

Wound healing properties and kill kinetics of *Clerodendron splendens* G. Don, a Ghanaian wound healing plant

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ABSTRACT

As part of our general objective of investigating indigenous plants used in wound healing in Ghana, we hereby report our findings from some *in vitro* and *in vivo* studies related to wound healing activities of *Clerodendron splendens* G. Don (Verbanaceae). Methanolic extract of the aerial parts of the plant was tested for antimicrobial activity against Gram positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Micrococcus flavus*, as well as resistant strains of *Staph. aureus* SA1199B, RN4220 and XU212), Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*) and *Candida albicans* using the micro-well dilution method. Survivor-time studies of the microorganisms, radical scavenging activity using 2,2'-diphenylpicrylhydrazyl (DPPH) and various *in vivo* wound healing activity studies were also conducted on the extract. The extract exhibited biostatic action against all the test microorganisms with a Minimum Inhibition Concentration (MIC) ranging between 64 and 512 $\mu\text{g/ml}$ and a free radical scavenging property with an IC_{50} value of 103.2 $\mu\text{g/ml}$. The results of the *in vivo* wound healing tests showed that upon application of *C. splendens* ointment, there was a reduction in the epithelization period from 26.7 days (control) to 13.6 days along with a marked decrease in the scar area from 54.2 mm^2 (control) to 25.2 mm^2 . Significant increase in the tensile strength and hydroxyproline content were also observed as compared to the control and was comparable to nitrofurazone. The above results appear to justify the traditional use of *C. splendens* in wound healing and treatment of skin infections in Ghana.

Key words: *Clerodendron splendens*, antimicrobial, antioxidant, biostatic, wound healing

INTRODUCTION

More than 80% of the world's population depends upon traditional medicines for the management of various diseases including skin infections.^[1] Recently, the traditional use of plants for wound healing has received attention by the scientific community.^[2] Wound healing is a complex process characterized by homeostasis, re-epithelization, granulation tissue formation and remodeling of the extracellular matrix.^[3] Although the healing process progresses naturally, an infection can seriously delay this healing process by prolonging the inflammatory phase, disrupting the normal clotting mechanisms, promoting disordered leukocyte function and ultimately delaying

angiogenesis.^[4] Therefore, a variety of activities such as tensile strength and collagen estimation of healing tissue, antioxidant and antimicrobial effects can explain the traditional use of a plant for helping the wounds to heal.^[2]

Clerodendron splendens G. Don (family: Verbenaceae) is a climbing shrub, mostly found growing on cultivated lands between food crops in Ghana and other West African countries. Carbohydrates, glycosides, unsaturated sterols, triterpenoids and flavonoids are reported to be present in the leaves,^[5] in addition to volatile oil in the flowers.^[6] The plant is used ethnomedicinally in Ghana for the treatment of vaginal thrush, bruises, wounds and various skin infections.^[7] In this study, we provide scientific evidence for the use of this plant in the traditional treatment of some infectious conditions and in wound healing.

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MATERIALS AND METHODS

Collection of plant material

The plant was collected from Kumasi in the Ashanti

Region of Ghana in July 2007 and was authenticated at the Department of Pharmacognosy, Kwame Nkrumah University of Science and Technology, Ghana. A voucher specimen (FP/07/0032) has been kept in the herbarium of the Department.

Extraction and phytochemical screening

The plant material was air-dried at room temperature for 7 days and milled into coarse powder using a Laboratory Mill Machine (Type 8, Christy & Norris, UK). Two hundred grams of the powder was cold-macerated with one litre methanol (Sigma-Aldrich, USA) over 48 h. The extract was concentrated under reduced pressure using the rotary evaporator and dried over nitrogen to give a yield of 12.3 g.

The methanolic extract was phytochemically screened for carbohydrates, tannins, saponin, anthraquinones, cardiac and cyanogenic glycosides, flavonoids and alkaloids using the procedures outlined by Wall *et al.*^[8] and Harbon.^[9]

Test organisms

The bacteria used for the tests were obtained from the National Collection of Type Cultures (NCTC), UK. The Gram positive bacteria used were *Bacillus subtilis* (NCTC 10073), *Staphylococcus aureus* (NCTC 4163) *Streptococcus faecalis* (NCTC 775), *Micrococcus flavus* (NCTC 7743), as well as resistant strains of *S. aureus* SA1199B, RN4220 and XU212. The Gram negative bacteria used were *Escherichia coli* (NCTC 9002), *Pseudomonas aeruginosa* (NCTC 10662), *Proteus mirabilis* (NCTC 10975) and *Klebsiella pneumoniae* (NCTC 418). *Candida albicans* (NCPF 3179) was also used.

Minimum inhibitory concentration determination

Inocula of the microorganisms were prepared from a 24 h Mueller-Hinton Broth (Sigma-Aldrich, St. Louis, MO, USA) cultures and suspensions were adjusted to 10⁵ colony forming units per ml (cfu/ml). Minimum inhibition concentration (MIC) values of the extract were determined based on a micro-well dilution method.^[10] The 96-well sterile plates were prepared by dispensing 180 µl of the inoculated broth plus a 20-µl aliquot of the plant extract made up in broth or 20 µl broth in the case of negative control in each well. Neomycin and tetracycline (Sigma-Aldrich, USA) were included as positive controls for bacteria and clotrimazole for *C. albicans*. Plates were covered and incubated for 24 h at 37°C. Bacterial growth was determined after the addition of 50 µl *p*-iodonitrotetrazolium violet (0.2 mg/ml, Sigma).

Survival-time studies

Mid-logarithmic phase cultures of the organisms were transferred into 50-ml portions of prewarmed Mueller-Hinton broth containing MIC, 2× MIC and 4× MIC concentrations of the extract, to yield final concentrations of 10⁵ cfu/ml. The broths were maintained, with agitation, on a water bath at 37°C.

Aliquots (1 ml) were withdrawn after 0, 1, 2, 3, 4, 5, 6, 12 and 24 h and appropriately diluted in Mueller-Hinton broth to neutralize the effect of the extract and viable counts determined by pour plate techniques. Each of the diluted culture suspensions (1 ml) was added to sterile petri dishes and approximately 20 ml, melted and cooled (45°C), Mueller-Hinton agar was added and mixed. Control broths containing 1% v/v Dimethyl Sulfoxide (DMSO) were also set up for each test organism. All experiments were performed in triplicates. Colonies were counted and the number of survivors was calculated after incubation at 37°C for 48 h. Survivor-time curves were drawn for the test organisms, exposed to the various concentrations, as well as the control.

Antioxidant activity

The method of Yoshida *et al.*^[11] as outlined in detail^[12] was used to determine the free radical scavenging activity of the *C. splendens* extract. Quantities of 0.5 ml of various concentrations of the extract in methanol were added to 5 ml of a 0.004% methanol solution of 2,2'-diphenylpicrylhydrazyl (DPPH). This was incubated at room temperature for 30 min after which absorbance (A_i) was read against a blank (A_0) at 517 nm on a UV Spectrophotometer (Cecil, 2000 series). L-ascorbic acid was employed as a control and the inhibition of free radical DPPH, in percentage was calculated as: Scavenging activity (%) = $(A_0 - A_i / A_0) \times 100$.

The IC₅₀ value was calculated through extrapolation from linear analysis, using the Prism Software (version 4, Santiago California). This denoted the concentration of extract required to scavenge 50% of DPPH radicals.

Animals

Male Sprague-Dawley rats (170–190 g) were purchased from Noguchi Memorial Research Institute, Accra, Ghana. They were kept at 26 ± 2°C and a relative humidity of 44–55%, light and dark cycles of 10 and 14 h, respectively, for 1 week before the experiment. Animals were given the rodent diet and water *ad libitum*. All studies were conducted in accordance with the National Institute of Health's guideline for Survival Rodent Surgery (1985) after approval from the Institutional Ethics Committee. All surgical procedures were carried out under thiopentone sodium (25 mg/kg, i.p.) anesthesia. Animals were allowed to recover and were housed individually in metallic cages containing sterilized paper cuttings.

In the experiment, the rats were divided into three groups ($n = 6$). Group 1 was the control group which received simple ointment BP base. Group 2 was treated with the reference drug (0.2% w/w nitrofurazone, a standard antimicrobial agent used in topical wound dressings). Group 3 received *C.*

splendens ointment (33.3% w/w *C. splendens* extract in Simple Ointment BP) topically on wounds that were created on the dorsal back of rats daily until the wounds completely healed.^[13,14] One hundred milligrams of ointment was spread over 500 mm² area.

Excision wound model

An impression was applied on the dorsal thoracic region 1 cm away from the vertebral column and 5 cm away from the ear using a biopsy punch (Acuderm, Florida, USA) of 2.5 cm diameter, on the anesthetized rat. The skin of the impressed area was excised to its full thickness to obtain a wound area of about 500 mm². Hemostasis was achieved by blotting the wound with a cotton swab soaked in normal saline.

Wound area

Contractions, which contribute to wound closure in the first 2 weeks, were studied by tracing the raw wound. The wound area was measured after specific time intervals by retracing the wound on a millimetre-scale graph paper. The difference in the area of the wound indicated the degree of wound healing.^[15]

Collagen estimation

Hydroxyproline constituent of collagen was measured.^[16] Healed tissues of the wounds were cut and dried in hot air oven at 60–70°C to constant weights and hydrolyzed in 6 M HCl at 130°C for 4 h in sealed tubes. The hydrolyzate was neutralized (to pH 7.0) and subjected to chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4 M perchloric acid and color was developed with the help of Ehrlich reagent at 60°C and measured at 557 nm using the spectrophotometer (Cecil, 2000 series).

Incision wound model

Rats were anesthetized and two paravertebral long incisions made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on each side of their depilated back. Aseptic techniques were not applied and no local or systemic antimicrobial was used throughout the experiment.^[17] Each of the three groups of animals was treated in the same manner as for the excision wound model. The parted skin was kept together by stitching with a black silk surgical thread (No. 000) and a curved needle (No. 11). Continuous threads on both wound edges were tightened for good wound closure. Group 1 animals (controls) were treated topically with simple ointment base BP whilst groups 2 and 3 received *C. splendens* extract [CS] and nitrofurazone ointments respectively twice a day for 9 days.

Tensile strength

The tensile strength of a wound represents the degree of wound healing, so wound healing agents usually provide

a gain in tensile strength.^[18] The sutures were removed on the ninth day of the wound and the tensile strength measured on the 10th day. The animals were anesthetized and strips of healing tissue (8 mm width and 20 mm long) along with normal skin at the two ends were excised. The tensile strength was measured by loading a strip between the upper and lower holders of the Tensile Testing Machine TKG-20 (Fine Testing Machines, India) in such a way that the effective load bearing size was 8 × 8 mm with the wound remaining in the centre.^[19] The total breaking load was measured in Newtons and the tensile strength was calculated from the following equation:

Tensile strength = total breaking load/cross-sectional area

The mean tensile strength of the two paravertebral incisions on both sides of the animals was taken as the measure of the tensile strength of the wound for an individual animal. The tensile strength of *C. splendens* ointment-treated wounds was compared with the control and nitrofurazone ointment as the standard. Further, epithelization period and scar area were measured daily for 25 days after tensile strength determination.^[15]

RESULTS AND DISCUSSION

Preliminary phytochemical analysis showed the presence of reducing sugars, phytosterols, tannins, terpenoids, alkaloids and flavonoids in the extract of *C. splendens*. The extract exhibited very good antibacterial and antifungal activities against the microorganisms tested with the MIC ranging from 64 to 256 µg/ml against the wild-type organisms [Table 1].

The MIC of the extract against the resistant organisms were however, higher. The controls used (tetracycline and neomycin) recorded very low MICs thus confirming their superiority over the plant extract as an antimicrobial agent.

Table 1: Antimicrobial activity of *C. splendens* expressed as MIC (µg/ml)

Organism	CSE	TET	NEO	CLO
<i>B. subtilis</i>	128	2	2	–
<i>Staph. aureus</i>	128	2	1	–
<i>Strept. faecalis</i>	256	4	8	–
<i>M. flavus</i>	64	1	1	–
<i>E. coli</i>	128	2	4	–
<i>P. mirabilis</i>	256	4	4	–
<i>K. pneumoniae</i>	128	2	2	–
<i>P. aeruginosa</i>	256	2	4	–
SA1199B	512	8	8	–
RN4220	512	4	4	–
XU212	512	128	4	–
<i>C. albicans</i>	64	–	–	2

CSE, *C. splendens* extract; TET, tetracycline; NEO, neomycine; CLO, clotrimazole; n = 5

The means of survivors obtained at each concentration plated for each organism are graphically presented and samples are as shown in Figure 1 for *S. aureus*, *P. aeruginosa* and *C. albicans*. The graphs of the other test organisms (not shown) followed similar pattern. All the organisms exhibited a decline in survivors in the first 4–6 h of exposure to the extract in concentration-dependent manner whilst the controls showed a steady rise in population within these same periods, followed by some level of recovery of the organisms even at higher concentrations. This depicts biostatic mode of action of the extract. The recoveries may be due to inactivation of the bioactive constituents of the extract with time when in the nutrient medium. This might have necessitated the daily collection of this herb for treatment by the local herbal practitioners.

DPPH free radical scavenging test conducted on the extract recorded an IC₅₀ of 103.2 µg/ml. This value is about 5 times higher than what was recorded for L-ascorbic acid (IC₅₀ 21.4 µg/ml) the standard antioxidant^[20] employed in this study but it is an indication that *C. splendens* possesses antioxidant

property. Free radicals such as molecular oxygen play a vital role in the pathogenesis and therapy of wounds. Overproduction of these reactive oxygen species in the body results in oxidative stress thereby causing cytotoxicity and delayed wound healing.^[21] *C. splendens* therefore appears to play a useful role in promoting wound healing due to its antioxidant and antimicrobial properties.

The results of the *in vivo* wound healing tests showed that upon application of *C. splendens* ointment, there was a reduction in the epithelization period from 26.7 days (control) to 13.6 days along with a marked decrease in the scar area from 54.2 mm² (control) to 25.2 mm² [Table 2]. Significant increase in the tensile strength and hydroxyproline content were observed as compared to the control. The percentage closure of excision wound area, which was an indication of degree of wound contraction, showed that *C. splendens* extract significantly promoted wound contraction (69.2%) in 7 days as compared to the control (46.2%) and nitrofurazole (67.5%) [Table 3].

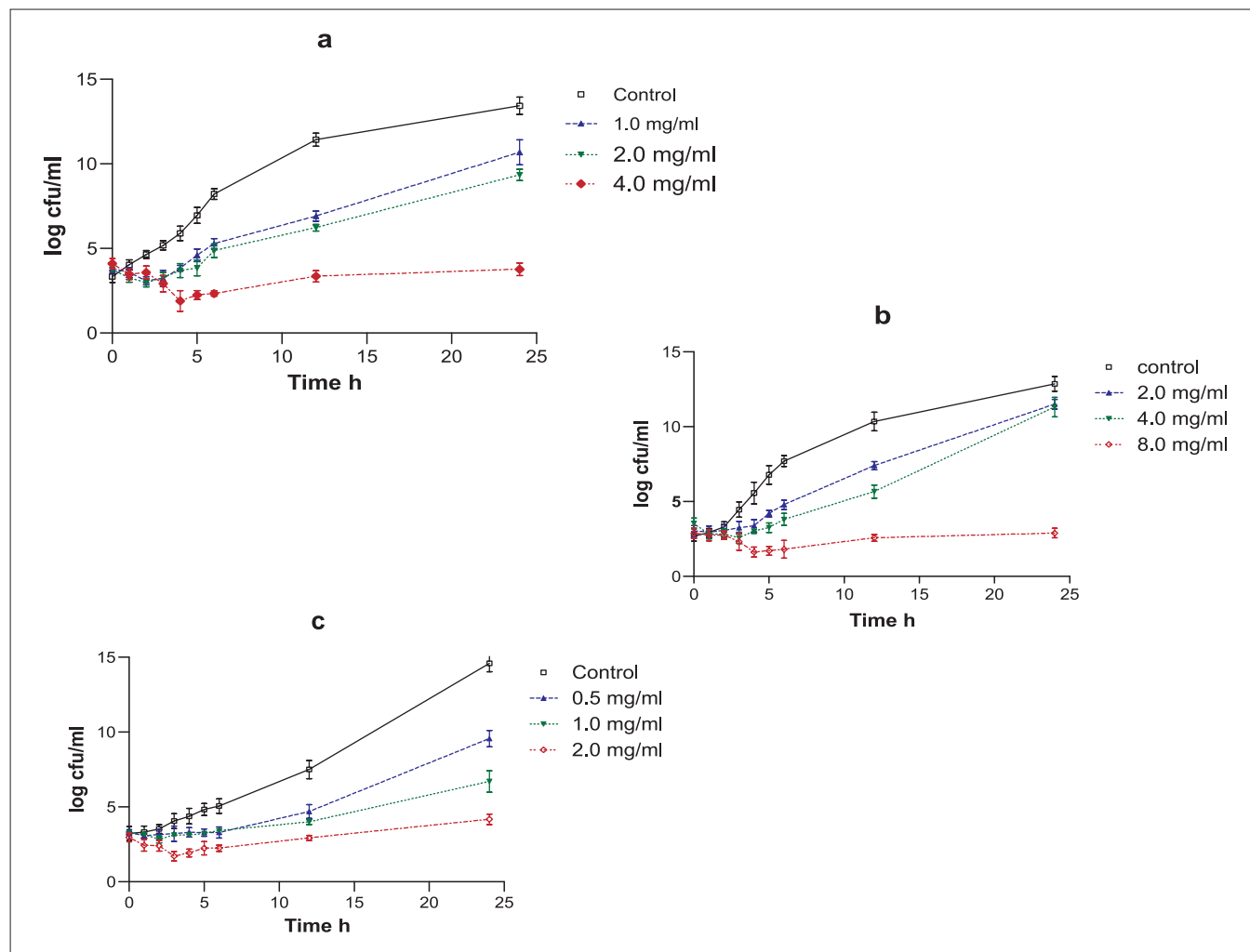


Figure 1: Survival of (a) *Staph. aureus* (b) *P. aeruginosa* and (c) *C. albicans* in the presence of various concentrations of *C. splendens* extract

Table 2: Effect of *C. splendens* extract ointment on incision wound

Topical treatment	Epithelization period (days)	Tensile strength (g)	Scar area (mm ²)	Hydroxyproline (mg/100 mg tissue)
Control	26.7 ± 1.2	287.5 ± 17.3	54.2 ± 3.8	7.28 ± 0.34
CS ointment (100 mg/500 mm ²)	13.6 ± 1.1	426.2 ± 12.4	25.2 ± 3.4	11.10 ± 0.45
Nitrofurazone (2% ointment) [100 mg/500 mm ²]	11.5 ± 1.4	428.2 ± 21.3	27.9 ± 2.9 ^c	11.7 ± 0.45

Values are mean ± SEM for six rats; Statistically significant difference in comparison with control group: $P < 0.05$

Table 3: Effect of *C. splendens* extract ointment on excision wound

Topical treatment	Percentage of closed excision wound area after days			
	4	7	15	21
Control	26.4 ± 2.6	46.2 ± 3.1	68.3 ± 3.5	75.8 ± 3.6
CS ointment (100 mg/500 mm ²)	36.5 ± 2.9	69.2 ± 3.7	96.7 ± 4.8	98.2 ± 4.1
Nitrofurazone (2% ointment) [100 mg/500 mm ²]	37.2 ± 3.1	67.5 ± 4.1	96.9 ± 4.7	99.3 ± 4.8

Values are mean ± SEM for six rats. Statistically significant difference in comparison with control group: $P < 0.05$

As the wound healing process involves various phases, the use of a single model is inadequate and no *in vitro* experiment exists that can collectively represent the various components of wound healing. Because of this, *in vivo* assays are highly recommended to confirm the *in vitro* observations. Some of the *in vivo* assays of significance include the determination of tensile strength, which is an indication of quality of the healing, and the determination of hydroxyproline content. Collagen is a major protein in the extracellular matrix and is the component that ultimately contributes to wound strength. Breakdown of collagen liberates free hydroxyproline. Measurement of the hydroxyproline could be used as an index for collagen turnover. In the present study, a significant increase in the hydroxyproline content of the granulation tissue of the animals treated with *C. splendens* extract was recorded compared to the control group, thus indicating the positive effect of the extract on collagen synthesis and hence, on wound healing. The increase in tensile strength of the granulation tissue indicated enhanced collagen maturation by increased cross-linking. The increase in tensile strength, as well as the epithelization, could be attributed to the increased hydroxyproline content in the wound tissue.

CONCLUSIONS

It has been demonstrated that *C. splendens* extract exhibits antibacterial, antifungal and antioxidant properties and this has provided scientific basis for its folkloric use in the treatment of various infectious conditions and wound healing. The wound healing potential which was confirmed by the *in vivo* experiments further supports the ethnomedicinal use of the plant.

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