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Original Research

Evaluation of the antioxidant activity and the healing action of the ethanol extract of *Calotropis procera* bark against surgical wounds

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ABSTRACT

The objective of the present study was to evaluate the antioxidant potential and the wound healing effect of the ethanolic extract of the bark of *Calotropis procera*. The antioxidant study was evaluated *in vitro,* using 2,2-diphenylpicrylhydrazyl (DPPH) and deoxyribose degradation assays. Wound healing was studied using excision and incision wound on normal and dexamethasone-suppressed wound healing rodent models. Alkaloids, flavonoids, proteins and phenols were screened in the extract used whereas saponins and true tannins were absent. The extract contains only 12.5 gallic acid equivalent and 399.54 rutin equivalent. It was found to inhibit DPPH and deoxyribose oxidation (IC_{50} = 24.24 and 5.40 respectively). *In vivo* study demonstrated a significant reduction in the epithelialization time ($P < 0.001$) to 17-18 days in normal and dexamethasone treated rats following the ethanolic extract of the bark of *C. procera* application. The same extract also significantly increased the breaking strength in dexamethasone treated rats. Histological examination of incision wounds of treated group showed matured extracellular matrix, numerous fibroblasts. This study illustrated an excellent potential of the bark of *C. procera* therapy on dermal wound healing, with a tentative mechanism of action related to improved collagen deposition and reduced inflammatory reaction.

KEY WORDS: antioxidant, breaking strength, *Calotropis procera*, epithelialization, skin

Introduction

Many medicinal plants are claimed to be useful for wound healing in the cameroonian traditional system of medicine. Among these plants, *Calotropis procera* (Asclepiadaceae) originated from the Afro-Asian monsoonal regions and spread to the Indian subcontinent and subtropical America, Mascarene Islands and drier parts of Australia. The latex and the leaves are said to be valuable for skin injuries [1]. These properties are accompanied by toxic effects following oral administration or dermal contact of latex in animals. The root bark is known to promote secretion and to be useful in treating skin disease, among other ailments [2]. But the wound healing potential of the stem bark has not been evaluated yet.

The biological mechanism associated with wound healing is complex. Platelet aggregation during hemostasis liberates a number of soluble mediators, which initiate the healing process. Hemostasis is followed by an early inflammatory phase that is characterized by vasodilatation, increased capillary permeability, complement activation and polymorphonuclear and macrophage migration into the wound within three days. Macrophages are actively phagocytic, and secrete regulatory factors that are responsible for the proliferation by fibroblasts and endothelial cells (granulation tissue) around the 5th day post-injury heralds the "proliferative phase." Fibroblasts synthesize collagen and ground substance (proteoglycans and fibronectin), which support new cells, and the fragile capillary buds, which appear around this time (angiogenesis). Epithelialization requires the migration of epithelial cells across the granulation tissue, to close the epidermal defect. Collagen synthesis continues for many months after wound closure but also undergoes continual lysis, so a delicate balance exists between the two processes. This final remodeling phase, accompanied by increasing tensile strength of the wound, and a decreasing cellularity, may continue for up to a year [3]. Wound healing is currently a clinical challenge due to inconsistencies encountered in the healing processes and the financial burden [4]. Therefore, medicinal plants have generated much interest for the treatment of skin ailments as they are affordable and purportedly safe from hypersensitive reactions [5]. The present study was designed to evaluate the antioxidant potential and the wound healing effect of the ethanol extract of *C. procera* bark.

Material and Methods

Plant Material and Extraction

Leafy plants of C. procera were obtained from the city of Maroua. The identity was confirmed by plant taxonomist Todou G., from the University of Maroua. A voucher specimen was deposited at the Herbarium of the University Campus. Powdered stem bark of C. procera was extracted with ethanol (70°) at room temperature. The extract solution was filtered, and the solvent was evaporated under reduced pressure. The dark green mass obtained 4.50% yield based on dry bark was considered as the ethanol extract used in the study.

Drugs

2-deoxy-d-ribose was from Sisco Research Laboratories PVT LTD (India). Trichloroacetic acid was from Sigma (Germany). Thiobarbituric acid was from Titan Biotech LTD (India). 1, 1- diphenyl-2-picrylhydrazy (DPPH) was from Fluka (Switzerland). Ketamine (Rotexmedica-Trttau-Germany), diazepam (Renaudin-France), dexamethasome (Guangdong Medecine and Health Products I/E corp) and nylon surgical treat size 1 (Agary Pharmaceutical Ltd) were purchased from a local pharmacy store). All other chemicals were of laboratory grade and used as received.

Quantitation of Total Phenols

Total phenolics content (TPC) was measured using the Folin–Ciocalteu method [6]. A standard curve was prepared using 0.625 μg/mL - 10 μg/mL gallic acid. TPC content was expressed as gallic acid equivalent (GAE) per mg of extract.

Quantitation of Flavonoids

Flavonoids content (FC) was measured using the method described by Makkar *et al.* [7]. A standard curve was prepared using 10-100 μg/mL rutin. FC content was expressed as rutin equivalent (RE) per mg of extract.

Antioxidant Activity

All determinations were done in triplicate. The concentrations of samples that provide 50% inhibition (IC50) were obtained by interpolation from linear regression analysis.

DPPH Radical Scavenging Assay

Scavenging of the stable DPPH radical was assayed in vitro according to the method described by Obame *et al.* [8]. Various concentrations of the ethanol extract of the bark of *C. procera* (0.5, 1, 2 and 4 μg/mL) were added to a 0.5 mL solution of DPPH (0.125 mM in 95% ethanol). The mixture was shaken and allowed to stand at room temperature for 30 min, and the absorbance was measured at 517 nm in a spectrophotometer. Percent inhibition was calculated from the control. Ascorbic acid and gallic acid at doses lower than that which have been reported to act as pro-oxidant [9,10] were used as a standard compound in this assay.

Degradation of Deoxyribose (Fenton's reaction)

The ability of the ethanolic extract of the bark of *C. procera* to prevent Fe_2^+/H_2O_2-induced decomposition of deoxyribose was carried out using the method described by Hinneburg *et al.* [11]. Briefly, the reaction mixture contained 100 μL of extract dissolved in water, 500 μL of 5.6 mM 2-deoxy-D-ribose in KH_2PO_4-NaOH buffer (50 mM, pH 7.4), 200 μL of premixed 100 μM $FeCl_3$ and 104 mM EDTA (1:1 v/v) solution, 100 μL of 1.0 mM H_2O_2 and 100 μL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 50°C for 30 min.

Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were added to each tube. The samples were vortexed and heated in a water bath at 50°C for 30 min. The extent of oxidation was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated as follows:

$$\text{Inhibition}(\%) = \frac{\text{Absorbance of the control-Absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

Animals

All animal procedures were in strict accordance with the NIH Guide for the care and Use of Laboratory Animals. Locally bred male albino-Wistar rats weighing 150-180 *g*, fed on a standard diet and allowed water ad libitum were used. Animals were caged under laboratory environment and 12-h dark and light cycles. Standard rodent chow pellets were given ad libitum with free access to water.

Determination of *In Vivo* Cicatrizant Activity

Grouping of animals

For each model, 20 animals were divided into 4 groups of 5 rats as follow: Group 1: H_2O; Group 2: Dexamethasone i.m (DX); Group 3: C. procera (50 mg) eosinophil cationic protein (ECP); and group 4: Dexamethasone + C. procera (DX+ECP).

Excisional Wound Model

Animals were anesthetized by intramuscular injection of ketamine/diazepam (ketamine 25 mg/kg and diazepam 10 mg/kg). An area (4 cm^2) was marked using a frame and marker pen. The required area of the dorsal fur of the animals was shaved with an electric clipper. The area was sterilized by spraying with 70% ethanol. A full thickness skin (4 cm^2) was excised from the predetermined area by removing the epidermis and dermis layer with a surgical blade (0.1 mm) until the subcutaneous fat (avoiding *panniculus carnosus* and the muscle layer). Group I was applied topically with distilled water (negative control), Group II with distilled water (positive control), Groups III and IV with the plant extract (dose approximately 50 mg g/wound) every two days until the wound was completely healed. Special care was taken to avoid variation in the dose given. Animals were monitored every day. Wound diameter was recorded in vertical and horizontal planes daily, as well as epithelialization time that indicate the formation of new epithelial tissue to cover the wound. The lesions on each rat were also rated using the following parameters: (1) The presence and type of exudates, (2) erythema, (3) swelling, (4) ulceration and (5) crust formation [12]. The degree of wound healing was calculated using formula:

$$\frac{\text{Wound area on zero day-wound area on corresponding day}}{\text{wound area on zero day}} \times 100$$

The number of days for complete epithelization was noted. Wounds were considered closed (completely healed) if moist granulation tissue was no longer apparent and the wound was covered with new epithelium.

Incisional Wound Model

A 5-cm incision was made perpendicular to the axis of symmetry of the animal and the two borders of the wound were stitched together at its center, with interrupted sutures at distance of 1 cm. Treatment was started immediately, and every 48 h the compound being tested was applied to the wound. On 10th day post wounding, animals were sacrificed by chloroform overdose and wound areas from each animal were dissected carefully. Stripes of equal size (width) from one side were cut, and the line was drawn on either side, 3 mm away from the wound, for breaking strength determination. One piece of tissue was fixed in 10% formalin for histopathological examination, and the other was used to quantify the wound breaking strength (WBS).

Determination of Wound Breaking Strength

Both ends of each skin stripe were fixed with a pair of steel clip, one clip was allowed hanging on a stand and other clip with a freely suspended polyethylene bag through a string run over a pulley. It was then gradually filled with water from a polyethylene reservoir till the wound stripe was broken at the site of the wound. The amount of water required to break the wound was noted and expressed as tensile strength of the wound in grams [12]. The tensile strength was calculated according to the following equation:

$$\text{Tensile strength} = \frac{\text{Total breaking load}}{\text{cross sectional area}}$$

For preliminary screening, an activity greater than 25% is considered significantly important, and the sample is described as having positive wound-healing activity. The percentage of activity was calculated according to the following formula:

$$\text{Activity}(\%) = \frac{\text{WBSc-WBSt}}{\text{WBSc}} \times 100$$

WBSt = Average of the force necessary to open the wound of a treated mouse.

WBSc = Average of the force necessary to open the wound of an untreated mouse (control).

Histomorphological Study

Skin specimens were immediately fixed in 10% (v/v) neutral formalin until the tissues hardened. Each specimen was embedded in a paraffin block, and thin sections (5 μm) were prepared and stained with hematoxylin and eosin (HE) (for general morphological observations). Slides were examined qualitatively under a light microscope, for collagen formation, fibroblast proliferation, angiogenesis, and epithelialization.

Statistical Analysis

All data were expressed as mean ± standard deviation. Statistical analyses were evaluated by one-way ANOVA followed by Dunett test. $P < 0.05$ was regarded as significant.

Results

Phytochemical Content and Antioxidant Activity of the Ethanolic Extract of *C. procera*

In the present study, the ethanolic extract of C. *procera* have shown strong DPPH radical scavenging (IC50 28.57 μg/mL). Total phenols in the ethanol extract were found to be 12.5g GAE/g extract, and total flavonoids were found to be 399.54 g RE/g extract. The ethanolic extract showed its ability to quench the stable DPPH radical and inhibit deoxyribose degradation. Table 1 shows the percent inhibition of DPPH with C. *procera* ethanolic extract and pure antioxidant compounds at different concentrations (0.625-5 μg/mL). The activity of plant extract was between14.90 and 69.65 %. Ascorbic acid and gallic acid showed the highest radical scavenging effectiveness. Activity of ascorbic acid acid was between 81.45 % and 83.79%. That of gallic acid was between 80.37 and 82.29. Calculated IC50 were 28.57 mg/mL, 2.13 μg/mL and 2.16 μg/ml for C. *procera* ethanolic extract, ascorbic acid and gallic acid respectively.

Table 2 shows the percent inhibition of deoxyribose degradation with C. *procera* ethanolic extract and pure antioxidant compounds at different concentrations (0.5-5 μg/mL). The activity of plant extract was between 27.68 and 43.75%. Ascorbic acid and gallic acid showed the highest antioxidant effectiveness. Activity of ascorbic acid acid was between 69.19% and 89.28%. That of gallic acid was between 82.14 and 90.18. Calculated IC50 were 3.50 μg/mL, 1.66 μg/mL and 1.60 μg/mL for C. *procera* ethanolic extract, ascorbic acid, and gallic acid, respectively.

Table 1: Antiradical activity of ethanolic bark extract of *C. procera* on DPPH *in vitro*

Inhibition (%)			
Concentration (μg/mL)	Ascorbic acid	Gallic acid	*C. procera*
0.625	81.45	80.37	14.90
1.25	81.87	80.79	50.91
2.5	82.45	81.45	63.74
5	83.79	82.29	69.65

C. procera: Calotropis procera

Table 2: Antioxidant activity of ethanolic bark extract of *C. procera* on deoxyribose *in vitro*

Inhibition (%)			
Concentration (μg/mL)	Ascorbic acid	Gallic acid	*C. procera*
0.5	69.19	82.14	27.68
1	82.14	83.93	35.27
2	83.03	87.50	37.05
4	89.28	90.18	43.75

C. procera: Calotropis procera

Wound Contraction and Epithelialization Time

Results of the wound contraction rate and epithelialization time are shown in Table 3 and Figure 1 respectively. The extract-treated group demonstrated significantly higher wound contracting ability ($P < 0.001$) than the control group from day 4 post-wounding. In the extract treated, group complete healing was observed on 17th-18th day, while untreated group (control) and Dexamethasone treated animals took more than 20 days for healing of wounds.

Wound Breaking Strength

In incision wound study, tensile strength was 672.90 g/cm^2 in the

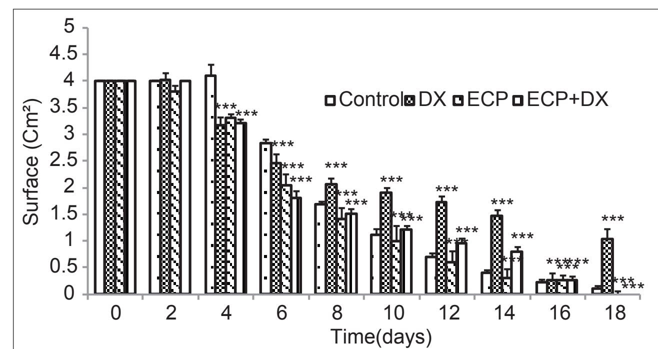

Figure 1: Effect of the ethanol extract of the stem bark of *Calotropis procera* on wound contraction in rats each value represents the mean ± standard error of mean, *n* = 5. ****P* < 0001: Difference significant when compared to control, DX: dexamethasone, ECP: Ethanol extract of the stem bark of *Calotropis procera*, ECP+DX: Ethanol extract of the stem bark of *Calotropis procera* combined with dexamethasone

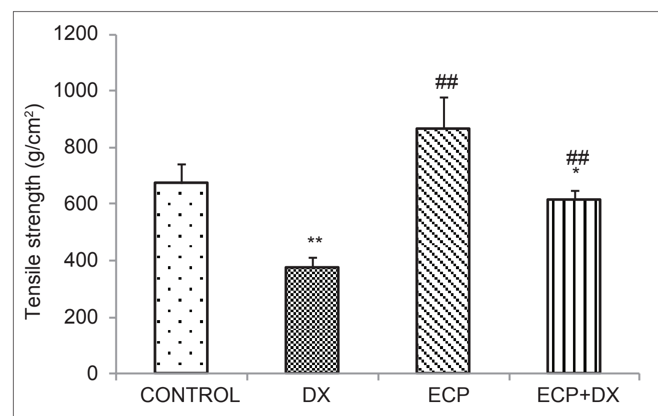

Figure 2: Effects of the ethanol extract of *Calotropis procera* on the tensile strength of rats. Each value represents the mean ± standard error of mean, n = 5. ##*P*<0.01: Difference significant when compared to control, DX: dexamethasone, ECP: ethanol extract of the stem bark of *Calotropis procera*, ECP+DX: ethanol extract of the stem bark of *Calotropis procera* combined with dexamethasone

Table 3: Effects of the ethanol extract of *C. procera* on epithelialization time in rats

	Control	DX	ECP	ECP+DX
Epithelialization time (days)	21.00±1.12	31.00±1.12***	17.00±1.55###	18.00±0.00###

Each value represents the mean±SEM, *n*=5, ****P*<0.001: Difference significant when compared to control, ###*P*<0.001, Difference significant when compared to dexaméthasone. Dx: Dexaméthasone, ECP: Ethanol extract of the stem bark of *Calotropis procera*, ECP+DX: Ethanol extract of the stem bark of *Calotropis procera* combined with dexamethasone, SEM: Standard error of the mean, *C. procera: Calotropis procera*

control group. *C. procera* enhanced the tensile strength of 10 days old wounds as compared with wounds of the untreated group. In this study, dexamethasone decreased this force by 44.44%, while the plant extract exerted an activity of 28.41% when compared to control animals. Finally, the plant extract prevents dexamethasone inhibition on the tensile strength by 64.08 % [Figure 2].

Increase in tensile strength may be due to increase in collagen concentration per unit area and stabilization of fibers. Tensile strength was improved in all groups treated with the ethanol extract of the stem bark of *C. procera*. This may be due to promotion of collage nation and cross-linking.

Histomorphological Study

Figure 3 shows the various hitomorphological features of the skin tissue obtain with different treatments. Histological sections of scar tissue from extract-treated rats showed increased and well-organized dermis, more fibroblasts than control rats. Scar tissue sections obtained from dexamethasone treated rats revealed fewer collagen fibers and fibroblasts than all the other groups. Blood vessels of dexamethasone treated rats were dilated compared to the other groups of rats, and the epithelium layer was poorly organized.

Discussion

Despite the large number of researches, wound healing is still challenging investigators. The healing process consists of different phases including contraction, epithelialization, granulation, collage nation and scar maturation which are concurrent but independent to each other. In this study, the antioxidant activity of the ethanolic extracts was tested against two important oxygen radicals, DPPH and hydroxyl radical. Oxygen radicals are toxic waste products which produce oxidative stress during the inflammatory phase of wound healing [13]. Scavengers application to the injury site has been reported to be effective in inflammatory conditions and wound healing [14,15]. These health effects have been traditionally attributed to the antioxidant property and is associated with the development of matured collagen fibers and fibroblasts with better angiogenesis [16,17]. Accordingly, natural accelerators of wound healing with antioxidant action are of great interest for surgery, dermatology and modern cosmetology [14].

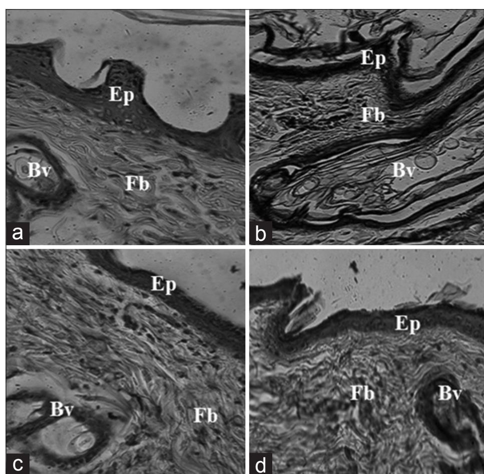

Figure 3: Histological section of control wound (a), dexamethasone (b), *Calotropis procera* (c), and dexamethasone + *Calotropis procera* (d) (HE stain, ×400). Bv: Blood vessel; Cg: collagen; Ep: epithelial cells; Fb: fibroblasts

The DPPH assay validated the free radical scavenging activity of the ethanolic extract. Oxidation products of deoxyribose by hydroxyl radical, upon heating with thiobarbituric acid under acid conditions, would yield a pink chromogen with the maximum absorbance wavelength of 532 nm. Added hydroxyl radical scavengers compete with deoxyribose for hydroxyl radicals and diminish chromogen formation [18]. Phytochemical screening of CPE revealed the absence of saponins and glycosides. Tannins were also likely absent since the potassium dichromate test was negative, even though incidental properties of tannins were indicated because the gelatin test was positive. In fact, gelatin is not only precipitated by tannins, but also by gum arabic, starch inulin and methyl gallate [19]. But, phenols, flavonoids, alkaloids, reducing sugar and proteins were detected in the extract used. The scavenging activity of the extract in this study is expected due to its phenolic content, beside other phytochemicals. In effect, it is known that the wound healing properties of plants, in most cases, are associated with their significant antioxidant activities [20]. Two models were used to assess the effect of an ethanol extract of the stem bark of *C. procera* on acute wounds in rats. Prohealing action of the extract was observed in normal and dexamethasone-induced healing delay in acute wounds. This effect was demonstrated by an increase in the rate of wound contraction and by a reduction of epithelialization time. Significant increase in tensile strength (> 25%) compared to dexamethasone was observed, which were further supported by histopathological analysis, indicating improved collagen maturation. In normal tissues, strength, integrity, and structure are provided by collagen [21]. Hence, bark extract of *C. procera* can restore skin structure and function. The molecular mechanism involved in the extract effect was not studied; nevertheless, the reduction in epithelialization time may be related to an anti-inflammatory effect of the extract during wound repair. This was supported by the presence of inflammatory signs in dexamethasone treated rats 10 days after wounding (dilated blood vessels, reduction in erythema and wound contraction 48 h after injury) [22]. Reversely, more intense granulation tissue was evident in the treated groups. Interestingly, in this study we observed that the *C. procera* administered in combination with dexamethasone at higher dose, significantly deteriorated the wound healing rather than improving it when compared with dexamethasone treatment alone, indicating complex drug-herb interaction(s) and/or other effective compound(s) responsible for the wound healing effectiveness of *C. procera* regimen. The ethanolic extract of the bark of *C. procera* also exerted its curative effect through enhancement of the tensile strength as described in this study. The healing effect observed with our extract may be due the plant action, at least partly, to its antioxidant potential, as well as the improvement of collagen deposition. Antioxidants and flavonoids have been reported to improve wound healing through increasing collagen cross-linking and then breaking strength [23].

conclusion

On the basis of the considerations obtained in the present study, the bark of *C. procera* possess considerable antioxidant property as evident from the result of antiradicalar assay. This extract demonstrated wound healing effect by accelerating wound closure and epithelialization *in vivo*. This effect may be due the antioxidant potential of the extract and may be attributed at least partly on its improvement of collagen deposition. Results obtained provided additional data for the use of the bark of *C. procera* as wound healing agent. However, to understand fully the process of wound healing by a *C. procera* extract, it is essential to study the basic cell biology, immunology and biochemistry involved in the processes of inflammation and collagen metabolism, and how these pathways are regulated.

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