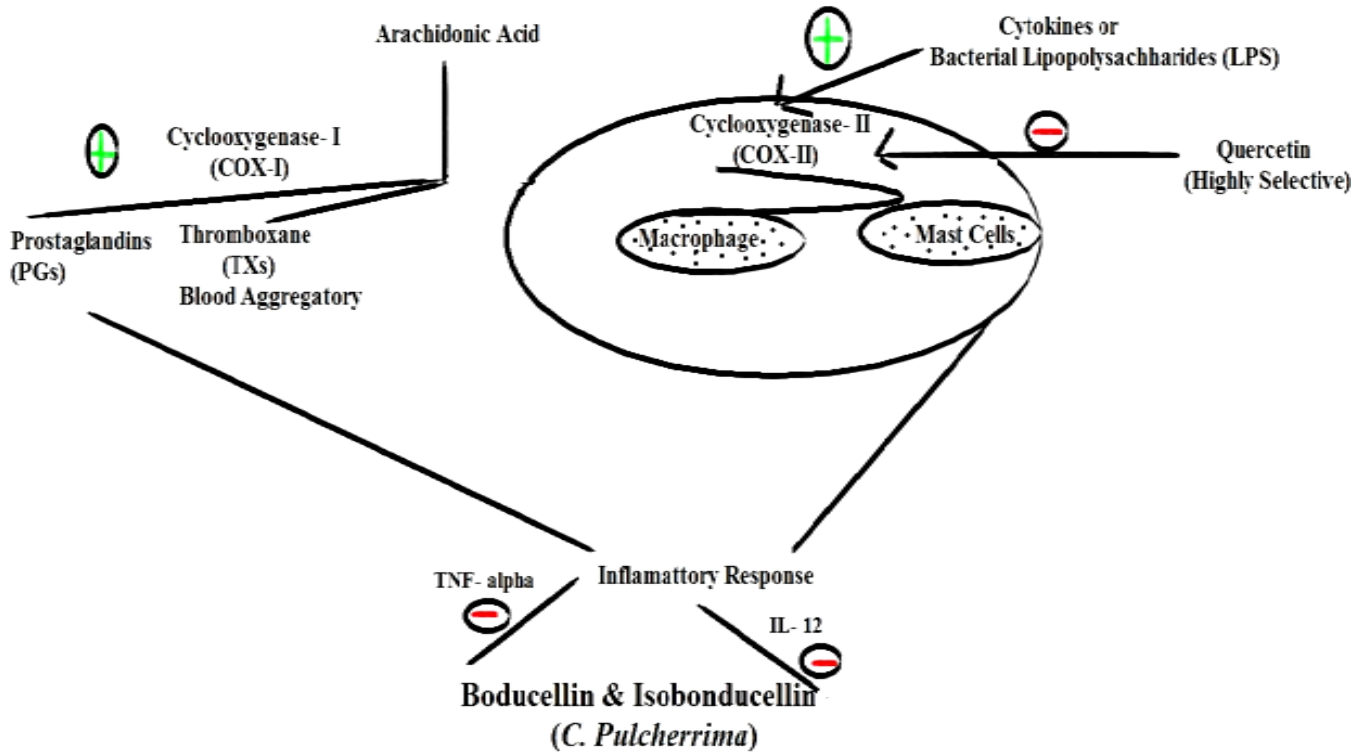


Figure 3- Anti-inflamatory role of Natural Flavonoids

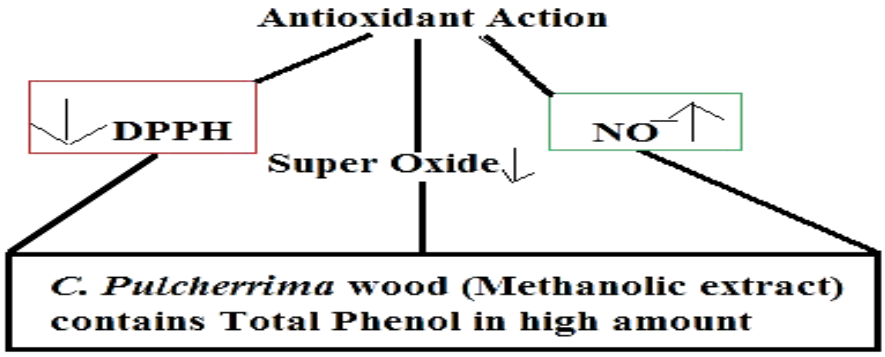


Figure 4- Antioxidant mechanism by Total phenolic contents

DISCUSSION

Wound is referred as the disruption of normal anatomical structure and functions. It is caused due to the breaking and opening of the skin. The most common symptoms of wound are bleeding, swelling, redness, loss of function. Wound healing is a complex factor which involves various biochemical and cellular mechanisms. The aim of these mechanisms is to regenerate and reconstruct the disrupted anatomical continuity and functional status of the skin. Healing process is a natural body reaction and it occurs immediately after wounding and it takes place in 4 stages. The first stage is coagulation which controls excessive blood loss from damaged blood vessels. The second stage includes inflammation and debridement of wound, which is followed by re-epithelization. Re-epithelization includes various stages, such as proliferation, migration and differentiation of squamous epithelial cells of the epidermis. In final stage of healing collagen deposition and remodeling occurs within the dermis. [9]

Molecular oxygen and microbial infection play a central role in the pathogenesis of chronic wounds. Over production of reactive oxygen species and microbial infections causes cytotoxicity and delayed wound healing. Flavonoids, a group of naturally occurring benzo-γ-pyrone derivatives have the ability to show various biological properties like antibacterial, antioxidant, anti-inflammatory and antiviral activities. Lipid peroxidation is one of the processes which occur in injuries and so inhibition of lipid peroxidation has shown an increase in strength of collagen fibers by increasing circulation and preventing cell damage. Flavonoids are known to reduce lipid peroxidation and prevent cell necrosis. They improve vascularity and also help in wound healing.[10]
Inflammation causes a continuous generation of reactive oxygen species such as superoxide radical or non-radical hydrogen peroxide. An imbalance between the reactive oxygen species and anti-oxidant defense mechanism of cell due to excess production of oxygen metabolites, leading to a condition called oxidative stress.
It has been reported antioxidants play a significant role in wound healing and stimulation of production of these anti-oxidants would produce a favorable environment for wound healing. Flavonoids are reported to scavenge the reactive oxygen species and free radicals. Flavonoids like quercetin and rutin are reported to produce antioxidant activity by their hydrogen donating capability and by scavenging reactive oxygen species.[11]

Cyclooxygenase (COX) that produces prostaglandins (PGs) and thromboxanes (TXs) from arachidonic acids. COX has two isoform of enzymes mainly, COX-1 affording to the cytoprotective PGs and blood aggregatory TXs. While COX-2 mainly expressed into the inflammatory cells including mast cells and macrophages. COX-2 stimulated by pro inflammatory cytokines or by bacterial lipopolysaccharides (LPS). Quercetin was the first flavonoid reported for inhibiting phospholipase A_2 (PLA_2) in human neutrophils.[12] Quercetin and xanthomicrol were reported to inhibit COX-1 in sheep platelet preparation.[13] In order to find out the selectivity of the COX enzyme inhibition, quercetin was found to be more selective for COX-2 inhibition as compared to COX-1.[14] The acetone extract of the aerial parts of *C.pulcherrima* has resulted in two new flavonoids; 5,7-dimethoxy-3′,4′-methylenedioxyflavanone (2) and *cis*-(*Z*)-7-hydroxy-3-(4-methoxybenzylidine)chroman-4-one (isobonducellin,3), along with 5,7-dimethoxyflavanone (1),2′-hydroxy-2,3,4′,6′-tetramethoxychalcone (4), and the homoisoflavanoid,bonducellin (5).[15] In addition, isolated flavanoids of aerial parts posses bonducellin and isobonducellin from *C. Pulcherrima* attributes potent in vitro inhibitory effects of TNF α and IL- 12 (Figure- 3).[16]

The total phenolic content of methanolic and aqueous extract of *C. pulcherrima* wood was studied for the DPPH (1,1-diphenyl-2-picryl hydrazyl), nitric oxide (NO) and super oxide scavenging activity. An antioxidant action was found more with methanolicextract (Figure-4).[17]

A novel Pulcherrimins A, D from dried root of *C. Pulcherrima* was reported for its highly selective activity against DNA repair deficient yeast mutant (RAD 52 gene deletion). [18]

Tannins extract from immature fruits of *Terminalia chebula* Fructus Retz promotes wound healing through several cellular mechanisms and shown beneficial effects on wound contraction or healing.[19]

Appropriate suture size can provide proper holding power for a wound during healing. Keeping the wound sides close to each other by suture during early healing is optimal for recovery. Besides this physical effect, some studies have shown that local stress provided by sutures can cause inflammatory signs[20], change extracellular matrix synthesis and influence scar formation.[21] Suture size also effects on the skin wound healing strength in rats. The recovery index of the 4-0 nylon sutured wounds was found significantly higher than that of the 6-0 nylon sutured wounds. The 6-0 sutured wound provided less holding power in the rat model; it also prolonged the inflammatory process and delayed overall healing. The proper suture size should be an important consideration in selecting different tissue closures.[22]

To exact mechanism of action in wound healing contributed by the *C. pulcherrima* is still unclear and certainly needs to be more explored with various biomarkers associated during wound healing process.

CONCLUSION

In both, incision and excision skin wound models- medicated ointment formulation of hydro-alcoholic extract of *C. pulcherrima* (stem bark) has shown wound healing activity as compared to that of control.

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