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ANTIBACTERIAL, ANTI-INFLAMMATORY AND ANTIOXIDANT PROPERTIES OF *GOUANIA LONGIPETALA* HEMSL

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ABSTRACT

Background: *Gouania longipetala* Hemsl. (Rhamnaceae), has ethnomedicinal use in the treatment of wounds, gonorrhoea, abdominal pain, lumbago, ophthalmia, conjunctivitis, and rickets. However, no scientific investigation of these effects has been established. Our aim was to verify the antibacterial, antioxidant and anti-inflammatory effects of the stem of *Gouania longipetala.*

Methods: The antibacterial effect of the 70% ethanolic extract was studied using the agar well diffusion and micro dilution assays. The extract was also tested *in vitro* for its free radical scavenging effect, total antioxidant capacity and total phenol content. The extract activity in acute inflammation was assessed using the carrageenan-induced foot oedema in chicks.

Results: The 70% ethanollic extract showed antibacterial effect against the test organisms with the lowest MIC of 125 µg/mL against *Bacillus subtilis* NCTC 10073. The total antioxidant capacity was 0.80 mg/g ascorbic acid equivalent. The extract demonstrated free radical scavenging activity yielding IC_{50} value of 0.004 mg/mL. In the total phenol content assay, tannic acid equivalent was 52.02mg/g and correlated highly with its total antioxidant capacity. In the anti-inflammatory assay, the extract gave a maximal inhibitory effect of total oedema by 93.78% at 300 mg/kg.

Conclusions: The results indicated that the extract possessed antibacterial, antioxidant and anti-inflammatory properties and may give credence to some of its ethnopharmacological uses.

INTRODUCTION: Infectious diseases, caused by bacteria, fungi, viruses and parasites, and inflammatory disorders continue to pose a public health threat particularly in developing countries. Africa's biodiversity has the potential to be a major resource for developing innovative therapeutic agents. Medicinal plants especially, those used in ethnomedicine still play important roles in the management of various ailments including infectious diseases and inflammatory disorders.

Gouania longipetala Hemsl. (Rhamnaceae) is a scandent shrub or liane mainly present in closed-forests and jungle regrowths. In traditional medicine, it is used to treat various ailments such as venereal diseases, oliguria, gout, abdominal pain, lumbago, wounds and rickets. The leaf juices are used as drops or wash for sore eyes, conjunctivitis, iritis, opthalmia and trachoma [1]. The leaf is used for dropsy, swellings, stomach troubles, as a genital stimulant/depressant, laxative, as a febrifuge and as an antidote for venomous stings, bites, etc [2].

Personal communication with some herbalists in the Ashanti Region, Ghana indicates the stem decoction is used for the treatment of various infectious ailments, skin disorders and as an analgesic. The plant is considered a general "cure-all". However, no scientific investigation to the best of our knowledge of these effects has been established. Furthermore, some of the ailments mentioned are inflammatory and infectious in nature and accompanied with oxidative stress. Therefore, the present study aimed to verify the anti-inflammatory, antibacterial and antioxidant effects of the stem of the plant.

MATERIALS AND METHODS:

Plant material collection and extract preparation: The stem of *Gouania longipetala* Hemsl. was collected in the Ashanti Region of Ghana in June 2009. A voucher specimen (No. KNUST/HM1/2010/S005) has been retained in the herbarium of the Department of Pharmacognosy, College of Health Sciences, Kwame Nkrumah University of Science and Technology. The dried and ground stem of *Gouania longipetala* (50 g) was soxhlet-extracted with 0.5 L 70% ethanol for 12 h.The extract was evaporated to a brown syrupy mass under reduced pressure in a rotary evaporator, air-dried and kept in a dessicator till required. The yield obtained was 11.4%w/w.

Chemicals: All chemicals used were of analytical grade and purchased from Sigma Aldrich Co Ltd. Irvine, UK. Organic solvents were also of analytical grade and purchased from BDH Laboratory Supplies (England). Precoated aluminium-backed silica gel F_{254} TLC plates (0.25 mm thickness), product code OB 315394 were purchased from Merck KGaA, Germany. Carrageenan sodium salt was purchased from Sigma Chemicals, St. Louis, MO, USA. Diclofenac and dexamethasone were purchased from Troge, Hamburg, Germany and Pharm-Inter, Brussels, Belgium respectively.

Test organisms: The Gram-positive bacteria species used were *Enterococcus faecalis* ATCC 29212, *Bacillus thurigiensis* ATCC 13838, *Staphylococcus aureus* ATCC 25923 and *Bacilllus subtilis* NCTC 10073. Gram–negative bacteria species used were *Salmonella enterica* (*Salmonella typhi*) NCTC 6017*, Escherichia coli* NCTC 9002*, Proteus vulgaris* NCTC 4635 and *Pseudomonas aeruginosa* ATCC 27853.

The test organisms were obtained from the University of Ghana Medical School.

Animals: The animals used in this study were one-day post-hatch cockerels (*Gallus gallus*; strain shaver 579). They were maintained in stainless steel cages at 29^{o}C on a 12 hour light-dark cycle. Feed (Chick Mash, GAFCO, Ghana) and water were available *ad libitum.* Chicks were tested at 7 days of age. All studies were carried out by using 5 chicks in each group. All procedures and techniques used in these studies were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication No.85-23, revised 1985) and were approved by the Ethics Committee of the Department of Pharmacology, KNUST.

Antimicrobial Assays

Agar Well Diffusion Method: The antimicrobial activities of the different extracts were determined using the Vanden Berghe and Vlietnick agar well diffusion method [3]. Inocula of the microorganisms were prepared from 24 h Mueller-Hinton broth cultures and suspensions were adjusted to 10^5 CFU/ml following a 0.5 McFarland turbidity standard. Wells of 9 mm diameter were made in 20 mL nutrient agar (Oxoid) inoculated with 20µL of a suspension of test organisms. The wells were filled with 100 µL of the extracts (5 mg/mL), and incubated at 37^{o}C for 24 hours, after which they were examined for zones of inhibition. Amoxycillin (200 µg/mL) was included as positive control. The test results are the mean of 3 replicates.

Micro Dilution Assay: Minimal inhibitory concentration (MIC) was considered as the least concentration of the extract that inhibited visible growth of the test organism was determined based on a micro-well dilution method [4]. The inocula of microorganisms were prepared from 24-hour Mueller Hinton broth cultures.

The 96-well sterile plates were prepared by dispensing into each well 100 µL of double strength nutrient broth and 100 µL of plant extract (7.8 µg/mL - 1000 µg/mL) together with 20 µL of the inoculum (10^5 CFU/mL). The microplates were incubated at 37^{o}C for 24 hours. Bacterial growth was determined by adding 20 µL of a

5% solution of *p*-iodonitrotetrazolium salt (MTT) and incubating for further 30 minutes. Dark wells indicated the presence of microorganisms as the dehydrogenase enzymes in the live bacteria reacts to form a dark complex with the *p*-iodonitrotetrazolium salt. Amoxycillin (7.8 µg/mL - 1000 µg/mL) was included as positive control. All experiments were carried out in 3 replicates.

Anti-inflammatory Assay

Carrageenan-induced Oedema: Anti-inflammatory activity was determined by the method of Roach and Sufka [5], modified by Woode *et al.,* [6]. Three groups of cockerels containing 5 chicks in each group received the plant extract (30, 100 and 300 mg/kg, *p.o.*), the standard groups received diclofenac (5, 15 and 50 mg/kg, i.p.) and dexamethasone (1,3 and 10 mg/kg, i.p.) and the control animals received the vehicle only. All the treatments were given 30 minutes for i.p. route and 1 hour for p.o prior to the subplantar injection of carrageenan (10 µL of a 2% w/v solution). Foot volumes were measured by water displacement plethysmography at 0, 1, 2, 3, 4, and 5hour [7].

Statistical Analysis: Raw scores for right foot volumes were individually normalized as percentage of change from their values at time 0 then averaged for each treatment group. The time-course curves for foot volume was subjected to two-way (treatment × time) repeated measures analysis of variance with Bonferroni's *post hoc t* test. Total foot volume for each treatment was calculated in arbitrary unit as the area under the curve (AUC). The inhibition percentage of oedema was calculated for each animal group in comparison with its vehicle-treated group.

Differences in AUCs were analyzed by ANOVA followed by Newman-Keul's *post hoc t* test. ED_{50} (dose responsible for 50% of the maximal effect) for each drug was determined by using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation:

$$Y = \frac{a+(b-a)}{[1+10^{(LogED_{50}-X)}]}$$

Where X is the logarithm of dose and Y is the response. Y starts at a (the bottom) and goes to b (the top) with a sigmoid shape.

GraphPad Prism for Windows version 5.00 (GraphPad Software, San Diego, CA, USA) was used for all statistical analysis and ED_{50} determinations. $P < 0.05$ was considered statistically significant.

Antioxidant Assays:

Rapid screening for Antioxidants: Extract (5 µL, 0.1 mg/mL) was monitored initially for antioxidant activity on TLC (solvent system: chloroform, methanol 9:1) using 20 mg/L of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol and antioxidant compounds in the extracts gave clear zones against a purple background [8].

Free Radical Scavenging Activity of the Ethanolic Extracts: The stable 1, 1-diphenyl-2-picryl hydrazyl radical(DPPH) was used for determining the free radical scavenging activity of the extract [9]. Different concentrations of the extracts (3, 1.5, 0.75 and 0.375 mg/mL) were added at an equal volume, to methanolic solution of DPPH (20 mg/L). After 30 minutes, the absorbance was measured at 517 nm. Inhibition of radical scavenging was calculated according to the following equation.

$$\text{DPPH scavenging activity (\%)} = [(A_o-A_1)/A_o] \times 100$$

With A_o being the absorbance of the control and A_1 is the absorbance in the presence of the test sample.

IC_{50} values represent the concentration of sample, which is required to scavenge 50% of the DPPH free radicals. The test results are the mean of 3 replicates.

Total Phenols Determination: Total phenols were determined by Folin-Ciocalteau's reagent using tannic acid as a standard [10]. Different doses were tested for both tannic acid and the plant extracts: tannic acid (0.03-0.1 mg/mL); extract (0.125-2.5 mg/mL). 1mL of plant extract or tannic acid (standard phenolic compound) was mixed with 1mL Folin-Ciocalteau's reagent and aqueous Na_2CO_3 (1 mL, 2%). The absorbance of reaction was measured at 760 nm after 2 hours of incubation at room temperature. Results were expressed as tannic acid equivalents (mg/g of dry mass).

Total Antioxidant Capacity: Total antioxidant capacity of extract was determined as described by Prieto [11]. Ascorbic acid served as positive control. Three mL of

reagent solution (0.6 M H_2SO_4, 28 mM Na_2HPO_4 and 4mM ammonium molybdate) was pipetted into test tubes. A total of 1mL extract (0.125-2.5 mg/mL) was then added to the same test tubes, and incubated at 95^{o}C for 90 min and centrifuged. Absorbance of the supernatant was determined at 695 nm. Total antioxidant values are expressed in terms of ascorbic acid equivalent (mg/g of dry mass).

Phytochemical Screening: The preliminary phyto-chemical screening was performed by the standard methods [12].

RESULTS:

Antimicrobial Effects:

Agar Well Diffusion: The extract produced varying zones of growth inhibition against all the test organisms. The largest diameter of zone of inhibition, 24.00 mm was given by the extract against *Bacillus subtilis* NCTC 10073 The least diameter of zone of inhibition,12.68 mm was against *Proteus vulgaris* NCTC 4635 (**Table 1**).

Micro-Dilution Assay: Minimum inhibitory concentrations were observed for the extracts that showed activity in the agar well diffusion assay. The extracts showed activity with MICs from 125 µg/mL to more than 1000 µg/mL (Table 1).

TABLE 1: ANTIBACTERIAL ACTIVITY OF 70% ETHANOLIC EXTRACT OF *GOUANIA LONGIPETALA* GROUND STEM AGAINST BACTERIA

Microorganisms	ZOI ±SEM (mm)	MIC (µg/mL)
Gram positive		
Bacillus subtilis NCTC 10073	24.00±1.00	125
Bacillus thurigiensis ATCC 13838	16.33±1.15	1000
Staphylococcus aureus ATCC 25923	16.33±1.15	500
Enterococcus faecalis ATCC 29212	14.33±0.57	1000
Gram negative		
Proteus vulgaris NCTC 4635	12.68±0.58	>1000
Pseudomonas aeruginosa ATCC 27853	14.67±0.58	>1000
Salmonella enterica (Salmonella typhi) NCTC 6017	14.33±0.58	500
Escherichia coli NCTC 9002	15.00±0.00	>1000

ZOI = Zone of Inhibition, MIC = Minimum Inhibitory Concentration, SEM = Standard Error of Mean, NT = Not Tested.

Anti-inflammatory Activity:

Carrageenan-induced Oedema: Figure 1 shows the time course curve and AUC for the effect of diclofenac, dexamethasone, *Gouania longipetala* stem on carrageenan-induced oedema in chicks. When compared with the control, the extract, diclofenac and dexamethasone significantly reduced the foot oedema. The effect was dose-dependent for the extract.

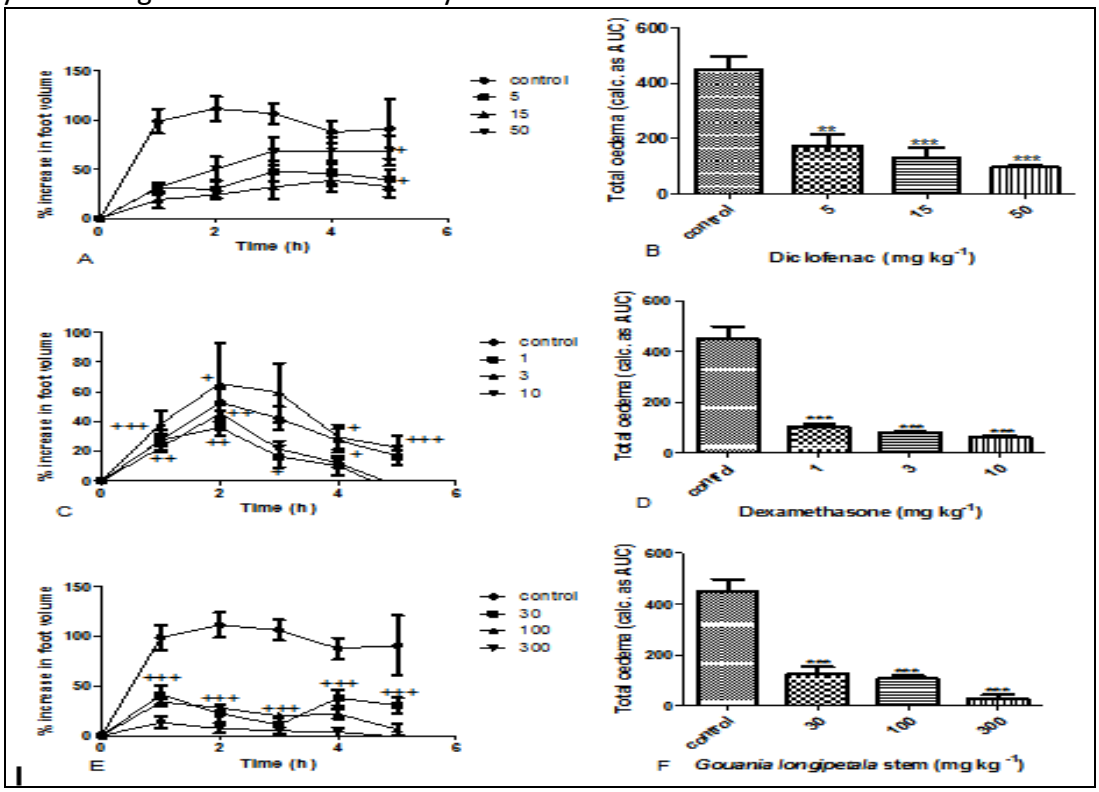

Figure 1: Effect of diclofenac (10 – 100 mg/kg; i.p.), dexamethasone (1 – 10 mg/kg; i.p.) and extract (30 – 300 mg/kg; p.o) on time course curve (A, C, F) and the total oedema response (B, D , F, respectively) in carrageenan-induced oedema in chicks

Values are means ± SEM. (n =5). $^{+++}P < 0.0001$; $^{++}P < 0.001$; $^{+}P < 0.05$ compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni's *post hoc* test). $^{***}P < 0.0001$; $^{**}P < 0.001$; $^{*}P < 0.05$ compared to vehicle-treated group (One-way ANOVA followed by Newman-Keul's *post hoc* test.

Based on the ED_{50} values (**Table 2**) obtained from the dose response curves (Figure 1), the standard drugs, diclofenac and dexamethasone were four to five times as effective as *Gouania longipetala* stem as an anti-inflammatory agent.

TABLE 2: ED_{50} VALUES FOR THE EFFECT OF *GOUANIA LONGIPETALA* STEM, DICLOFENAC AND DEXAMETHASONE IN CARRAGEENAN-INDUCED OEDEMA IN CHICKS

Drug	ED_{50} (mg/kg)
Gouania longipetala stem	15.45
Dexamethasone	3.43
Diclofenac	4.42

Antioxidant Effects:

TLC-screening for Antioxidant Compounds: The active compounds were detected as yellow spots on a violet background. The extract was subjected to further testing.

Free Radical Scavenging Activity: The extract reduced DPPH to the yellow coloured product, diphenylpicrylhydrazine, and the absorbance at 517 nm declined. The extract as well as n-Propyl gallate exhibited concentration dependent scavenging activity. The rank order of potencies, as defined by EC_{50} in mg/mL is shown in Table 3. The extract was found to be equally potent as the n-propyl gallate.

TABLE 3: IC_{50} VALUES (MG/ML) FOR FREE RADICAL SCAVENGING ACTIVITY BY EXTRACT

Extract	IC_{50} DPPH
Gouania longipetala stem	0.004
Propyl gallate	0.0039

Total Phenol Contents: Total phenol content is reported as tannic acid equivalents by reference to the standard curve, y= 0.1465x + 0.2223. The total phenol was 52.02 mg/g dry weight in the stem extract of *Gouania longipetala* stem.

Total Antioxidant Capacity: Total antioxidant capacity is reported as ascorbic acid equivalents by reference to the standard curve, y= 2.314x + 0.6330. The total antioxidant capacity of the stem extract of *Gouania longipetala* was 0.804 mg/g dry weight

Phytochemical Screening: The results of the phytochemical screening on the powdered plant parts are as shown in **Table 4**.

TABLE 4: RESULTS OF THE PHYTOCHEMICAL SCREENING FOR *GOUANIA LONGIPETALA* GROUND STEM

Plant secondary metabolites	*Gouania longipetala* stem
Phenolics	+
Reducing sugars	+
Alkaloids	-
Phytosterols	+
Triterpenoids	+
Saponins	+
Flavonoids	+

- absent, + = present

DISCUSSION: The present study establishes the antibacterial, antioxidant and anti-inflammatory effects of the 70% ethanolic extract of the stem of *Gouania longipetala*. The extract was active against all 8 microorganisms employed in the antibacterial assay with MIC ranging 125 µg/mL to more than 1000 µg/mL. The largest diameter of zone of inhibition 24.00 mm observed for the agar well diffusion method was against *Bacilllus subtilis* NCTC 10073 (Table 1). The extract recorded a very low MIC, 125 µg/mL against the same microorganism (Table 1).

Bacterial infections are implicated among the conditions treated with *Gouania longipetala* in traditional medicine. These ailments include chronic ulcers, trachoma, conjunctivitis, iritis and venereal diseases. The role of anti-infectives in the management of these conditions has been well documented.

Different classes of secondary metabolites have been shown to possess antimicrobial effect. These include alkaloids, tannins, saponosides, diterpenoids, triterpenoids and steroids [13]. Phytochemical tests established the presence of one or more of these metabolites and may be responsible for the antibacterial effects observed (Table 4).

Carrageenan-induced oedema, an animal model of acute inflammation, involves the synthesis and/or release of histamine, serotonin, kinins, prostaglandins and cyclooxygenase-2 [14]. These mediators cause the

symptoms of pain, oedema, redness, fever and loss of function. Inhibition of these mediators normally relieves the inflammation. This study has shown that the stem of *Gouania longipetala* gave a maximal inhibitory effect of total oedema by 93.78% at 300 mg/kg. Consequently, their anti-inflammatory activity is backed by inhibiting the synthesis, release or action of the inflammatory mediators.

Glycosides and flavonoids have been reported to exhibit anti-inflammatory activities by inhibiting the release and/or action of the mediators of inflammation. The presence of these secondary metabolites in *Gouania longipetala* may be responsible for the observed anti-inflammatory activity [15-16].

Free radical formation occurs due to both enzymatic and non-enzymatic reactions. These reactions include phagocytosis, prostaglandin synthesis, non-enzymatic reactions of oxygen with organic compounds and those initiated by ionizing radiations [17]. Free radicals are part of the defense mechanisms against infections. However, excess free radicals may damage tissues. Antioxidants are capable of counteracting the damaging effects of free radicals. The scavenging effect of *Gouania longipetala* in the DPPH assay was 0.004 mg/mL. The total antioxidant capacity expressed as ascorbic acid equivalent was 0.804 mg/g dry weight of extract. In the total phenol content, the extract gave a value of 52.0174 mg/g dry weight of extract expressed as tannic acid equivalents. The results obtained suggest the potential of the extracts as antioxidant agents.

Phenolic and flavonoid components are reported to function as good electron and hydrogen atom donors and therefore, are able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products [18-19]. Thus, the presence of these secondary plant metabolites in the plant may be responsible for the antioxidant effects observed. Phenolic content of the extract correlated highly, R = 0.93, with their total antioxidant capacities.

CONCLUSIONS: In conclusion, the extract of *Gouania longipetala* stem possesses antibacterial, antioxidant and anti-inflammatory properties which are likely to contribute to their beneficial effect in the various ailments mentioned early on.

Further isolation of the various compounds responsible for these activities is in progress in our laboratories.

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