Antimicrobial, Wound Healing and Antioxidant potential of Couroupita guianensis in rats

Sanjay.Prahalad Umachigi*, Jayaveera K.N[1]., Ashok kumar C.K[2]., G.S. Kumar[3]
*,[3] Rural College of Pharmacy, D.S.Road, PO Box-10, Devanahalli-562110, Bangalore Rural Dist,Karnataka, India.
[1]Department of Chemistry, Jawaharlal Nehru Technological University College of Engineering, Ananthpur- 01, Andhra Pradesh, India
[2] Department of Pharmacognosy, Sree Vidyanikethan College of Pharmacy, A.Rangamepeta, Tirupathi, Chitoor Dist. Andhra Pradesh, India

Summary

Wound healing potential of ethanolic extract of Whole plant of Couroupita guianensis (CGEE) (barks, leaves, flowers and fruits) for treatment of dermal wounds in rats was studied on excision and incision wound models. HPTLC of the total extract was recorded for the purpose of standardization. Various parameters of incision wound, viz. epithelization period, scar area, tensile strength and hydroxyproline measurements along with wound contraction, were used to evaluate the effect of Couroupita guianensis on wound healing. The results obtained indicate that Couroupita guianesis accelerates the wound healing process by decreasing the surface area of the wound and increasing the tensile strength. Nitrofurazone ointment was used as a positive control. Complete epithelization was observed within 15 days with CGEE. Measurements of the healed area and the hydroxyproline level were in agreement. The antimicrobial activity of CGEE was studied against Gram positive (*Staphlococcus aureus*) and Gram Negative Bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) compared to erythromycin and tetracycline. Moderate activity was observed against all organisms. The present study provides a scientific rationale for the traditional use of *Couroupita guianesis* in the management of skin diseases such as sores, boils and itching.

Key words : *Couroupita guianesis*(Fagaceae), wound healing activity, Antimicrobial activity, Antioxidant activity, HPTLC

Author correspondence:

Sanjay.Prahalad .Umachigi
Assistant professor, Department of Pharmaceutics,
Rural College of Pharmacy, Post Box No. 10, D.S.Road,
Devanahalli-562110, Bangalore Rural District,
Karnataka, India.
e-mail: umachigisanjay@yahoo.co.in

Introduction

Wounds are physical injuries that result in an opening or breaking of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. It is a product of the integrated response of several cell types to injury. Wound healing is a complex multifactorial process that results in the contraction and closure of the wound and restoration of a functional barrier (1). Injury of the skin induces repair mechanism that restores its functions in protecting the individual against environmental factors that might be harmful. Three different phases constitute the physiologic process of wound-healing; (i) substrate phase, (ii) proliferative phase and (iii) remodeling phase (2).All these steps are orchestrated in controlled manner by a variety of cytokines including growth factors (3).

Inflammation is often associated with pain and fever, which may be due to release of histamine, kinins, serotonin and prostaglandin (4). Inflammation, which constitutes a part of the acute response, results in a coordinated influx of neutrophils at the wound site. In spite of tremendous advances in the pharmaceutical drug industry, the availability of drugs capable of stimulating the process of wound repair is still limited (5). Moreover, the management of chronic wounds is another major problem due to the high cost of therapy and the presence of unwanted side effects (6,7).

Inflammatory diseases are accompanied by the chronic release of cytokines and reactive oxygen and nitrogen species, which may be involved in increased tissue injury (8). Much evidence has shown that the production of reactive species such as superoxide anion radical (O^2) , hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and peroxynitrite occurs at the site of inflammation and contributes to tissue damage (9).

In acute and chronic inflammation, high concentrations of reactive oxygen species (ROS) are produced (mainly O^2), which generate an oxidative imbalance and decrease the capacity of the endogenous antioxidant enzymes (such as superoxide dismutase (SOD)) to remove them, contributing to tissue damage. Some important pro-inflammatory roles for O^2 include endothelial cell damage and increased microvascular permeability, formation of chemotactic factors such as leukotriene B^4, lipidic peroxidation and oxidation and DNA single strand damage (10).

Couroupita guianensis Aublet (Family: Lecythiadaceae) commonly known as Naglingam or a Canaball tree, found throughout India in plains. The flowers are used to cure cold, intestinal gas formation and stomachache (Wealth of India). Little work has been reported on phytochemical constituents (11). and pharmacological actions (12,13,14)

Proanthocyanidins or condensed tannins are a group of biologically active polyphenolic bioflavonoids that are synthesized by many plants. Proanthocyanidins and other tannins facilitate wound healing (15). Since the role of free radicals in the physiology of wound is clearly defined and the plant has been reported to contain tannoid principles with potent antioxidant activity, we have studied the wound healing potential of the *Couroupita guianesis* extract. Also, since the plant has been used traditionally in the treatment of skin diseases, antimicrobial activity of the extract has been also studied for scientific validation of ethnobotanical claims. The antioxidant activity was also investigated to reveal the mechanism behind the wound healing activity.

EXPERIMENTAL

Plant material and Extraction

The *Couroupita guianesis* was collected from Western Ghats of Karnataka, India in October 2005. The plants were identified and voucher specimens (RCP/PCOG/07/2005-06) have been deposited in Pharmacognosy Department Herbarium, Know for future reference. The plant material was air dried at room temperature and powdered coarsely. The powder obtained was macerated with 50% aqueous ethanol for a period of 24 h and filtered. The extracts were pooled, concentrated at reduced temperature on a rotary evaporator (Büchi, USA) and then freeze-dried (Freezone Labconco, USA) under high vacuum and at a temperature of –40 ± 2 °C to get 18.2 g (6.5%) of the dry extract (CGEE). 300 mg of CGEE was incorporated into a simple ointment base to make up 1 g (24). CGEE dissolved in 50% ethanol (100 µg mL^{-1}) was used for antimicrobial screening.

High Performance thin layer chromatography

The high performance thin layer chromatography (HPTLC) studies of CGEE were carried out on a pre-coated silica gel plate (0.2 mm, Merck 60 F 254, Germany) as the stationary phase, and Chloroform: Ethyl acetate: Formic acid (2.5:2:0.8) as the mobile phase. The extract was spotted as a band using a Camag Linomat V applicator (CAMAG, Switzerland). The plates were observed in the visible region after derivatization by using Vannilin Sulphuric acid reagent at 110^{o} C for 5 mins and were scanned on a CAMAG TLC scanner III using the Wincats software.

Animals

Inbred house Wistar rats of either sex were used in the study. The range of the weight of the animals was between 200-250 g. they were housed individually in standardized environmental condition. All the animals were provided with water food ad libitum. Gold Mohur Lipton India Ltd supplied the standard rat pellet food. Ethical clearance for the animal study was obtained from the institutional animal ethics committee. In the experiment, the rats were divided into three groups (n = 6): group 1 was the control group that received simple ointment base, group 2 was treated with reference standard (0.2%, w/w nitrofurazone ointment) and group 3 received CGEE ointment (100 mg/500mm^2) topically on wound created on the dorsal back of rats daily till the wounds completely healed (16). For antioxidant activity, rats were divided into two groups of control and experimental rats. Experimental rats received 1.25 ml (250 mg/kg b.w.) of CGEE while control rats received normal saline.

Excision wound model

An impression was made on the dorsal thoracic region 1 cm away from vertebral column and 5 cm away from ear using a round seal of 2.5 cm diameter on the anaesthetized rat. The skin of impressed area was excised to the full thickness to obtain a wound area of about 500 mm^2 diameter. Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. Contractions, which contributed to

wound closure in the first 2 weeks, were studied by tracing the raw wound. Wound area was measured by retracing the wound on a millimeter scale graph paper. The degree of wound healing was calculated (17) and hydroxyproline was measured using the method of Neuman and Logan (18).

INCISION WOUND MODEL

Rats were anaesthetized and two paravertebral-long incisions were made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on each side of the depilated back of the rat. Full aseptic measures were not taken and no local or systemic antimicrobial was used throughout the experiment (5). All the groups were treated in the same manner as mentioned in the case of the excision wound model. No ligature was used for stitching. After the incision was made, the parted skin was kept together and stitched with black silk at 0.5 cm intervals. Surgical thread (No. 000) and a curved needle (No. 11) were used for stitching. The continuous thread on both wound edges were tightened for good closure of the wounds. The wound was left undressed; CGEE ointment, along with water soluble base ointment (control) and nitrofurazone ointment were applied topically twice a day for 9 days. When wounds were thoroughly cured, the sutures were removed on the 9th day and tensile strength was measured with a tensiometer.

Tensile strength

The tensile strength of a wound represents the degree of wound healing. Usually wound healing agents promote a gain in tensile strength. The sutures were removed on the 9th day after wounding and the tensile strength was measured on the 10th day. The herbal ointment along with standard and control were applied throughout the period, twice daily for 9 days. The mean tensile strength on the two paravertebral incisions on both sides of the animals were taken as the measures of the tensile strength of the wound for an individual animal. The tensile strength of CGEE ointment treated wounds was compared with control and nitrofurazone ointment as standard. The tensile strength

increment indicates better wound healing stimulated by the applied herbal formulation. Further epithelization period and scar area were measured daily for 25 days after determination of tensile strength (19).

Antimicrobial activity

The agar diffusion method (20).was used to evaluate the antimicrobial activity. Bacteria were cultured overnight at 37^{o} C in Mueller Hinton Broth (MHB, Oxoid) and fungi at 28^{o} C for 72 h in Potato Dextrose Broth (PDB, Oxoid) and used as inoculum. A final inoculum, using 100 µl of suspension containing 10^{8} CFV/ml of bacteria and 10^{4} spore/ml of fungi spread on Mueller Hinton Agar (MHA) and Potato Dextrose Agar (PDA) medium, respectively. The disc (6mm in diameter) was impregnated with 10 µl of 100 mg/ml (1 mg/disc) extracts placed on seeded agar. Gentamicin (10µg/disc), streptomycin (10 µg/disc) and tetracycline (10µg/disc) were used as positive controls for bacteria and fluconazole (10µg/disc), ketoconzole (10 µg/disc) and metronidazole (5 µg/disc) for fungi. The test plates were incubated at 37^{o}C for 24 h for bacteria and at 28 ^{o}C for 72 h for fungi depending on the incubation time required for a visible growth. MIC values were also studied for microorganisms, which were determined as sensitive to the extract in disc diffusion assay. Sterile filter paper discs (6mm in diameter) containing 2.5–1000 µg/disc of plant extracts were placed on the surface of a medium. MIC was defined as the lowest concentration of extract that inhibited visible growth on agar.

Antioxidant activity

Lipid peroxidation was assayed by the measurement of malondialdehyde (MDA) levels in liver on the basis of reaction with thiobarbituric acid (21). The activity of SOD was determined in liver by monitoring the inhibition of the autoxidation of pyrogallol (22). CAT activity in liver was determined according to the standard method (23). Proteins were determined according to Lowry method (24) using bovine serum albumin as a standard Values were represented as mean ± S.E.M and data were analyzed by paired *t*-test using SPSS software package.

Statistical Analysis

Pharmacological data were subjected to statistical analysis using SPSS 11.0 for Windows. The values are represented as mean ± S.E.M. for six rats. Paired t-test was used for reporting the p-value and significance with respect to the control group.

RESULT AND DISCUSSION

The results showed that upon administration of CGEE, there was a decrease in the epithelization period, along with a visibly decreased scar area (Table 1). There was a significant increase in the tensile strength and hydroxyproline content compared to the control group and comparable to the nitrofurazone group (Table 1). The observations and results obtained in this study indicate that the alcoholic extract of Couroupita guianesis significantly stimulated wound contraction (Table 2). CGEE also exhibited a potential inhibitory effect on all the pathogens examined, in the following order*: Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae and E. coli* MIC being 0.56, 0.78, 0.89, 1.33 mg mL–1, respectively. Erythromycin and tetracycline were used as standards (Table 3). The 50% aqueous ethanol (20 mL) served as a negative control and showed no inhibiting effect. Since the extract of *Couroupita guianesis* has been found to contain stigmasterol and Flavonoids are the major constituents (25). The significant antimicrobial effect of CGEE against all the four pathogens confirmed that the compounds present in the crude extract are responsible for the effective antimicrobial activity. Thin layer chromatography studies indicated the presence of different compounds (Fig. 1), further confirming the synergistic action.

Fig.1. HPTLC fingerprint profile of CGEE. Stationary phase: silica gel (Merck 60 F 254), mobile phase: Toluene/ethyl acetate/Formic acid (5:4:1).

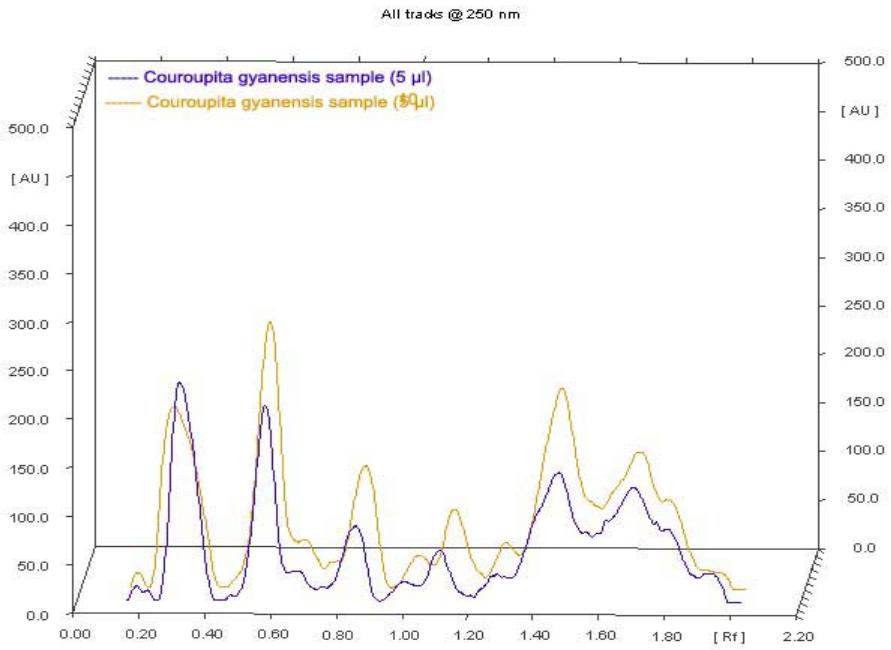

Table 1. Effect of *Couroupita guianesis* extract ointment on incision wounds

Topical treatment	Epithelization period (day)	Tensile strength (g)	Scar area (mm^2)	Hydroxyproline (mg per 100 mg tissue)
Control	25.7 ± 1.7	289.5 ± 21.3	55.2 ± 4.2	6.96 ± 0.3
CGEE(33%) ointment [b]	15.0 ± 0.95 [d]	413.5 ± 21.7 [e]	30.2 ± 3.1 [e]	9.28 ± 0.55 [e]
Nitrofurazone(0.2 %) ointment [c]	13.9 ± 1.5 [e]	420.2 ± 21.6 [e]	31.6 ± 3.8 [f]	9.85 ± 0.46 [e]

[a] Values are mean ± SEM for six rats; [b] 60 µg mm^{-2}; [c] 0.4 µg mm^{-2} Statistically significant difference in comparison with the control group: [d] $p < 0.01$, [e] $p < 0.001$, [f] $p < 0.02$.

Table 2. Effect of *Couroupita guianesis* extract ointment on excision wounds

Topical treatment	Percentage of close of excision wound area after days			
	4	7	15	21
Control	26.4 ± 2.46	46.9 ± 3.67	64.2 ± 3.95	73.4 ± 4.00
CGEE(33%) ointment [b]	36.7 ± 3.00 [d]	64.7 ± 4.28 [d]	90.06 ± 5.21[d]	96.2 ± 4.9 [e]
Nitrofurazone(0.2%) ointment [c]	38.4 ± 3.20 [d]	64.5 ± 4.17[d]	91.6 ± 4.80 [d]	96.2 ± 5.0 [e]

[a] Values are mean ± SEM for six rats; [b] 60 µg mm^{-2}; [c] 0.4 µg mm^{-2}
Statistically significant difference in comparison with the control group: [d] $p < 0.01$, [e] $p < 0.001$.

Table 3. Antimicrobial activity of *Couroupita guianesis* extract

	Inhibition zone (mm)		
Microorganisms	CGEE (100 µg mm^{-1}) in 50 % Et OH	Erythromycin (10µg mm^{-1})	Tetracycline (10µg mm^{-1})
Escherichia coli	16	20	19
Pseudomonas aeruginosa	15	25	21
Klebsiella pneumoniae	12	23	25
Staphylococcus aureus	11	18	17

CONCLUSION

The use of *Couroupita guianesis* in Indian traditional systems of medicine for various skin diseases, has been justified by this work, as it showed a wound healing potential and commendable activity against several microorganisms. These findings could justify, at least partially, the inclusion of this plant in the management of wound healing in folk medicine. Since the role of free radicals and antioxidants in wound healing are very clearly defined, wound healing potential *Couroupita guianesis* may be partly due to the potent antioxidant activity of the plant. Further experiments are needed to test the effect of this plant in the treatment of chronic wounds.

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