5-Methylresorcinol and Stigmasterol Glycoside from *Acanthus montanus* (Nees) T. Anderson Root Extract and Their Antimicrobial Activities

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ABSTRACT: This work presents the isolation and characterization of two natural products from the root extract of *Acanthus montanus*. Extract of the root was acquired by means of maceration using *n*-hexane and ethyl acetate sequentially. The ethyl acetate extract was fractionated via vacuum liquid chromatography and further purification through column chromatography provided two compounds (one crystalline and one amorphous). Characterization of the compounds was achieved through IR spectroscopy and NMR experiments while biological activities of the two isolates were confirmed using agar well diffusion assay. The two isolated natural products were characterized as 5-methylresorcinol and glycosylated stigmasterol having 2.6% and 1.7% yields, respectively. 5-Methylresorcinol and glycosylated stigmasterol showed biological activities against eight bacteria and seven fungal species. Zones of inhibition ranged from 25 to 39 mm against bacteria and 18 to 34 mm against fungi at 200 µg/ml. 5-Methylresorcinol and glycosylated stigmasterol exhibited better antibacterial properties.

Key words: 5-Methylresorcinol, glycosylated stigmasterol, *Acanthus montanus*, antibacterial, antifungal, secondary metabolites.

INTRODUCTION

Plants have been very essential in the existence of man, both for food and medicine. Their medicinal values and activities are dependent on their phytochemical compositions.[1] These phytochemicals include sterols, phenolics and so many others. Sterols are a subgroup of steroids with a hydroxy group at the 3-position of the A-ring. They are found in microorganisms, animals and plant membranes, also called mycosterols, zoosterols and phytosterols, respectively. Cholesterol is known as the main zoosterol, but sterols in plants usually exist as mixtures with β-sitosterol, campesterol, and stigmasterol regarded as major phytosterols.[2] It is imperative to note that sterols are significant for countless biological processes necessary for reproduction and survival.[3]

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Resorcinol, a 1,3-isomer (or meta-isomer) of benzenediol having the formula $C_6H_4(OH)_2$, is a phenolic compound found in dermatologic medications for treatment of skin disorders and infections such as warts, eczema acne, calluses, seborrheic dermatitis, corns and psoriasis and used in several industrial applications like photography, tanning, tire manufacturing among others.[4]

Acanthus montanus belongs to the Acanthaceae family. In tropical Africa, *A. montanus* has been used as vegetable for most families in rural areas due to its availability and affordability.[5] The plant is rich in phytochemicals and hence finds a place in traditional medicine. The leaves and roots are used for treatment of respiratory tract infections, skin diseases, venereal diseases, spontaneous abortion, gastritis, arthritis and rheumatism.[5,6] Compounds such as saponins and the gammaceranes-acanthusol and its 3-O-β-D-glucopyranoside have been isolated from the plant.[6] In our earlier studies, we reported that the ethyl

acetate extract of *A. montanus* root possessed outstanding wide spectrum antimicrobial properties.[7] We had also reported the isolation of two new antimicrobial compounds (a flavanone and a coumarin) from the root of *A. montanus*.[8] The current study presents the isolation and characterization of a phenolic compound (resorcinol) and a sterol (glycosylated stigmasterol) from the ethyl acetate extract of *A. montanus* root as well as their biological activities.

MATERIALS AND METHODS

Reagents, instrumentation and organisms. All solvents (*n*-hexane, ethyl acetate and ethanol) utilized in chromatography were redistilled. Other reagents used were hydrochloric acid 37.5%, DMSO 98.0% and conc. H_2SO_4. Terbinafine and ceftriaxone were antifungal and antibacterial standard drugs used for the antimicrobial assays.

Transmittance method was used to obtain infrared spectra (in the range 4000 – 650 cm^{-1}) from IR spectrometer (Agilent Technologies Cary 630 FTIR). Samples (crystals and amorphous solids) were prepared for analysis with potassium bromide pellets. NMR (Nuclear magnetic resonance) spectrometer (Agilent Technologies 400 MHz Premium +AR) was employed for 1D and 2D NMR experiments. The NMR data acquired were used for structural elucidation. For NMR experiments, the compounds (I and J) were dissolved in deuterated chloroform. TLC was performed on silica gel precoated aluminium plates (F_{254} Merck) and detections done under UV light (254 nm) and using H_2SO_4-MeOH (1:10) as spray reagent accompanied by 3 minutes heating at 105 °C.

Stock cultures of previously characterized clinical isolates of fungi and bacteria utilized in this study were obtained from the Federal Medical Centre Owerri, Nigeria. The fungi were Fusa equisetti, Saccharomyces cerevisiae, Aspergillus fumigates, Candida albicans, Rhizopus oligastus, Aspergillus niger and Aspergillus flavus. While Salmonella typhi, Shigella dysenteriae, Salmonella paratyphi, Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli, Shigella sonniea and *Staphylococcus aureus* were bacteria used for this work.

Extraction. Roots of *A. montanus* were collected from Rivers State, Nigeria. The washed roots were dried in the shade and triturated. The triturated root (1.5 kg) was successfully defatted using *n*-hexane before macerating with ethyl acetate for 48 hours with intermittent stirring. The solvent was completely removed from the extract using a rotary evaporator.

Isolation. Previously observed biological activities of ethyl acetate extract of *A. montanus* root necessitated its selection for isolation.[7] The extract (11.32 g) and silica gel (15 g) were blended to afford a light brown powder. A vacuum liquid chromatography (VLC) set up comprising of separatory funnel and sintered glass funnel was connected to a vacuum pump via a hose. TLC grade silica gel (100 g, 400-800 mesh size) was evenly poured into the funnel to the height of 3.5 cm. Subsequently, the column bed was protected with a layer of treated sand and the prepared mixture (of extract and silica gel) poured unto the column. Gradient elution (employing separate mixtures of *n*-hexane, ethyl acetate and EtOH as mobile phase) was utilized and fractions (200 ml each) collected. Thirty-nine fractions were collected via chromatography.[9,10]

VLC Fractions 6 - 10 and 31 - 39 were purified with column chromatography (80 x 2.3 cm glass column). Wet packing procedure was followed in loading the columns.

VLC Fractions 6 - 10 (eluted with EtOAc-*n*-hexane (7:3 and 8:2) were well mixed together and concentrated to give light brown crystals. Silica gel (4000 mg, 60-200 mesh size) was properly blended to powder with 760 mg of the concentrated fraction. A slurry of silica gel (30 g, 60-200 mesh size) and 100% *n*-hexane was gradually poured into the column with the effluent tap opened while tapping the column. Sand (treated) was sprinkled evenly on the column bed after which the blend of crystals and silica gel was gently poured into the column. Gradient elution was done using EtOAc-*n*-hexane (1:9, 1.5:8.5, 2:8, 2.5:7.5 and 3:7). Cumulatively, one

hundred and thirty-five fractions (20 ml each) were collected. Fractions 36 – 50 (eluted with EtOAc-*n*-hexane (2:8)) were merged and concentrated to afford compound I (295 mg).

VLC Fractions 31 – 39 eluted with ethyl acetate-*n*-hexane (9:1), 100% ethyl acetate and ethyl acetate-ethanol (9:1) were combined to afford a brownish yellow solid weighing 3.25 g after concentration. Silica gel (5000 mg, 60-200 mesh size) was thoroughly mixed with 3.25 g of the VLC fraction to give a brown powder while 100 g of the same silica gel was used to prepare the slurry for the column. Treated sand was used to protect the column bed while the prepared mixture was gently poured into the column. Gradient elution was conducted using EtOAc-*n*-hexane (1:9, 2:8, 3:7, 4:6, 1:1), EtOAc-*n*-hexane (6:4, 7:3, 8:2, 9:1), 100% EtOAc, MeOH-EtOAc (0.2:9.8, 0.5:9.5, 1:9, 1.5:8.5). A total of 115 fractions (20 ml each) was collected. Fractions 14-55 (eluted with MeOH-EtOAc (0.5:9.5)) were merged and concentrated to give 192 mg of compound J.

Spectroscopic analyses (FTIR and NMR) were conducted and the melting points of compounds I and J were recorded.

Antimicrobial analysis. Compounds I and J were subjected to antimicrobial assays against 8 bacteria and 7 fungal species via standard protocols where the agar well diffusion assay was utilized.[11,12] The bacteria were reestablished for viability and purity using selective media (mannitol salt agar and eosin methylene blue agar). Hot agar was poured into Petri dishes and left to cool and solidify. The organisms were incorporated and spread evenly on the sterile agar medium. Uniformly spread holes of 5 mm diameter were created in each dish using a sterile instrument. Compounds I and J (100 and 200 µg/ml each) were then aseptically poured into the created holes and left for 1 hour pre-diffusion. Similarly, a known medication (ceftriaxone, 30µg/ml) was incorporated into a single hole in each of the dishes. The Petri dishes were individually tagged and incubated at 37°C. Zones of inhibition (ZOI) were measured after 24 hours. ZOIs of 6 mm and below were regarded as not significant.

On the other hand, potato dextrose agar medium was utilized for antifungal experiments. The fungi were taken from previously incubated cultures and were aseptically incorporated into sterile Petri dishes. Warm agar was also poured into the sterile dishes and the agar allowed to cool and solidify. A sterile cup borer was used to create uniform holes (5 mm) in each dish. Compounds I and J (200 and 100 µg/ml each) along with a known medication (terbinafine, 30 µg/ml) were aseptically incorporated into the prepared holes and left for 1 hour for pre-diffusion. The Petri dishes were then incubated at 37°C. The ZOIs were measured after 48 hours.

RESULTS AND DISCUSSION

Isolated Compounds I and J. Compound I was colourless crystals, having a yield of 2.6% (295 mg) and R_f 0.725 (*n*-hexane-EtOAc (4:6)). It was purple under the UV light and pink when treated with 10% sulphuric acid in methanol. Its melting point was 110 °C (Table 1). The IR absorption peaks for compound I suggested the existence of an aromatic ring showing their stretches at 1604 cm^{-1} for aromatic ring, 3121 cm^{-1} for aromatic C-H stretch and 787 cm^{-1} for disubstitution (meta) in the aromatic ring. Phenolic OH stretching was also seen at 3406 and 3541 cm^{-1}. The ^{13}C NMR spectrum showed seven carbon signals (Table 2). From ^{1}H NMR spectrum proton signals at δ 7.10 (H-2), 7.18 (H-4) and 7.13 (H-6) were identified as aromatic protons (Table 2). HSQC spectrum showed that proton signals at δ7.07 (H-a) and 7.23 (H-b) were not attached directly to carbons. The ^{1}H and ^{13}C NMR assignments aided by HSQC and verified by HMBC showed only one primary carbon at δ 109.1 (C-7). Compound I structure was ascertained using ^{1}H and ^{13}C NMR supported by connection seen in HSQC and verified with HMBC experiment. Carbon signals observed at δ 144.2 (C-1), 110.0 (C-2), 129.9 (C-3), 124.0 (C-4), 156.0 (C-5) and 123.0 (C-6) (Table 2) were found to be similar to reported signals of aromatic ring of resorcinol.[13] Long range connectivity from HMBC (Figure 1) indicated that δ 7.07 (H-a) is correlated to δ 144.2 (C-1) while δ 7.23 (H-b) is correlated to δ 129.9 (C-3), corroborating the position of the hydroxy group. The

signals observed were similar to resorcinol.[13,14] The position of the methyl group (C-7) was also established from HMBC correlation (Figure 1). Compound I was confirmed as 5-methylresorcinol which was colourless crystals having a melting point of 110°C.[13,15]

Table 1. Compounds I and J profiles.

Property	Compound	
	I	J
Nature	Crystalline	Amorphous
Colour	Colourless	White
Weight (mg)	295	192
% Yield	2.6	1.7
Melting point (°C)	110	272
Polarity	Semi-polar	Semi-polar
Solubility	Soluble in chloroform	Soluble in chloroform
R_f value	0.725	0.875
TLC mobile phase	EtOAc-*n*-hexane (6:4)	MeOH-EtOAc (0.5:9.5)
Spot under UV light	Purple	Nil
Colour of spot (after spray reagent)	Pink	Pink

Table 2. Carbon-13 and proton NMR data of compound I.

Position	δ Carbon-13		δ Proton	
	Experimental	Literature [13]	Experimental	Literature [14]
1	154.3	156.6	-	-
2	110.0	104.9	7.10	6.95
3	156.6	156.6	-	-
4	124.0	118.7	7.15	6.97
5	129.9	132.0	-	-
6	123.0	118.7	7.13	6.97
7	109.1	-	1.65	-
a			7.07	6.71
b			7.03	6.71

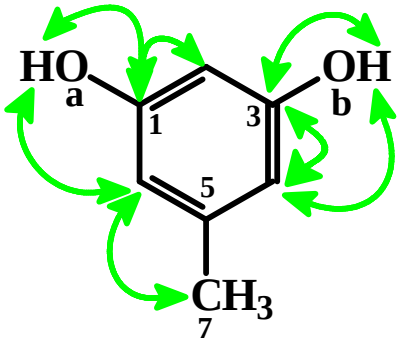

Figure 1. Structure of compound I. The green arrows indicate HMBC correlations.

Compound J was a white amorphous powder, and its melting point was found to be 272 °C. It had a yield of 1.7% with R_f value of 0.875 using solvent system of 5% methanol in ethyl acetate. It was colourless under the UV light and pink when treated with 10% sulphuric acid in methanol. Thirty-five carbons were detected from the ^{13}C NMR data (Table 3); these comprised of one anomeric carbon signal at δ 101.5 (C-1′) and five signals δ 74.0 (C-2′), 76.5 (C-3′), 70.5 (C-4′), 76.5 (C-5′), 61.5 (C-6′) associated with a sugar moiety. From the ^{1}H NMR data (Table 3), proton signal at δ 4.18 (H-1′) was identified as anomeric proton. Compound J structure was

established using ^{1}H and ^{13}C NMR experiments supported by HSQC and verified with COSY and HMBC (Figures 2 and 3). The observed signals for compound J are similar to those observed in stigmasterols and their striking characteristics.[16,17] Compound J was confirmed as a glycosylated stigmasterol.[16,17] Some of the reported activities exhibited by stigmasterol and its derivatives include antioxidant, anti-diabetic, anti-tumor, antimicrobial, hypoglycaemia, osteoarthritis, antianthelmintic, anti-inflammatory and anti-hypercholestrolemia.[18-20]

Table 3. Carbon-13 and proton NMR data of compound J.

Position	δ Carbon-13		δ Proton	
	Experimental	Literature [17]	Experimental	Literature [17]
1	37.0	37.3	1.76, 0.97	1.76, 0.96
2	30.0	31.9	1.78, 1.48	1.52, 1.37
3	76.8	77.4	3.42	3.46
4	38.0	39.6	2.33, 2.09	1.89, 1.14
5	141.0	140.9	-	-
6	121.5	121.7	5.29	5.32
7	31.5	29.7	1.92, 1.49	1.92, 1.46
8	31.4	31.9	1.51	1.51
9	50.0	50.1	0.86	0.95
10	36.1	36.7	-	-
11	20.5	21.6	1.47, 1.40	1.22, 1.16
12	39.5	38.8	1.94, 1.60	1.92, 1.13
13	42.0	42.2	-	-
14	56.0	55.8	0.97	1.09
15	24.0	24.4	1.51, 1.03	1.48, 0.97
16	28.5	29.0	1.64, 1.23	1.63, 1.13
17	57.0	56.7	1.12	1.04
18	12.0	12.4	0.63	0.67
19	19.8	19.3	0.92	0.98
20	39.9	40.0	2.02	2.02
21	21.0	21.4	0.97	0.99
22	138.5	138.5	5.12	5.17
23	129.0	129.3	4.99	5.04
24	51.0	51.1	1.50	1.50
25	31.5	31.8	1.39	1.63
26	17.5	21.1	0.77	0.82
27	20.5	19.6	0.82	0.80
28	25.0	25.5	1.35, 1.12	1.01, 1.03
29	11.9	12.6	0.77	0.81
1′	101.5	101.2	4.18	4.22
2′	74.0	70.6	2.86	3.37
3′	76.5	77.2	3.09	3.11
4′	70.5	73.9	3.03	3.06
5′	76.5	73.0	3.06	3.37
6′	61.5	61.6	3.61, 3.39	3.57, 3.46
a			4.82	4.89
b			4.84	4.92
c			4.83	4.85
d			4.38	4.43

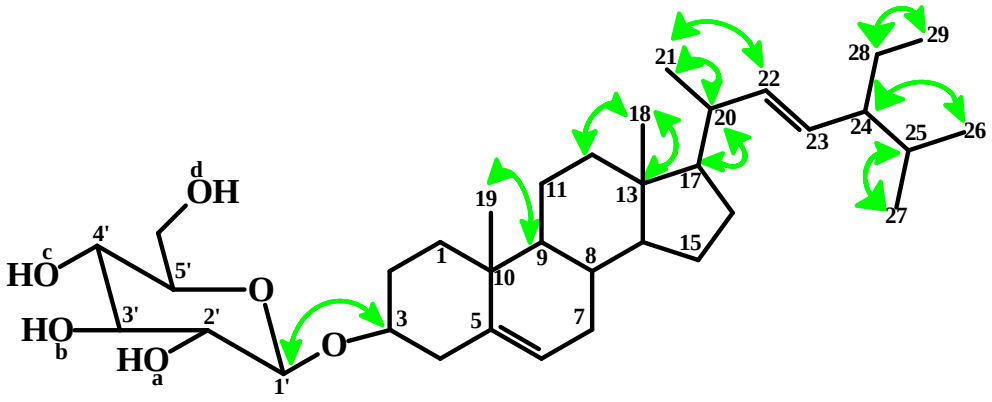

Figure 2. Structure of compound J. The green arrows indicate HMBC correlations.

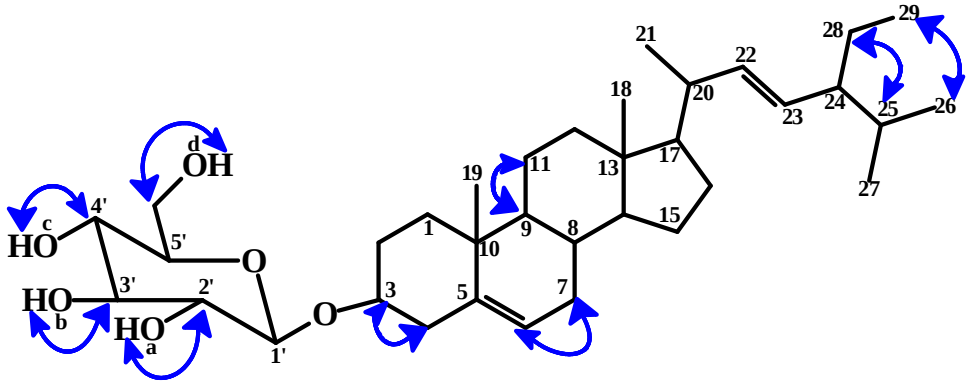

Figure 3. Structure of compound J. The blue arrows indicate COSY correlations.

Biological activities of compounds I and J. Compound I showed good antibacterial activities against all the test organisms at 200 µg/ml with ZOIs ranging from 29 to 39 mm. *Staph. aureus* exhibited the highest sensitivity with 39 mm zone of inhibition while *E. coli* showed the least sensitivity with 29 mm zone of inhibition at 200 µg/ml (Table 4). Compound J demonstrated slightly less activities against the test organisms at 200 µg/ml with 25-32 mm ZOI range. *S. sonniea* had the highest sensitivity to compound J with 32 mm zone of inhibition while *E. coli* was the least sensitive with 25 mm zone of inhibition (Table 4).

Table 4 also shows the antifungal activities of compounds I and J. The two compounds displayed dose-dependent antifungal activities. Compound J displayed antifungal activities with zone of inhibition of 24 - 32 mm at 200 µg/ml. Susceptibility was high for *A. niger* and *R. oligastus* with 32 mm ZOI while *F. equsette* was the least susceptible with 24 mm zone of inhibition at 200 µg/ml. Compound J had activity against the test fungi with 18 - 34 mm zone of inhibition at 200 µg/ml. *R. oligastus* and *A. fumigates* had the highest susceptibility with 34 mm ZOI while *C. albicans* had the least susceptibility with zone of inhibition of 18 mm at 200 µg/ml (Table 4).

However, other biological activities have been reported for compound I; these include antidepressant/anxiolytic, antioxidant, antiproliferative, anticytotoxic and antimutagenic activities.[18,19,21] Compound J had higher antifungal activities against all the test organisms than the standard (Table 4). Compound J, which is a glycosylated stigmasterol, has been reported to have other properties aside from antimicrobial activity.

These properties include antioxidant, antidiabetic, anti-inflammatory and anti-hypercholes-terolemia.[18-20] The antimicrobial activities of compounds I and J suggest that they can be studied further in developing drugs for the treatment of diseases caused by any of the test bacteria and fungi.

Table 4. Antimicrobial activities of compounds I and J.

Organism	ZOI (mm)					
	I (µg/ml)		J (µg/ml)		Ceftriaxone (standard)	Terbinafine (standard)
	200	100	200	100		
S. typhi	33 ± 0	23 ± 2	26 ± 2	20 ± 0	32 ± 0	-
S. paratyphi	32 ± 0	24 ± 1	28 ± 1	18 ± 1	32 ± 0	-
P. aeruginosa	33 ± 0	24 ± 1	28 ± 1	20 ± 0	29 ± 0	-
S. sonniea	33 ± 1	24 ± 0	32 ± 1	20 ± 0	34 ± 0	-
S. dysenteriae	33 ± 0	24 ± 0	25 ± 1	18 ± 1	28 ± 0	-
E. coli	29 ± 0	19 ± 0	26 ± 0	18 ± 0	30 ± 0	-
Staph. aureus	39 ± 1	23 ± 0	31 ± 1	20 ± 0	32 ± 1	-
S. pyogenes	32 ± 1	23 ± 0	26 ± 0	16 ± 1	36 ± 1	-
C. albicans	26 ± 2	10 ± 0	18 ± 0	0	-	18 ± 0
S. cerevisiae	30 ± 2	9 ± 8	32 ± 1	10 ± 0	-	30 ± 0
R. oligastus	32 ± 2	11 ± 1	34 ± 1	10 ± 0	-	23 ± 0
A. flavus	26 ± 1	10 ± 0	32 ± 0	10 ± 1	-	26 ± 0
A. fumigates	29 ± 1	10 ± 1	34 ± 0	12 ± 1	-	28 ± 1
F. equisetti	24 ± 0	0	24 ± 0	12 ± 1	-	20 ± 0
A. niger	32 ± 0	10 ± 0	32 ± 2	10 ± 0	-	24 ± 0

Data are presented as mean±standard deviation

CONCLUSION

5-Methylresorcinol and a glycosylated stigmasterol were successfully isolated from *A. montanus* root extract. They are known compounds which have not been previously isolated from *A. montanus*. The isolated 5-methylresorcinol and glycosylated stigmasterol strongly exhibited both antibacterial and antifungal activities against fifteen pathogens.

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