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Anti-inflammatory, antioxidant and antimicrobial activities of the stem bark of *Psydrax subcordata*

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ABSTRACT

Psydrax subcordata (DC.) Bridson (Rubiaceae) is used by traditional medical practitioners in Ghana for treating piles, haemorrhoids, enteritis and stomach ulcer. This study investigated the anti-inflammatory, antioxidant and antimicrobial properties of the methanol stem bark extract of Psydrax subcordata. The extract at 30, 100 and 300 mg/kg body weight, showed a dose-dependent reduction ($p < 0.001$) in the carrageenan-induced footpad oedema in seven-day old chicks with of 61.21 ± 2.16% at the highest dose. Considerable antimicrobial activity was observed against selected microorganisms with the highest sensitivity towards Streptococcus pyogenes. The extract exhibited moderate DPPH scavenging activity with an IC_{50} of 226.5 ± 0.86 µg/ml compared to ascorbic acid (IC_{50} of 24.37± 0.33µg/ml). The findings of this study has highlighted the anti-inflammatory, antioxidant and antimicrobial properties of Psydrax subcordata stem bark and therefore gives scientific credence to its use in folklore medicine.

Key words: *Psydrax subcordata*, anti-inflammatory, antioxidant, antimicrobial

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INTRODUCTION

Inflammation is an important defensive response of the body to noxious stimuli such as toxins, and pathogens. Though a protective mechanism, unregulated or exaggerated inflammation can induce or aggravate a number of diseases. The production of copious amounts of reactive oxygen species (ROS) during inflammatory process has also been shown to underline the pathogenesis of many chronic disease conditions including rheumatoid arthritis, cancer, cardiovascular and neurodegenerative diseases [1, 2]. Anti-inflammatory and antioxidant drugs are therefore pivotal in preventing and treating many human diseases. Unfortunately, the currently used anti-inflammatory drugs such as the Non-Steroidal Anti-inflammatory Drugs are associated with several adverse effects limiting their use [3]. Consequently, the development of potent anti-inflammatory drugs that possess fewer adverse effects is necessary.

In Ghana, the stem bark of *Psydrax subcordata* (DC.) Bridson (Rubiaceae) is used traditionally for the treatment of haemorrhoids, piles, stomach ulcer and intestinal infections [4], suggesting possible antimicrobial and anti-inflammatory properties of the plant. Despite the numerous claims of biological activity by traditional healers, only three reports exist on the bioactivity and phytochemical constituents from this plant. Previous studies reported the anti-plasmodial and antimicrobial activities of the stem bark and identified the major constituents of the stem bark and fruits as iridoid type compounds [5, 6]. Continuing our search for potent antimicrobial, anti-inflammatory and

antioxidant Ghanaian medicinal plants [7-10], we investigated the stem bark of *Psydrax subcordata* for anti-inflammatory, antioxidant and antimicrobial activities.

MATERIALS AND METHODS

Plant Material Collection and Processing

The stem barks of *Psydrax subcordata* (Rubiaceae) were collected from Kwahu Asakraka (N 06º 38, 045 W 000º 41, 719) in the Eastern region of Ghana. The plant was authenticated by Mr. Clifford Asare, of the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Ghana where a voucher specimen was deposited (voucher number: KNUST/HM/2016/12). The barks were cut into pieces and shade dried for 14 days. They were then pulverized into powder.

Extraction

The powdered bark (3 kg) was Soxhlet extracted with 8 L of methanol for 6 hours. A concentrate of the extract was obtained by evaporation on a rotary evaporator (R-114, Buchi, Switzerland) and further dried in a desiccator to afford a dark brown gummy extract (yield = 15.6 % $^{w}/_{w}$).

Drugs and Chemicals

All chemicals except standard drugs were bought from Sigma Aldrich Co Ltd., Irvin, UK. The organic solvents used were of analytical grade purchased from BDH laboratory supplies (England). Diclofenac sodium, dexamethasone sodium phosphate, carrageenan, DPPH, tannic acid, Folin-Ciocalteau's reagent, ascorbic acid, ammonium molybdate, disodium hydrogen phosphate (Na_2HPO_4), ciproflaxacin and nutrient agar were used in the experiments.

Animals

Cockerels (*Gallus gallus*, strain Shaver 579; weight: 45-50 g) were obtained 1-day post hatch from Akate farms, Kumasi, Ghana and housed in stainless steel cages (34 x 57 x 18 cm^3) at a population density of 5 chicks per cage. Food (Chick Mash, GAFCO, Tema, Ghana) and water were available *ad libitum.* Room temperature was maintained at 28 ºC. The experiment procedures were done in accordance with the National Institute of Health guidelines for care and use of laboratory animals (NIH Department of Health Services publication number: 83-23, revised 1985; Committee, 2011) and were approved by the Ethics Committee, Department of Pharmacology, College of Health Sciences of the Kwame Nkrumah University of Science and Technology.

Microorganisms

The following typed and clinical strain microorganisms were obtained from the Department of Pharmaceutics, KNUST: two Gram positives (*Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (Clinical strain)), two Gram negatives (*Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC4853)) and one fungus; *Candida albicans* (Clinical strain).

Anti-inflammatory activity

Carrageenan-induced foot oedema in chicks

The anti-inflammatory activity was assessed using the carrageenan-induced footpad oedema in 7-day old chick model with modification [11]. The initial foot thicknesses of the chicks were measured and chicks were randomly placed into 10 groups consisting of 5 chicks each. The treatment groups received oral (p.o.) doses of PSE at 30, 100 or 300 mg/kg suspended in 2 % tragacanth. The positive control groups received dexamethasone (0.3, 1, and 3.0 mg/kg) and diclofenac (10, 30, and 100 mg/kg) administered intra-peritoneally (i.p.) while the negative control group received the vehicle, 2 % tragacanth in normal saline (p.o.). Carrageenan (10 µL of 2 %$^{w}/_{v}$ suspension in saline) was injected sub-plantar into the right footpads of the chicks. The increase of foot thickness was recorded at an hourly interval for 5 hours using vernier calliper. The oedema induced by the inflammation was quantified by measuring the difference in foot thickness before carrageenan injection and at the various time intervals. The values were individually normalized as a percentage change in foot oedema relative to the corresponding values at time zero, and then averaged for the respective treatment groups. Total foot thickness for each treatment was calculated in arbitrary unit as the area under the curve (AUC). The percentage inhibition for each treatment was determined using the equation:

$$\% \text{ inhibition of oedema} = \left(\frac{AUC\ control - AUC\ treatment}{AUC\ control}\right) \times 100$$

Antimicrobial activity

Antimicrobial activity was determined by the agar well diffusion method [12]. The crude extract of *P. subcordata* (PSE), (20 mg/ml, 10 mg/ml and 5 mg/ml) were prepared using sterile water. Ciprofloxacin (5 mg/ml) and ketoconazole (5 mg/ml) were used as the positive controls. Twenty millilitres (20 mL) of sterile molten agar was seeded with 0.1 mL of overnight broth culture of test organism with the aid of a sterile pipette, swirled to mix uniformly and poured into a sterile petri dish. The agar was allowed to solidify after which four wells (holes) of 8 mm depth were drilled in the agar by the use of number five cork borer, ensuring proper distribution of holes in the periphery. The wells of each plate were filled with 100 µL of the test samples or positive control. The plates were then left at room temperature for 1 hour to allow diffusion of the test samples and incubated at 37 ^{0}C for 24 hours. The assay was performed in triplicates for all test samples. The results of the antimicrobial activities were expressed as the diameter of the zones of inhibition in millimetres (mm).

Antioxidant Activity

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH is a free stable radical which can receive a proton (hydrogen) from a suitable free radical scavenger (reducing agent) to form the non-radical DPPH-H. [13]. The experiment was carried out as previously described in literature with few modifications [10] Different concentrations of PSE (500, 250, 125, 62.5 and 31.25 µg/ml) was mixed with methanol solution of DPPH (20 g/L) in a ratio of 1:3. The mixtures were kept in the dark at room temperature for 30 minutes, and the amount of free radical scavenged was quantified by measuring the absorbance of the residual DPPH at 517 nm with a Cecil UV/VIS Spectrophotometer (Model: CE 7200, Milton, England). Methanol was used as a negative control and ascorbic acid (100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/ml) as the positive control. All the tests were performed in triplicate and the % DPPH scavenging activity was determined by the following formula:

$$\% \text{ DPPH scavenging activity} = \left(\frac{\text{ABSORBANCE } control - \text{ABSORBANCE } test}{\text{ABSORBANCE } control}\right) \times 100$$

The concentration required to scavenge 50 % of the DPPH molecule (IC_{50}) was estimated using an iterative computer least square method, with the following non-linear regression (three- parameter logistic) equation:

$$Y = \frac{a - (b-a)}{1+10^{(\log EC50 - X)}}$$

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

Total antioxidant capacity (TAC) assay

The assay was based on the reduction of molybdenum, Mo^{+6} to Mo^{+5} by the antioxidant substance and the formation of a green phosphate-molybdate (Mo^{+5}) complex at acidic pH [14]. Different concentrations of PSE (500, 250, 125, 62.5 and 31.25 µg/ml, 1m L) were mixed with 3 mL of the reagent solution (0.6 M H_2SO_4, 28 mM Na_2HPO_4 and 4 mM Ammonium molybdate) and incubated at 95 ºC for 90 minutes. The solutions were then cooled to room temperature and the UV absorbances were measured at 695 nm. Ascorbic acid (100, 50, 25, 12.5, 6.25 and 3.125 µg/ml) was used as standard to construct a calibration curve from which the TAC of extract was extrapolated. The antioxidant capacity was thus estimated as ascorbic acid equivalent (AAE) in milligram of per gram (mg/g) of dried extract.

Total phenol content

The total phenolic content in PSE was determined using the Folin-Ciocalteau reagent [15]. Different concentrations (500, 250, 125, 62.5 and 31.25 µg/ml) of PSE (1 mL) were mixed with Folin-Ciocalteau reagent (0.5 mL) and 2 % ^{w}/v of Na_2CO_3 (2.5 mL ^{w}/v). The mixture was incubated at room temperature for 2 hours, after which it was centrifuged at 3000 rpm for 10 minutes to get a clear supernatant. The absorbance of the supernatant was measured at 760 nm using a Cecil UV/VIS spectrophotometer (Model: CE 7200, Milton, England). Tannic acid (50, 25, 12.5, 6.25, 3.125 µg/ml) was used reference drug while distilled water served as the negative control. All experiments were carried out in triplicate. The total phenolic content of extract was estimated as tannic acid equivalents (TAE) in mg/g of dried extract.

RESULTS AND DISCUSSION

In this study, the stem bark of *P. subcordata* was investigated for anti-inflammatory, antioxidant and antimicrobial activity based on its claimed medicinal properties in traditional medicine.

Following the induction of oedema in chicks, the foot thickness of the negative control group increased gradually and reached its peak within 2-3 hours and was sustained during the 5th hour of observation (Fig. 1). The standard drugs, diclofenac and dexamethasone exhibited a dose and time dependent anti-inflammatory effect as expected and in line with our previous reports (Figs. 2- 3) [7-10, 16] . As demonstrated by the time course curves of groups treated with PSE (30 – 300 mg/kg body weight) (Fig. 1), a drastic reduction of peak oedema response during the 2nd hour and slowly throughout the period of the experiment was observed. This effect was dose dependent and significant ($p < 0.001$) compared to the negative control group. From the analysis of AUCs, the highest dose of PSE inhibited oedema by 61.2 %. At 100 mg/kg body weight, PSE exhibited an anti-inflammatory effect (53% inhibition of oedema) which was comparable to diclofenac (55 %) at the same dose (Table 1). Inflammation induced by carrageenan has been described as a biphasic event which begins with the release of histamine, bradykinin and serotonin in the early phase (0-1 h) and prostaglandins during the later phase (1-6 h) [17, 18]. From the time course curves of treatment groups, it may be postulated that the anti-inflammatory activity of *P. subcordata* stem bark extract may be by the inhibition of mediators involved in the later phase of the inflammatory process since reduction of footpad oedema was observed from the 2nd to the 5th hour. On the basis of these results, the traditional use of the plant in the treatment of inflammatory conditions is scientifically justified to an extent.

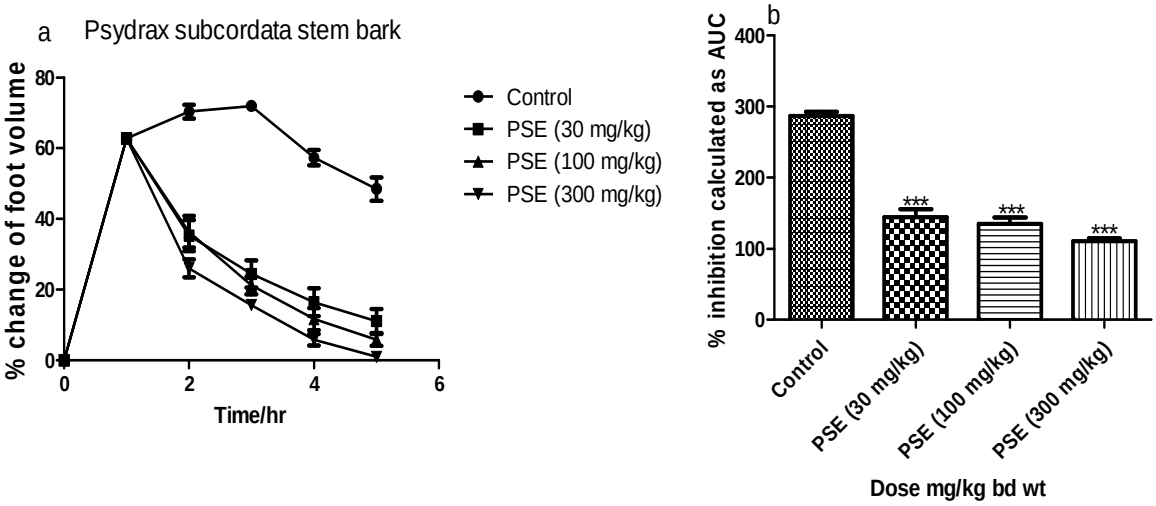

Figure 1: (a) Effect of PSE (30, 100, 300 mg kg-1 *p.o*), on the progression of inflammation (b) Total oedema response, calculated AUCs; values are mean ± SEM (n=5) *** p < 0.001 compared to vehicle - treated group

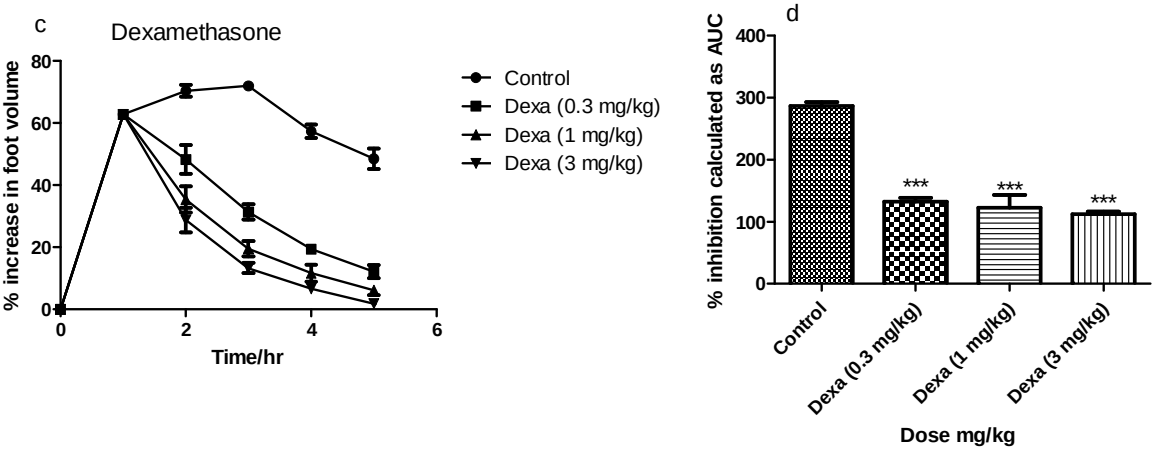

Figure 2: (c) Effect of Dexamethasone (0.3, 1, 3 mg/kg; *i.p.*) on the progression of inflammation (d) Total oedema response, calculated as AUCs; values are mean ± SEM (n=5). ***P < 0.001; compared to vehicle-treated group

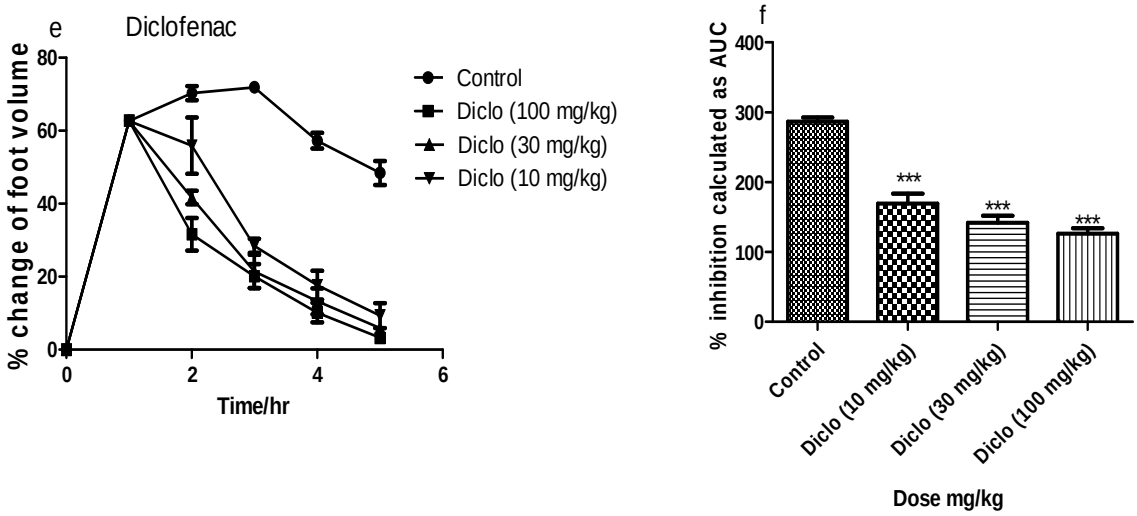

Figure 3: (e) Effect of Diclofenac (10-100 mg kg-1; i.*p.*) on the progression of inflammation (f) Total oedema response calculated as AUCs; values are mean ± SEM (n=5). ***P < 0.001 compared to vehicle-treated group

Table 1: Anti-inflammatory activity of PSE and the standard drugs

Extract/ Standard drug	Dose (mg/kg body weight)	% Inhibition of oedema
PSE	300	61.21 ± 2.16
	100	53.00 ± 2.67
	30	49.54 ± 3.92
Diclofenac	100	55.73 ± 3.22
	30	50.13 ± 4.17
	10	41.92 ± 3.94
Dexamethasone	3	61.33 ± 1.94
	1	57.59 ± 7.38
	0.3	53.57 ± 3.10

In antimicrobial activity studies, PSE showed a concentration dependent inhibition of the growth of both Gram positive and Gram negative clinically significant human pathogens including *S. aureus*, *S. pyogenes*, *E. coli*, *P. aeruginosa* and *C. albicans* (Table 2). The results give some scientific credence to the use of the plant in the treatment of common infectious diseases in traditional medicine.

Table 2: Zone of inhibition of *P. subcordata* extract (PSE)

Microorganisms	Mean zone of growth inhibitions (mm) ± SEM				
	20 mg/mL	10 mg/mL	5 mg/mL	Cipro (5 mg/mL)	Keto (5 mg/mL)
Pseudomonas aeruginosa	14.7 ± 0.3	12.7 ± 0.3	12.0 ± 0.0	47.3 ± 1.2	-
Escherichia coli	13.7 ± 0.3	11.7 ± 0.7	NI	47.3 ± 1.3	-
Streptococcus pyogenes	20.7 ± 0.3	13.0 ± 0.6	10.7 ± 0.3	43.0 ± 0.6	-
Staphylococcus aureus	17.3 ± 0.3	12.7 ± 0.3	NI	17.7 ± 1.7	-
Candida albicans	17.7 ± 0.3	10.7 ± 0.3	NI	-	12.3 ± 0.3

n= 3, Cipro=ciprofloxacin; Keto = ketoconazole; NI = No inhibition

As earlier established by previous studies, unregulated inflammation and infection are inseparably linked to the release of reactive oxygen species, leading to oxidative stress and tissue damage [1, 2]. Scavenging reactive oxygen species is therefore pivotal in attenuating the inflammatory cascade and alleviating many other diseases. From the results of antioxidant activity testing, both PSE and ascorbic acid gave a concentration dependent radical scavenging activity with IC_{50} values of 226.50 and 24.37 µg/ml respectively (Fig. 4). The extract was found to contain a total phenol content of 457.02 mg/g (tannic acid equivalent) (Fig. 5) and a high antioxidant capacity (347.08 mg/g ascorbic acid equivalent) which increased with increasing concentration (Fig. 6). A strong correlation between total

phenolic content and total antioxidant capacity (r^2 = 0.85) (Fig. 7) suggested that about 85 % of the total antioxidant capacity of the crude extract may be due to the phenolic constituents of the stem bark.

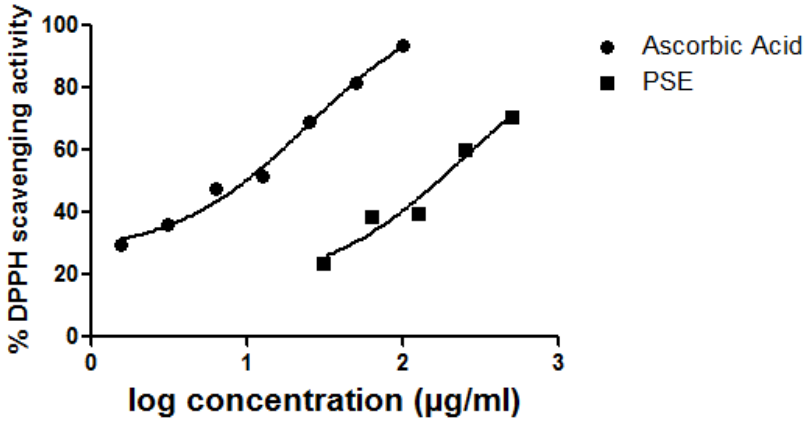

Figure 4: DPPH radical scavenging activity of PSE and Ascorbic Acid

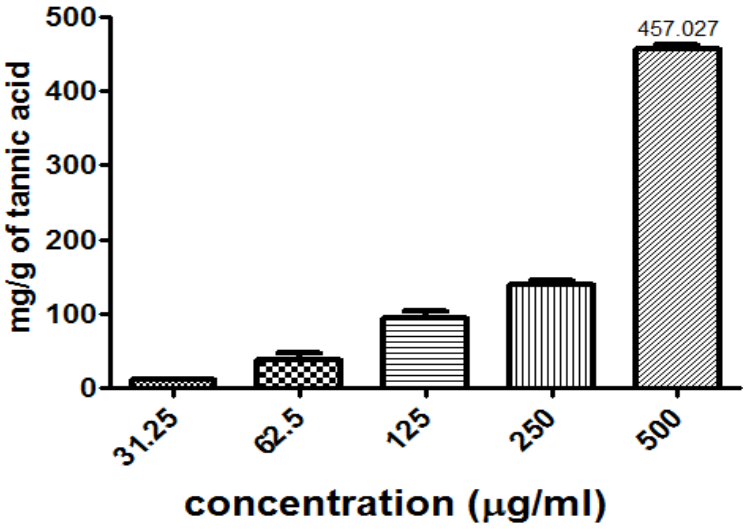

Figure 5: Total phenol content of the methanol extract of *P. subcordata* expressed as the tannic acid equivalent

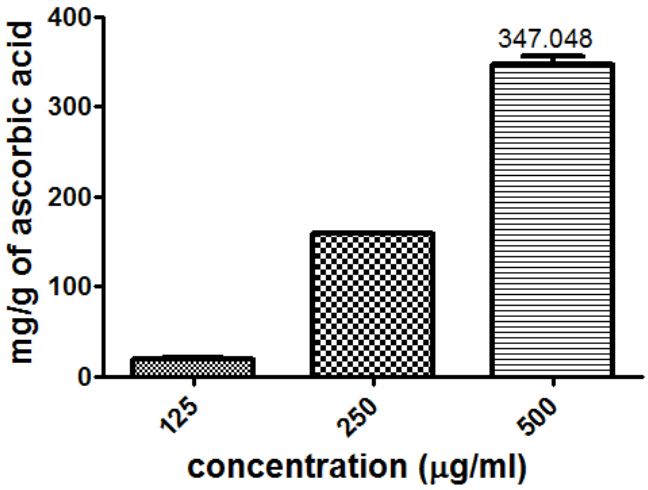

Figure 6: Total antioxidant capacity of the methanol extract of *P. subcordata* expressed as ascorbic acid equivalent

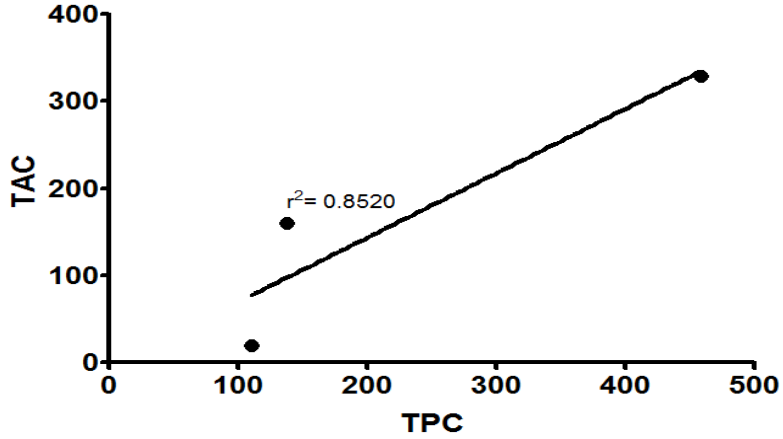

Figure 7: Correlation between the total phenol content (TPC) and the total antioxidant capacity (TAC) of the bark extract of *P. subcordata*

Preliminary phytochemical screening of the powdered plant material revealed the presence of polyphenols such as tannins, coumarins and flavonoids, triterpenoids, anthraquinones, cardiac glycosides and alkaloids. These plant constituents have been previously reported to possess significant antimicrobial, antioxidant and anti-inflammatory activities [19-21]. Their presence in the plant may therefore contribute to the observed antimicrobial, antioxidant and anti-inflammatory effects in traditional medicine.

CONCLUSION

The results of bioactivity and preliminary phytochemical screening of *P. subcordata* stem bark have shown that the plant possess significant anti-inflammatory, antioxidant and antimicrobial activities which are attributed to the presence of some important classes of phytochemical constituents. This gives scientific credence to the folklore uses of the plant in traditional medicine and gives room for further studies to identify the specific compounds responsible for the observed biological activities.

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