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Chemical Constituents from the Stem Bark of *Pentaclethra macrophylla* Benth (Fabaceae)

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

Abstract

Background: Plants have served as source of lead discovery in drug development; there is the need to look into our ethnomedicinal plants for the purpose of identifying bioactive antibacterial agents to combat the growing antibacterial resistance to current drugs.

Objective: The present study investigated the antibacterial activity of the extract and soluble fractions of *Pentaclethra macrophylla* against some selected pathogens and isolation of active constituents using chromatography and spectroscopic techniques.

Materials and methods: The pulverized stem bark of *P. macrophylla* was extracted to exhaustion with 70% methanol and the combined crude methanol extract after removal of solvent was partitioned with ethyl acetate and n-butanol to give ethyl acetate and n-butanol soluble fractions. Antibacterial activity was evaluated on the crude methanol extract,ethylacetate and n-butanol fractions against five pathogenic bacteria using agar diffusion assay method. The active ethylacetate and n-butanol fractions were subjected to Column chromatography and subsequent purification over sephadex LH-20 afforded compounds I, II and III. The structures of these compounds were elucidated using NMR and MS and are reported in this plant for the first time.

Results: Antibacterial activity showed that the extract and fractions at 10mg/mL showed activity against *B. subtilis* with zones of inhibition of 8.0 ± 2.89, 16.0 ±1.73 and 9.5 ± 1.78 respectively for 70% methanol extract, ethyl acetate, and n-butanol fractions. Ethyl acetate soluble fraction at (10mg/mL) had a good activity against *B. subtilis* and *S. aureus* with inhibition zones of 16.0 and13.5mm compared with Levofloxacin (1.5µg/mL) having zones diameter of 21.1 and 16.6mm, however, none of the extract or fractions showed activity against *K. pneumonae*.
Compound I was identified as methyl gallate, compound II: Bergenin and compound III: 11-O-galloylbergenin. The structures of these compounds were elucidated using NMR and ESI-MS and compared with literature.

Conclusion: Finding from this work showed that the ethylacetate fraction is the most active and compounds I, II were isolated from the fraction, while the n-butanol furnished compound III. These compounds are being reported for the first time in this plant and have been known to possess antibacterial activity, thereby given credence to the ethnomedicinal use of this plant against infections.

Keywords: *Pentaclethra macrophylla*, Antibacterial, Methyl gallate, Bergenin, 11-O-galloylbergenin.

INTRODUCTION

Plants have continued to be a source of medicine especially in the developing parts of the world where about 80% of the population depend on natural products for their health needs (Rates, 2001; Calixto, 2000; Raskin *et al*., 2002). This has necessitated intensive studies into bioactive secondary metabolites using methods such as extraction, isolation, purification and chemical characterization of plant extracts which could unfold a novel chemical compounds suitable for drug development (Butler *et al*, 2004; Lahlou *et al*, 2007 Ebada *et al*, 2008).The therapeutic efficacy of medicinal plants in combating diseases lies in presence of phytoconstituents present in them. These include alkaloids, flavonoids, steroids and saponins among others (Harbone, 1998; Busse, 2000; Newmann *et al*, 2000). Plants used as medicines have been considered safe, less toxic and also as a source of fantastic biomolecules unequalled by any synthetic procedure (Bhat, 2012; Katiyar *et al*, 2012).

Pentaclethra macrophylla (Fabaceae) also known as African oil bean tree (Ugba in Igbo) language of Eastern Nigeria, has both economic and ethnopharmacological importance. The stem bark has been used to treat diabetes, cough, diarrhea, convulsions, inflammation and gonnorhea and other infectious diseases in some communities in Nigeria (Iwu *et al*, 2014).

Pentaclethra macrophylla occurs in forest zone of West and Central Africa especially, Nigeria, Angola (Keay *et al.*, 1989). It is a large leguminous woody plant that belongs to the sub family Mimosoidae. Previous studies for various biological activities like anti-nociceptive (Okorie *et al*., 2009), hypoglycemic (Fomekong *et al*., 2008;Igbe *et al*., 2012), antidiarrheal (Akah *et al*., 1999) have all been reported. Olaitan *et al*, (2009) had reported the antimicrobial activity of the seed extracts of this plant, while the antimicrobial activity of the oil and stem bark extracts of this plant have been reported (Babatunde *et al*, 2015). From the chemical point of view, not much work have been done, except (Iwu *et al*, 2014) who reported the isolation of a steroid. In this present study, as part of effort to screen our ethnomedicinal plant for bioactive plant metabolites as lead in the discovery of anti-infective agents, we report here in the antibacterial activity of the methanol extract,ethylacetate and n-butanol soluble fractions of *Pentaclethra macrophylla* stem bark and isolation and structure elucidation of three compounds namely: Methyl gallate(I) ,Bergenin (II) and 11-O-galloyl bergenin from the active ethyl acetate and n-butanol soluble fractions of the ethanol extract of the stem bark of *Pentaclethra macrophylla*.The structure of these compounds were elucidated using spectroscopic technique and is reported here for the first time from this plant.

MATERIALS AND METHODS

Materials

Materials

Column chromatography was performed on silica gel G (200-400 mesh, Silicycle), while TLC was carried on pre-coated silica gel G (0.25 mm) Silicycle. Gel filtration was carried out using sephadex LH-20 (Sigma). NMR spectra were recorded at 600MHz (Advance III 600MHz), Bruker, Karlsruhe, Germany), in CD_3OD. Chemical shifts are recorded in ppm δ and referred to the residual solvent, coupling constant are given in Hz. The 2D-NMR experiments including COSY, HSQC and HMBC were performed using standard Bruker microprograms. The ESI-MS experiments were performed on a LT-Orbitrap XL (Thermo Scientific Company, Brehmen, Germany). Clinical isolates were used for the antibacterial study and the isolates were obtained from Department of Pharmaceutical Microbiology, University of Port Harcourt.

Plant Material

The stem bark of *Pentaclethra macrophylla* were collected in October, 2015 within the University of Port Harcourt, identified and authenticated by Mr. Ekeke Chimezie of the Department of Plant Science and Biotechnology (University of Port Harcourt). A voucher specimen with herbarium number-UPH/V/1237 was deposited in the herbarium of same institution. The stem bark was air dried for 12 days and pulverized.

Extraction

A 750 g quantity of the pulverized bark of *P. macrophylla* was extracted to exhaustion at room

temperature with 2x2.5L of 70% methanol for 7 days, the combined extract was concentrated in vacuo using a rotary evaporator to 10% volume and subsequently dried over water bath at reduced temperature to obtain 120 g of the dried extract a dark brown solid. 50 g of this extract was suspended in water (100 ml) and partitioned sequentially with 5x500 ml of ethyl acetate and 5x500 ml of n-butanol to give 4.6 g of ethyl acetate soluble fraction and 5.6 g of n-butanol soluble fractions respectively.

Antibacterial activity

The antibacterial activity of the methanol 70%, ethyl acetate and n-butanol crude extracts dissolved in dimethyl sulphoxide was carried out against *Staphylococcus aureus, Escherichia coli, Klebseilla pneumoneae, Bacillus subtilis*, and *Pseudomonas aeruginosa* by agar-well diffusion. Nutrient broth medium was used to prepare different fresh cultures of the test organisms. A 20mL of the Mueller Hinton Agar media was seeded with an aliquot (0.1mL) of the bacterial suspension (10^8 CFU/mL) and dispensed into sterile Petri dishes. This was allowed to set and using sterile cork borer (6mm diameter) of 6 wells were made in each seeded plate to be filled with 10mg/mL extracts and the test control. Levofloxacin (10 µg/mL) was used as the positive control while the negative control were prepared using dimethyl sulphoxide. Plates were incubated at 37 °C for 24 h. Antibacterial activity was expressed as the diameter of the inhibition zone (mm) produced by the extract and fractions and were measured to the nearest millimeter. All of the experiments were done in triplicate.

Isolation

A portion of the ethylacetate soluble fraction (3.0 g) was packed in a column (1.9x40.8 cm) and eluted gradiently with dichloromethane and dichloromethane and methanol mixtures as follow: 100.0,99:1,98:2,97:3,96:4,95:5,90:10,80:20,70:30 and 50:50). 20 ml aliquots were collected. The progress of separation were monitored on TLC using the solvent systems: Ethylacetate: dichloromethane: methanol and water (15:8:4:1 and 6:4:4:1) respectively to give 96 fractions. Fraction 13-15 eluted with 5% methanol in dichloromethane afforded compound I a buff solid (20 mg). Fractions 85-92 eluted with 10% methanol in dichloromethane were pooled together and was purified over sephadex LH-20 eluted with methanol to give compound II a white solid (10 mg).

The n-butanol soluble fraction (3.6 g) was packed in a column of (1.9 x40.8 cm) and eluted gradiently with dichloromethane and dichloromethane: methanol mixtures, 20 ml aliquots were collected and the progress of separation was monitored on TLC using the aforementioned solvent systems. Fractions 16-19 eluted with 10% methanol in dichloromethane were pooled together and further purified over sephadex LH-20 eluting with pure methanol to give compound III, a white solid (11 mg).

Results and Discussion

Antibacterial activity of the stem bark extracts of *P. macrophylla*

The observed antibacterial data as shown in Table 1 showed that at 10mg/mL the extract and fractions (methanol 70%, ethyl acetate and n- butanol) were active against three out of the five test bacteria (*E. coli, P. aeruginosa, S. aureus and B. subtilis*) with varying mean zones of inhibition but showed weak activity on *P. aeruginosa* and no activity against *K. pneumoneae*.

Table 1. Antibacterial activity of *Pentaclethra macrophylla* stem bark extract/fractions

		Inhibition zone diameter (mm)				
		Test organism				
Extract	Conc. (mg/ml)	B. subtilis	E. coli	P aeruginosa	S aureus	K. pneumonia
Methanol 70%	10	8.0 ± 2.89	3.5 ± 1.35	2.3 ± 0.58	7.2 ± 0.56	0.0
Ethyl acetate	10	16.0 ± 1.73	8.3 ± 1.53	3.2 ± 2.45	13.5 ± 2.08	0.0
n-butanol	10	9.5 ± 1.78	3.5 ± 1.41	4.3 ± 0.71	5.63 ± 1.15	0.0
Levofloxacin	1.5[a]	21.1 ± 2.12	17.2 ± 3.20	19.3 ± 1.52	16.6 ± 1.41	13.5 ± 1.00
DMSO	-	-	-	-	-	-

Values are Mean ± SD, n= 3. – indicates no visible growth ,a=µg/ml

Compound I, a buff solid (20 mg).
UV (MEOH) λmax (nm): 218, and 272
^{l}H-NMR (CD_3OD) δ(ppm):7.04(s,H-2,H-6);3.81 (s,OCH_3).
^{13}C-NMR (CD_3OD) δ(ppm):50.8 (C-8),108.6 (C-2,C-6),120.1 (C-1),138.3 (C-4),145.0(C-3,C-5),167.5 (C-7),208.7 (C=O) and 29.3 (CH_3).
ESI-MS: m/z (185) M^+ +1).

Compound II, a white solid (10 mg).
ESI-MS :m/z 327 $(M-H)^-$,373.07 $(M+FA-H)^-$ and 655.14$(2M-H)^-$.
^{1}H-NMR (600MHz) CD_3OD δ(ppm):3.47 (1H,t,H-3),3.73 (2H,m,H-11),3.84(1H,t,H-4),3.90(3H,s,OCH_3 H-12),4.04(1H,d,H-2),4.09(1H,d,H-4a),4.96(1H,d,10b) and 7.10(1H,s,H-7).
HSQC (Table 2).

Compound III, a white solid (11 mg)
ESI-MS: m/z 479 $(M-H)^-$
^{1}H-NMR (600MH_Z) CD_3OD δ(ppm :3.57 (1H,t,H-3),3.85 (1H,t,H-4),3.90(3H,s,OCH_3 H-12),3.95 (2H,m,H-11),4.15(1H,t,H-2),4.4(1H,m,H-4a),5.05(1H,d,10b) , 7.00(1H,s,H-7) and 7.10 (2H,d,H-2^1,6^1).
HSQC (Table 3).

Table 2. C→H Correlation of compound II (HSQC)#.

C Number	H	C
2	4.04 1H (d)	81.0
3	3.47 1H (t)	71.0
4	3.84 1H (t)	61.0
4a	4.09 1H (d)	80.0
7	7.10 1H (s)	112.5
10b	4.96.1H (d)	71.5
11	3.73 2H (m)	61.8
OCH_3	3.90 3H (s)	60.0

δ values in ppm.

Table 3. C→H Correlation of compound III (HSQC)#.

C Number	H	C
2	4.15 1H (t)	82.5
3	3.57 1H (t)	73.0
4	3.95 1H (t)	82.0
4a	3.85 1H (d)	77.0
7	7.10 1H (s)	110.0
10b	5.05 1H (d)	75.0
11	4.40 2H (m)	65.0
2^1,6^1	7.12 2H (s)	111.5
OCH_3	3.90	61.0

δ values in ppm.

Discussion

The antibacterial test at 10mg/ml showed activity against both Gram positive and negative organisms. The test organisms (*E .coli, P. aeruginosa, S.aureus and B. subtilis*) showed susceptibility to the extracts with varying zones of inhibition as an indication of antibacterial effect but have no effect on *K. pneumoneae*. The positive control – Levofloxacin showed activity on all the test organisms. The ethyl acetate extract showed its best activity against *B. subtilis* and *S. aureus*, and a weak activity against *E .coli* with zones of inhibition diameter (16.0mm ± 1.73 and 13.5mm ± 2.08 and 8mm ±1.53) respectively while the positive control- levofloxacin had (21.1mm ± 2.12 and 16.6mm ± 1.41) for the same organisms. The n-butanol and methanol 70% extracts had good activities against some of the test organisms. This result indicates that the ethylacetate fraction might contain active constituents as antibacterial agent.

Compound I was isolated from the ethylacetate fraction as a buff solid, its ESI-MS gave an M+1 and M-1 peaks at m/z 185 and 183 indicating a molecular weight of m/z 184 pointing to a molecular formula $C_8H_80_5$. The ^{1}H-NMR spectrum revealed an aromatic protons at δ=7.04 ppm assigned to H-2 and H-6 of benzene ring, and a methoxy protons at δ=3.86ppm, indicating a trihydroxy substituted benzene ring. The UV spectrum at λmax 218 nm and 272 nm confirming the presence of substituted benzene ring. Compound I was found to be methyl gallate by comparison of its spectral data with literature (Hussaini *et al*, 1979; Rao *et al*, 2012).

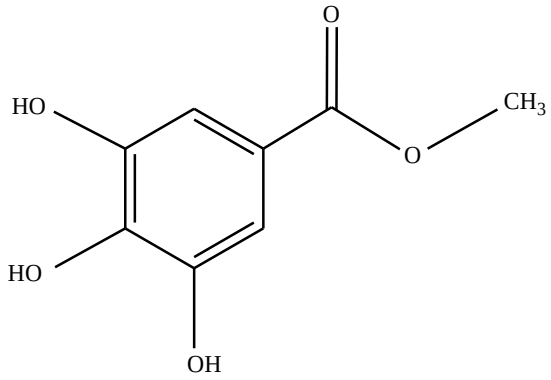
Compound II was also isolated from the ethylacetate fraction as a white solid. The HR- ESI-MS spectral data gave an $[M-H]^-$ and [2M-H]-ion peaks at m/z 327.07 and 655.14 respectively consistent with the molecular formula $C_{14}H_{16}O_9$. The ^{1}H-NMR spectrum revealed the presence of an aromatic singlet at δH=7.10ppm which correlated with a carbon signal at δ_C=112.5 assigned to H-7 of the bergenin nucleus which is characteristic of a pentasubstituted aromatic ring nucleus, the methoxyl proton at δ=3.90 ppm correlated with a carbon signal at δ=60.0ppm, and the location of this methoxyl group was established from the long correlation of the methoxyl hydrogen (δH 3.90) with the carbon signal at δc 142 ppm . The ester carbonyl signal was evident at δc

163.5ppm.Complete assignment of the C-H correlation was aided with the HSQC (Table 2) and HMBC. Based on the spectral data of compound II, NMR and HR-ESI-MS, compound II was found to be Bergenin by comparison of the spectral data with literature (Jahodar *et al*, 1992; Nunomura *et al*, 2009 and Subramanian *et al*, 2015).

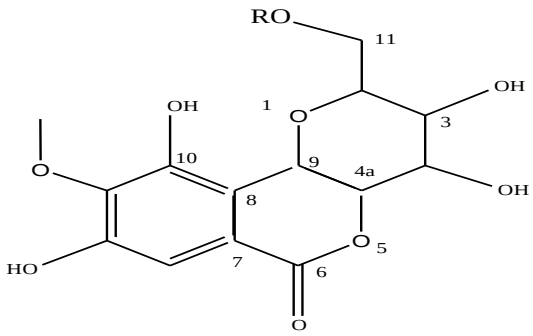
Compound III was isolated from the n-butanol soluble fraction as a white solid. The HR-ESI-MS spectral data gave an [M-H] ion peak at m/z 479 consistent with the molecular formula $C_{21}H_{20}0_{13}$. The spectral characteristics of compound III showed it was a derivative of bergenin (II).The ^{1}H-NMR of compound III showed similar pattern with compound II, except additional symmetric AA^1 type singlet protons at δ_H = 7.12 ppm assigned to H-2^1,H-6^1 of the galloyl moiety. This signal showed a correlation in the HSQC (Table 2) with the carbon at δc 111.0 ppm (C-2^1,C-6^1).The galloyl attachment to the bergenin nucleus was evident from HMBC correlation in which the proton signal at δH 4.40 (C-11) exhibited a long range correlation with the carbon signal at δc 166 (C-7^1) and 82.5 (C-2) .The position of the methoxyl group was established via the long range correlation of the methoxyl protons (δ_H 3.90) with the carbon signal at δc 144.0ppm (C-10).Compound III was found to be 11-O-galloyl bergenin by comparison of its spectral properties with literature data (Yoshida *et al*,1982).

Bergenin is a bioactive molecule with diverse pharmacological activity being reported such as its antioxidant activity which was more than that of ascorbic acid or quercetin (Lee *et al*, 2005),hepatoprotective activity(Lim *et al*,2000),its antifungal activity was reported by Prithiviraj *et al*,(2007). The wide spectrum pharmacological profile of bergenin still permits the investigation for its new therapeutic targets associated with molecular mechanism (Uddin *et al*, 2014).

11-O-galloyl bergenin (compound III) has also been reported to exhibit a very good antioxidant activity comparable to α-tocopherol and has also shown to exhibit an excellent antioxidant activity compared to bergenin and synthetic derivatives (Takahashi *et al*,2003).Compound II and III have been reported by Uddin *et al*, (2014) to exhibit anti-malarial activity against *P. falciparum* strain. The anti-inflammatory activity of methyl gallate (compound I) have been reported (Ahmadu *et al*, 2015). The presence of these compounds in *Pentaclethra macrophylla* might be responsible for some of its ethnomedicinal uses and can further be exploited for a lead discovery.



Compound I



Compound II R=H

Compound III R=galloyl

Fig 1: Structures of isolated compounds.

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