

ANTIMICROBIAL AND WOUND HEALING ACTIVITIES OF *CENTROSEMA PUBESCENS* (LEGUMINOSAE)

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ABSTRACT

Antimicrobial potential of *Centrosema pubescens* against a wide range of microorganisms was studied.

The ethanolic extract of this plant showed significant antibacterial and antifungal effect against most of the pathogenic organisms: *Bacillus subtilis*, , *Proteus mirabilis*, *Staphylococcus aureus*, *Escherichia coli*, *shigella dysenteriae*, *Proteus mirabilis*, *Salmonella typhi*, and two fungi *Candida albicans*, *Tinea capitis*, with especially good activity against the dermatophyte (*Tinea capitis*) and some infectious bacteria (*Escherichia coli* ,*Tinea capitis*, *Proteus mirabilis* and *Staphylococcus aureus*) with an MIC of 2.5 µg/disc.

Phytochemical screening of the extracts showed the presence of a number of bioactive constituents such as saponins, tannins, terpenes etc. wound healing activity test on albino rats with the crude ointment of the plant showed a certain degree of wound healing which is evident from wound contraction and increased tensile strength..

Key words: *Centrosema pubescens*, Phytochemical screening, wound healing, bioactive

1. INTRODUCTION

The manufacture and clinical evaluation of herbal remedies and/or their isolates have made it increasingly feasible to transform traditional medicine from an almost invisible trade into a modern industrial enterprise capable of making significant contribution to both healthcare deliveries and economic growth of developing countries ^[1].

Today, traditional medical practice has been recognized by the World health Organization (WHO) as a building block of primary healthcare ^[2]. But it emphasizes the fact that safety should be the overriding criterion in the selection of herbal remedies for use in healthcare ^[3]. Proof of safety should therefore take precedence over establishing efficacy. There is no longer any doubt regarding the value and potential of traditional remedies.

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 ^[4]. Repair of injured tissues occurs as a sequence of events, which includes inflammation, proliferation and migration of different cell types ^[5]. It is consented that reactive oxygen species (ROS) are deleterious to wound healing process due to the harmful effects on cells and tissues. Absorbable synthetic biomaterials are considered to be degraded via ROS ^[6]. Free-radical-scavenging enzymes (FRSE) are a cytoprotective enzymal group that has an essential role in the reduction, deactivation and removal of ROS as well as in the regulation of the wound healing process.

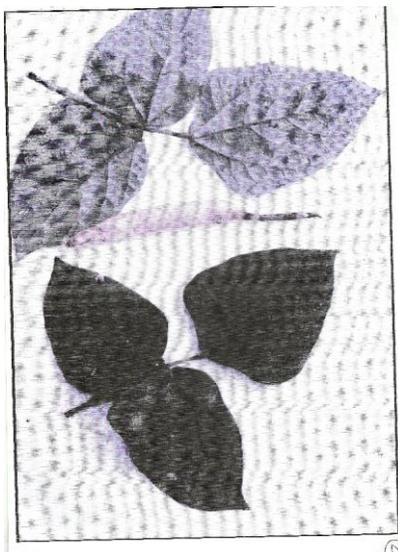
Centrosema pubescens is vigorous, trailing, twining and climbing perennial herb with trifoliolate leaves and is fairly drought tolerant ^[7]

Previous pharmacological work done on *C. pubescens* showed no insecticidal activity, the dose used was not stated, and the whole plant was used. Molluscicidal activity testing with the fresh entire plant, aqueous slurry of roots, leaves, fruits, showed the plant lacks molluscicidal activity. The extract of roots, fruits and leaves were also tested on female mice for oestrogenic effects, and no effect was recorded ^[8,9].

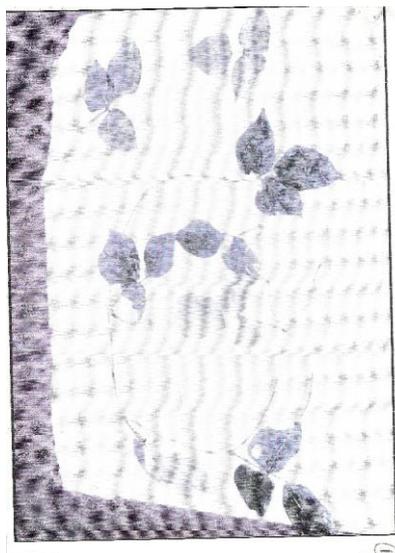
Much use has been made of the plants for the treatment of burns among the Ibibio Tribe of South South political Zone of Nigeria. However, there is no literature report to confirm its use in ethnomedicine.

The objective of this study was therefore, to evaluate the antimicrobial potential of *C. pubescens* against a wide range of microorganisms and to assess its potential in wound healing. Preliminary phytochemical screening was also conducted in order to identify the chemical profile of active substances.

2. MATERIALS AND METHODS



A. *Centrosema pubescens* showing the trifoliate leaves with pod



B *Centrosema pubescens* showing the creeping plant with trifoliate leaves

Plant material: The fresh plant was collected from a private garden of the first author, Dr Memfin Ekpo in Akwa Ibom State, Nigeria, in the month of July 2008. Identification and authentication was done by Dr (Mrs) U.A Essiet of the Department of Botany and Microbiology, University of Uyo, Uyo

Extraction: The solvents for extraction were 96% ethanol and distilled water at a volume ratio of 7:3 powdered leaves were macerated separately with sufficient quality of solvent and left for 72 hours (3 days). The extracts were filtered and concentrated to dryness *in vacuo* at 40°C.

Preliminary phytochemical screening: The plant material and extract were screened for the presence of various chemical constituents (alkaloids, tannins, cardiac glycosides, steroids, terpenoids, flavonoids, anthraquinones, phlobatannins, reducing sugars, anthocyanoside, and saponins) using standard procedures^[10, 11].

Animals: Albino rats (150-200g) of either sex at the laboratory animal centre of the University of Uyo, Nigeria were used. All the animals were housed in standard cages under laboratory condition in the Department of Pharmacology and Toxicology University of Uyo and were fed with pellet feed (Guinea feed) and water ad-labitum. All animal experiments were conducted in compliance with NIH guidelines for Care and use of laboratory animals. This study was approved by the Ethical committee of the Faculty of Pharmacy, University of Uyo, Nigeria.

Test microorganisms

Clinical isolates of *Shigella dysenteriae* (MTCC 1458), *Staphylococcus aureus* (MTCC 96), *Escherichia coli* (MTCC 443), *Salmonella typhi* (MTCC 733), *Bacillus subtilis* (MTCC 121), *proteus mirabilis* (MTCC 1429), *Candida albicans* (MTCC 183) and *Tinea capitis* (MTCC 7739) were obtained from the Department of Pharmaceutics and Pharmaceutical Microbiology, University of Uyo, Akwa Ibom.

Sterilization of materials

The Petri dishes and pipettes packed into metal canisters were appropriately sterilized in the hot air oven (Ov-335, Hareus) at 170°C for 1 h at each occasion. Solution of the extract and culture media were autoclaved at 121°C for 15 min.

Maintenance and standardization of test organisms

The microorganisms were maintained by weekly sub culturing on sabouraud agar slant. Before each experiment, the organism was activated by successive sub culturing and incubation. Standardization of the test microorganism was done according to previously reported method^[12, 13].

Antimicrobial activity

The agar diffusion method^[14] was used to evaluate the antimicrobial activity. Bacteria were cultured overnight at 37 °C in Mueller Hinton Broth (MHB, Oxoid) and fungi at 28 °C for 72 h in Potato Dextrose Broth (PDB, Oxoid) and used as inoculum. A final inoculum, using 100 μ l of suspension containing 10⁸ CFU/ml of bacteria and 10⁴ 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 μ l of 100 mg/ml (1 mg/disc) extracts placed on seeded agar. Gentamicin (10 μ g/disc), streptomycin (10 μ g/disc) and tetracycline (10 μ g/disc) were used as positive controls for bacteria and fluconazole (10 μ g/disc) and ketoconazole (10 μ g/disc) for fungi. The test plates were incubated at 37 °C for 24 h for bacteria and at 28 °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 μ g/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

Wound healing Activity

In the experiment, the rats were divided into three groups consisting of six rats each: 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 *Centrosema Pubescens* extract (CPE) ointment (100 mg/500mm²) topically on wound created on the dorsal back of rats daily till the wounds completely healed^[15].

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 mm² 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^[16] and hydroxyproline was measured using the method of Neuman and Logan^[17].

Incision wound model

Rats were anaesthetized with ether 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 taken and no local or systemic antimicrobial was used throughout the experiment^[18]. All the groups were treated in the same manner as mentioned in the case of the excision wound model. 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; CPE 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 CPE 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 ^[16].

Statistical analysis

Pharmacological data were subjected to statistical analysis using SPSS 17.0 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

3. RESULTS AND DISCUSSION

Table 1

Antimicrobial activity of *Centrosema pubescens* extract (1 µg/disc) inhibition zone in diameter (mm) standard antibiotic

	Microorganisms	Chloroform extract	Acetone extract	Ethanol extract	Water extract	Bactericide/ Fungicide S T G K F
	Bacterial strains	Zone of inhibition (mm)				
1	<i>Bacillus subtilis</i>	15.81 ±1.07	13.63±1.07	16.±1.86	15.67±0.75	26 33 32
2	<i>Escherichia coli</i>	17.33 ±1.07	17.±1.37	21 ±1.07	15 ±1.31	30 30 31
3	<i>Shigella dysenteriae</i>	12.67 ±0.75	15.5.±1.73	18.±1.07	16.67± 0.75	19 31 29
4	<i>Proteus mirabilis</i>	14.33±0.75	17.2±1	22±0.75	19±1	20 30 34
5	<i>Staphylococcus aureus</i>	17.5 ± 0.86	25.67±0.73	25.67±0.75	18.67±1.07	25 32 35
6	<i>Salmonella typhi</i>	16.9±1.31	11.67±0.75	18 ± 0	13.53± 0.75	29 39 30
	Fungal strains					
1	<i>Candida albicans</i>	17 ± 0	12 ± 0	19 ± 0	17 ± 0	24 23 17
2	<i>Tinea capitis</i>	14.33± 1.51	16.67±0.75	23.67±0.75	17.33± 1.07	32 24 23

Values are mean ± S.E.M of triplicates experiments

S: Streptomycin (10 µg/disc); T: Tetracycline (10 µg/disc); Zone of inhibition of solvents were the following: petroleum ether 8-10mm; chloroform, 7-9 mm; acetone, trace, and no inhibition in ethanol and water. The values of negative control were subtracted from the vales of samples and the corrected values are given

Table 2

The MIC values (µg/disc) of *Centrosema pubescens* extract against the microorganisms

	Microorganisms	Chloroform extract	Acetone extract	Ethanol extract	Water extract
	Bacteria strains	MIC(µg/disc)	MIC(µg/disc)	MIC(µg/disc)	MIC(µg/disc)
1	<i>Bacillus subtilis</i>	100	200	100	100
2	<i>Escherichia coli</i>	100	100	2.5	100
3	<i>Klebsiella pneumoniae</i>	200	100	10	100
4	<i>Proteus mirabilis</i>	100	100	2.5	5
5	<i>Staphylococcus aureus</i>	100	100	2.5	10
6	<i>Pseudomonas aeruginosa</i>	100	200	100	200
	Fungal strains				
1	<i>Candida albicans</i>	100	200	10	100
2	<i>Tinea capitis</i>	100	100	2.5	100

Table3

Effect of *Centrosema pubescens* extract ointment on incision wound

Topical treatment	Epithelization period (days)	Tensile strength (g)	Scar area (mm ²)
Control	26.5 ± 2.0	280.6 ± 16.1	60.09 ± 5.2
CPE ointment (100 µg/500mm ²)	17.7 ± 1.05*	379.5 ± 17.8*	34.75 ± 2.3*
Nitrofurazone (2%) ointment	12.5 ± 0.25*	408.8 ± 9.7*	30.5 ± 1.9*

Values are mean ± S. E. M for six rats,

* statistically significant difference in comparison with control group P<0.05

The disc diffusion method was used to determine zones of inhibition of *C. pubescens* extracts (organic and aqueous). The plant showed significant antibacterial and antifungal activity against almost all the organisms (Table 1) and especially good activity was found against *Staphylococcus aureus* and dermatophytes. However, the petroleum ether extracts of this plant showed little antimicrobial activity. Significant antimicrobial activity was observed in ethanolic and aqueous extracts. Amongst the test organisms used, *Staphylococcus aureus* was found to be most sensitive; *Tinea capitis* came next, followed by *Proteus mirabilis*, *Escherichia coli*, *Shigella dysenteriae*, *Salmonella typhi*, *Candida albicans*, and *Bacillus subtilis*. Increased inhibition was found at higher levels of extract concentration. MICs of these extracts are summarized in Table 2. Some of the extracts like the ethanolic extract of *C. pubescens* gave very low MIC values, and inhibited the growth of *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus* and *Trichophyton rubrum* with a concentration of 2.5 µg/disc.

Preliminary phytochemical screening of *C. pubescens* showed the presence of saponins, terpenes, sesquiterpenes glycosides, alkaloids and absence of anthraquinones and flavonoids. The antimicrobial activity could be due to the

presence of terpenes ^[19]. Since MIC of *C.pubescens* against *Staphylococcus aureus* was substantially low (2.5 µg/disc), the wound healing activity was done on it. The wound healing activity results showed that upon application of CPE ointment there was a decrease in the epithelization period, along with a visibly decreased scar area (Table 3). There was also a significant increase in the tensile strength and hydroxyproline content (Table 3).The crude extract of CPE significantly stimulated wound contraction.

4. CONCLUSION

Thus, the plant extract might be useful as a wound healing agent. The potent wound healing capacity of CPE as shown from the wound contraction and increased tensile strength has thus validated the ethnobotanical claim.

5. REFERENCES

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