EVALUATION OF WOUND HEALING ACTIONS OF *HOSLUNDIA OPPOSITA* VAHL, *ANTHOCLEISTA NOBILIS* G. DON. AND *BALANITES AEGYPTIACA* L.

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ABSTRACT
Hoslundia opposita, Anthocleista nobilis and Balanites aegyptiaca are widely used ethnomedicinally in Ghana in the treatment of skin diseases. In this context, antimicrobial potential of the three plant species against a wide range of microorganisms was studied. To validate the ethnotherapeutic claims of these plants in skin diseases, in vivo wound healing activity was studied, besides antioxidant activity to understand the mechanism of wound healing. Methanol extract of Hoslundia opposita showed significant antibacterial activity against all bacteria tested including some resistant strains of Staphyloccocus aureus, with MIC ranging between 64-256 µg/ml. The results show that all three extracts have potent wound healing activity as evident from the wound contraction, increased tensile strength and hydroxyproline content. The results also indicated that the three plant species possess potent antioxidant activity by inhibiting lipid peroxidation, bleaching DPPH radical and protecting against oxidant injury to fibroblast cells.

Keywords: *Wound healing; antioxidant; Hoslundia opposita; Anthocleista nobilis; Balanites aegyptiaca.*

INTRODUCTION

Wounds are physical injuries that result in an opening or breaking of the skin. Proper healing of wound is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin (Singh *et al*., 2006). Wound healing is a complex process characterized by inflammation, proliferation and migration of different cell types. (Priya *et al*., 2002). Fibroblast cells play a very important role in all these processes (Bodeker and Hughes, 1998). Though healing process occurs naturally, an infection, mostly from *Staph. aureus, E. coli*, *Psuedomonas spp.* and *Bacillus spp*. can seriously delay it by prolonging the inflammatory phase, disrupting the normal clotting mechanisms and ultimately delaying angiogenesis (Subramonium *et al*., 2001).

It is believed that reactive oxygen species are deleterious to wound healing due to their harmful

effects on cells and tissues. Topical application of compounds with free radical scavenging properties in patients has been shown to improve significantly wound healing and protect tissues from oxidative damage (Desnervis *et al*., 2005). *Hoslundia opposita* is used in ethnomedicine to treat sore throats, colds, sores, veneral diseases, herpes and other skin diseases (Abbiw, 1990); malaria, microbial infections (Gundidza *et al*., 1992), epilepsy, fever and inflammation (Olajide *et al*., 1998; Moshi *et al*., 2005). *Anthocleista nobilis* is used in local medicine in Ghana and other parts of West Africa for curing fever, stomach ache, diarrhoea, gonorrhoea and also as poultice for sores (Irvine, 1961; Dokosi, 1992). *Balanites aegyptiaca* is used in folk medicine for circumcision wounds, worm infestation and as abortifacient and contraceptive; to treat abdominal and chest pains (Liu and Nakanishi, 1982; Kamel *et al*., 1999).

In this study, we investigated the above-mentioned plants for their potential antibacterial activity since microbial infection can hamper the healing process. Antioxidant activity was determined since it has been found to have positive effect on wound healing whereas fibroblasts play a crucial role in wound healing by initiating the proliferative phase of repair (Mensah *et al*, 2001). The *in vivo* actions of the extracts on both incision and excision wounds were investigated to confirm the findings in the *in vitro* studies.

MATERIALS AND METHODS

Plant materials

Plant materials were collected in December 2003 and authenticated by Mr. Ofori Lartey, a senior research officer at the Centre for Scientific Research into Plant Medicine (CSRPM), Akwapim -Mampong, Ghana, where voucher specimens 10/03/004, 12/03/019, 12/03/016 for *Hoslundia opposita*, *Anthocleista nobilis* and *Balanites aegyptiaca* respectively, have been deposited.

Preparation of extracts

100 gm of each powdered plant material was packed into a cellulose thimble (28x100 mm) and soxhlet-extracted with 500ml methanol over 48 hours until the material was exhausted. Each extract was concentrated and dried in vacuum to give a yield of 1.93, 1.73 and 1.38 % for *Hoslundia opposita*, *Anthocleista nobilis* and *Balanites aegyptiaca* respectively.

Antibacterial assay

The bacteria used for the tests were obtained from the National Culture Type Collection (NCTC), UK and included both Gram positive and Gram negative bacteria. 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 *Staph. aureus* SA1199B, RN4220 and XU212. Gram negative bacteria used were *Escherichia coli* (NCTC 9002) and *Pseudomonas aeruginosa* (NCTC 10662).

Inocula of the microorganisms were prepared from the 24 h Mueller-Hinton broth (Sigma) cultures and suspensions were adjusted to 10^5CFU/ml. Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of the extracts were determined based on a micro-well dilution method (Eloff, 1998). 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. Tetracycline (Sigma) was included as positive control. Plates were covered and incubated for 24 h at 37^{o}C. Bacterial growth was determined after addition of 50 µl p-iodonitrotetrezolium violet (0.2 mg/ml, Sigma).

In vitro test for fibroblast growth stimulation

Confluent fibroblasts (142BR, Sigma) were trypsinised, centrifuged and resuspended in MEM/15%FBS/1% L-glutamine. The cells were counted using a haemocytometer and the suspension standardized at a concentration of 1 x 10^4 cells/ml in MEM/15%FBS/1%L-glutamine. Using a multi-channel pipette, the cells were seeded

at a density of 1 x 10^3 cells per well in 96-well plate excluding the first row. The plates were maintained at 37°C in a humidified incubator of 5%CO_2: 95% air atmosphere. The medium was replaced after 24 hours with MEM containing 0.5% FBS and a range of concentrations of the extracts (1-50µg/ml) except for two columns which were maintained at MEM/0.5% FBS and MEM/15%FBS to serve as serve as starting and positive controls respectively. The 0.5% FBS concentration is a maintenance dose needed for the production of healthy cells but does not significantly stimulate proliferation of cells. The cells were incubated and assayed after five days using the Neutral Red assay method (Weyermann *et al*., 2005; Fotakis and Timbrell, 2006) to assess the effect of the extracts on the growth of the cells.

Antioxidant activity

DPPH radical scavenging activity

The DPPH scavenging activity of the extracts was measured from the bleaching of a purple-coloured methanol solution of 2,2'-diphenyl-picrylhydrazyl (DPPH) which was used as a reagent in a spectrophotometric assay (Yoshida *et al*., 1989; Gyamfi *et al*., 1999). 50µl of various concentrations of the extracts and compounds in methanol were added to 5ml of a 0.004% methanol solution of DPPH. This was incubated at room temperature for 30 minutes after which absorbance was read against a blank at 517nm on a Thermo Spectronic UV spectrophotometer. L-ascorbic acid was used as positive control in these experiments.

Inhibition of free radical DPPH, in percentage was calculated as

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

A_0 is the initial absorbance of methanolic solution of DPPH at 517nm.

The IC_{50} value was obtained through extrapolation from linear analysis, using the Prism Software, and denoted the concentration of sample required to scavenge 50% of DPPH radicals.

Antioxidant activity of plant extracts on human skin fibroblasts

The method used for the hydrogen peroxide assay was the one described by Yamasaki *et al.*, (1994) and modified to evaluate the protective effect of the extracts on the cells against oxidant injury induced by hydrogen peroxide. Fibroblast cells were seeded at 5000 cells/well in a 96-well plate and incubated for five days until almost confluent. The growth medium was then discarded and the confluent cells subjected to three different types of experiment.

In the first experiment, the cells were pre-treated with different concentrations of the extracts overnight after which they were exposed to 10^{-4}M hydrogen peroxide in the standard growth medium and incubated for a further 3 hours. In the second protocol, fibroblast cells were pre-incubated with the extracts of different concentrations overnight, before exposure to the same concentrations of the extracts together with 10^{-4}M hydrogen peroxide in the growth medium. In the third experiment, different concentrations of were applied simultaneously with 10^{-4}M hydrogen peroxide in the growth medium and incubated for 3 hours at 37°C. Catalase (250 unit/ml), an antioxidant enzyme was used as positive control in all experiments.

After the incubation period, the fibroblast cells were stained with Neutral Red and observed microscopically for cell damage, followed by the Neutral Red assay to quantify the degree of protection of fibroblast cells by extracts against hydrogen peroxide damage.

In vivo wound healing assay

Animals used

Male Sprague-Dawley rats (160-180 g) were purchased from the animal house of the Central Drug Research Institute, Lucknow, India. They were kept at 26 ± 2°C and relative humidity of 44-55%, light and dark cycles of 10 and 14 hours respec-

tively, for one week before the experiment. Animals were given the rodent diet (Amruth, India) and water *ad libitum*. All studies were conducted in accordance with the National Institute of Health's guideline for Survival Rodent Surgery (1994). All surgical procedures were carried out under thiopentone sodium (25 mg/kg, i.p.) anaesthesia. Animals were allowed to recover and were housed individually in metallic cages containing sterilised paper cuttings.

In the experiment, the rats were divided into three groups (n=6). Group 1 was the control group which received the simple ointment BP base, group 2 was treated with the reference standard (0.2% w/w nitrofurazone, a standard antimicrobial agent used in topical wound dressings), group 3 received plant extract ointment (33.3% w/w methanol extract in Simple Ointment BP) topically on wounds, created on the dorsal back of rats daily until the wounds completely healed (Chatterjee and Chakravorty, 1993). 100mg of ointment was spread over 500 mm^2.

Excision wound model

An impression was applied on the dorsal thoracic region 1 cm away from the vertebral column and 5cm away from the ear using a biopsy punch (Acuderm, USA) of 2.5cm diameter, on the anaesthetized rat. The skin of the impressed area was excised to its full thickness to obtain a wound area of about 500mm^2. Haemostasis 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 two weeks, were studied by tracing the raw wound. The wound area was measured after specific time intervals by retracing the wound on a millimeter scale graph paper. The difference in the area of the wound indicated the degree of wound healing (Werner *et al*., 1994).

Collagen estimation

Hydroxyproline, which is a basic constituent of collagen was measured using the method of Shukla *et al*., (1999). Tissues were dried in a hot air oven at 60-70^oC to constant weight and were hydrolysed in 6M HCl at 130^oC for 4 hours in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4 M perchloric acid and colour was developed with the help of Ehrlich reagent at 60^oC and measured at 557 nm using the Pye Unicam spectrophotometer.

Incision wound model

Rats were anaesthetized 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 the depilated back of the rat. Full aseptic measures were not applied and no local or systemic antimicrobial was used throughout the experiment (Udupa *et al*., 1995). Each of the three groups of animals was treated in the same manner as for 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 the stitching. Continuous threads on both wound edges were tightened for good wound closure. The wound was left undressed and ointments from the plant extracts along with the water-soluble base ointment (control) and nitrofurazone ointment were applied topically twice a day for 9 days. When wounds were healed completely, the sutures were removed on the ninth day and tensile strength was measured with a tensiometer.

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 (Govindarajan *et al*., 2004). The sutures were removed on the ninth day after wounding and the tensile strength measured on the tenth day. On the tenth day after creating the wound, the animals were anaesthetised. Healing tissue along with

normal skin at two ends was excised for tensile strength measurement using Tensile Testing Machine TKG-20 (from Fine Testing Machines, India). Strips of 8mm width and 20mm length were cut out from the excised tissue in treated and control animals and were loaded between the upper and lower holder of the machine in such a way that the effective load bearing size was 8 x 8 mm with the wound remaining in the centre. The total breaking load is measured in Newtons and the tensile strength was calculated by the following equation;

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

Ointment from different plant extracts along with the 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 was taken as the measures of the tensile strength of the wound for an individual animal. The tensile strength of the plant extract ointment treated wounds was compared with the control and nitrofurazone ointment as the standard. The scar area were measured daily for 25 days after tensile strength determination (Werner *et al*., 1994).

Statistical analysis

One-way ANOVA was used for the comparison of the means. Results are expressed as mean ± SD (standard deviation) data, using the Prism Software.

RESULTS AND DISCUSSION

The methanol extracts of *Hoslundia opposita* and *Anthocleista nobilis* presented MICs > 512 µg/ml against all the selected bacteria including the resistant strains of *Staph. aureus SA1199B, RN4220* and *XU212*. *M. flavus* was the most susceptible with MIC of 32µg/ml (Table 1). Tetracycline, a standard antibiotic which was used as a positive control presented a very low MICs of 1-8 µg/ml except against *Staph. aureus XU212* (MIC 128 µg/ml) which is resistant to the tetracyclines and over expresses the Tet K efflux proteins. *Balanites aegyptiaca* however, did not show any activity against the tested bacteria. This observation is in line with previous screening for antimicrobial agents from these plants or related species where most had activity against Gram posi-

Table 1: Antibacterial activities of methanol extracts expressed as minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) in µg/ml

Bacteria	MIC (µg/ml)				MBC (µg/ml)		
	An	**Ho**	**Ba**	**Tet**	**An**	**Ho**	**Ba**
Staph. Aureus	128	64	NI	4	250	512	>1000
B. subtilis	256	128	NI	2	250	512	>1000
M. flavus	32	128	NI	1	250	256	>1000
E. coli	256	256	NI	8	512	512	>1000
P. aeruginosa	512	512	NI	8	512	>1000	>1000
SA 1199B (Nor A)	125	64	NI	8	>1000	>1000	>1000
XU212 (Tet K)	256	128	NI	128	>1000	>1000	>1000
RN4220 (MsrA)	256	128	NI	8	>1000	>1000	>1000

Key: NI- no inhibition, **An –***Anthocleista nobilis* stem bark; **Ho –** *Hoslundia opposita* leaves; **Ba –***Balanites aegyptiaca* stem bark; **Tet** - Tetracycline [positive control]; n=3

tive bacteria (Rabe, 1997; Koné *et al*., 2004). The two Gram negative bacteria that showed some degree of susceptibility were *E. coli* and *Pseudomonas aeroginosa*. This could be explained in terms of the nature of the cell wall of Gram negative bacteria which is comprised of various polysaccharides, proteins, lipids and so is much more complex than the cell wall of the Gram positive bacteria, coupled with the fact that the cell wall is surrounded by an outer membrane barely separated from the cell wall by a periplasmic space containing the periplasm which is believed to contain bacterial enzymes that destroy antibacterial substances before they affect the cell membrane (Lewis, 2000).

All the three plant extracts had no significant effect on the growth of human dermal fibroblast [142BR] up to concentrations 50 µg/ml. Doses of extracts above 50 µg/ml rather had toxic effects on the cells.

The quantitative DPPH test on the plant extracts revealed all of them having various degrees of antioxidant properties, with IC_{50} of 50.9, 14.6 and 32.3 µg/ml for *A. nobilis, B. aegyptiaca* and *H. opposita* respectively (Table 2). L-ascorbic acid which was used as a positive control recorded an IC_{50} value of 21.1µg/ml. This compared favorably with the literature value of 21.04µg/ml for L-ascorbic acid (Bizimenyera *et al*., 2007). The observed strong antioxidant action of these plant extracts may be attributed to the presence of phenolic compounds and flavonoids in the plants (Ngadjui *et al*., 1995) but these have not been tested for antioxidant activity. Some such compounds have been found by different workers to be highly antioxidant (Thang *et al*., 2001).

Table 2: DPPH scavenging activity of methanol extracts of selected plants

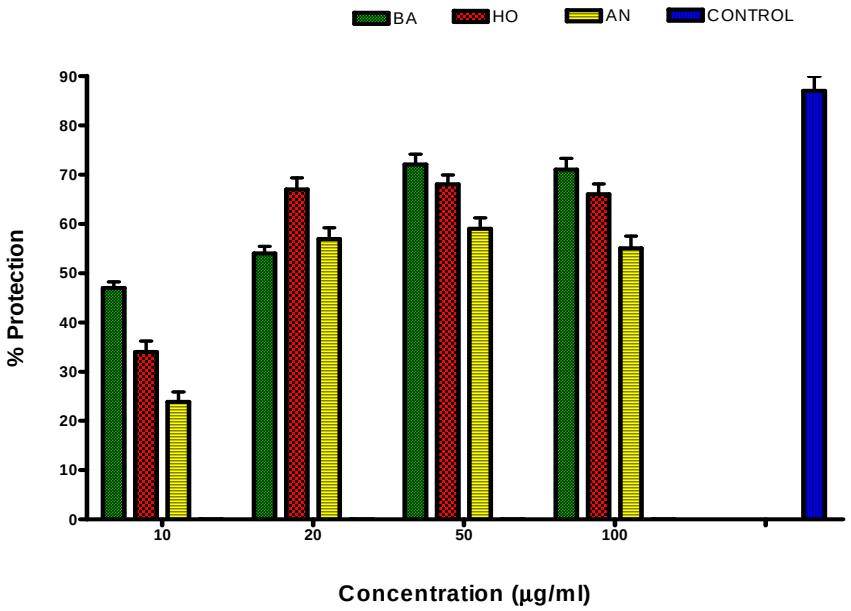
Plant material	IC_{50} (µg/ml) ±SD
Anthocleista nobilis	50.9 ±1.3
Balanites aegyptiaca	14.6 ±0.9
Hoslundia opposita	32.3 ±1.9
L- ascorbic acid	21.1 ±1.1

The scavenging effects were expressed as IC_{50} (±S.D., n=5) compared to the blank. L- ascorbic acid was used as a positive control.

Different protocols were used to assess the effects of the extracts on hydrogen peroxide induced damage on the fibroblast cells. In the first and second protocols, where the cells were pre-incubated with the extracts overnight before the application of the hydrogen peroxide, it was found by the Neutral Red assay that the cells were damaged and so were not protected. The cell damage however could be due to the inherent cytotoxicity of the extract rather than the effect of the hydrogen peroxide, due to the relatively high concentrations of the extracts used. In the third experiment, different concentrations of extracts were applied simultaneously with 10^{-4}M hydrogen peroxide in the growth medium and incubated for 3 hours at 37^{o}C. Here, it was observed that fibroblast cells were protected against hydrogen peroxide damage by various degrees by the extracts. *B. aegyptiaca* offered the highest protection against hydrogen peroxide-induced damage to cells with its activity (72% at 50µg/ml) almost comparable with that of catalase (control at 250 units/ml) [Fig .1]. *H. opposita* and *A. nobilis* also showed 68 and 57 % protection against oxidative damage to the fibroblast cells respectively.

The results of the *in vivo* wound healing tests showed that, upon administration of plant extracts, there was a decrease in the epithelisation period from 26.7 days (control) to 14.7, 16.4 and 13.3 days for *A. nobilis, B. aegyptiaca* and *H. opposita* respectively, along with a marked decrease in the scar area and a significant increase in the tensile strength and hydroxyproline content compared to the control and comparable to the nitrofurazone (Table 3).The percentage of close of excision wound area, which was an indication of degree of wound contraction, showed that *H.*

opposita extract significantly stimulated wound contraction with about 68.2% contraction in 7 days as compared to 46.2% given by the control and 67.5% by the nitrofurazone (Table 4). *A. nobilis* and *B. aegyptiaca* extracts also had significant effects on wound contraction.



Key BA- *B. aegyptiaca*; HO- *H. opposita*; AN- *A. nobilis*; Control- catalase (250 units/ml)

Fig. 1: Protection of fibroblast cells against hydrogen peroxide-induced damage by simultaneous application of extracts and hydrogen peroxide (10^{-4}M) [p<0.01]

Table 3: Effect of plants extract ointment on incision wound

Topical treatment	Epithelisation period (days)	Tensile strength (g)	Scar area (mm^2)	Hydroxyproline (mg/100mg tissue)
Control	26.7 ±1.2	287.5 ±17.3	54.2 ±3.8	7.22 ±0.34
A. nobilis	14.7 ±1.1[b]	420.9 ±19.8[a]	26.2 ±3.4[a]	10.11 ±0.45[c]
B. aegyptiaca	16.4 ±1.6 [a]	392.8 ±13.6 [a]	29.1 ±2.1 [a]	9.98 ±0.99 [a]
H. opposita	13.3 ±1.7 [a]	411 9 ±15.3 [a]	23.6 ±3.2 [b]	10.45 ±0.54 [b]
Nitrofurazone (2% ointment)	11.5 ±1.4[a]	428.2 ±21.3[a]	27.9 ±2.9[c]	11.7 ±0.45[b]

Values are mean ±SEM for six rats
Statistically significant difference in comparison with control group: [a]p<0.001, [b]p<0.01, [c]p<0.02.

Table 4: Effect of plants extract ointment on excision wound

Topical treatment	Percentage of closed excision wound area after days			
	4	7	15	21
Control	27.4 ±2.6	46.2 ±3.1	66.3 ±3.5	75.8 ±3.6
A. nobilis	36.5 ±2.9[a]	68.2 ±3.7[a]	94.7 ±4.8[b]	98.2 ±4.1[b]
B. aegyptiaca	33.9±3.1[a]	62.4±1.9[a]	88.9±1.8[a]	97.0±3.2[a]
H. opposita	34.8±2.9[a]	65.2±2.1[b]	92.5±3.0[b]	96.4±2.5[a]
Nitrofurazone (2% ointment)	37.2 ±3.1[a]	67.5 ±4.1[a]	96.9 ±4.7[b]	99.3 ±4.8[b]

Values are mean ±SEM for six rats.
Statistically significant difference in comparison with control group: [a]$p<0.01$, [b]$p<0.001$.

CONCLUSION

The results of the study indicated that the wound healing effects of *A. nobilis* and *H. opposita* could partly be attributed to their antibacterial and antioxidant properties as evidenced in their ability to inhibit bacteria growth and protect human fibroblast cells against oxidant injury. The wound healing effect of *B. aegyptiaca* however, could only be explained on the basis of its antioxidant properties. The increase in hydroxyproline content (indication of collagen synthesis) and tensile strength of healing tissue after the administration of the plant extracts confirmed the healing potential of the three plant species.

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