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Phytochemical constituents and antimicrobial activity of *Albizia Adianthifolia*

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Research Article

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ABSTRACT

The aim was designed to isolate, elucidate and carry out antimicrobial activity of the isolates against bacterial and fungal strains. Using several chromatographic separation techniques, three known flavonoids, 3',4',7-trihydroxy-5-methoxyflavone (5-methoxyluteolin) **1**, 3',4',3,4,7,8-hexahydroxyflavanol (melacacidin) **2**, and 3',4',7,8-tetrahydroxyflavanone (isookanin) **3** along with three other known constituents identified as, 2,3-epoxy-3-[4-hydroxy-3,4-dimethoxyphenyl]propan-1-ol **4**, D-*chiro inositol* (pinitol) **5** and protocatechuic acid **6** were isolated and reported for the first time *in Albizia adianthifolia*. The structure of these compounds were elucidated by means of spectroscopic methods such as UV, IR, MS, 1D and 2D (COSY, HMQC, HMBC, NOESY) NMR and by comparison of their data with those of published compounds. Using TLC plate agar overlay technique, the preliminary antimicrobial activity of these compounds were tested against some selected bacterial strains *E. coli*, *P. aeruginosa*, *B. subtilis*, *S. aureus* and a fungus *C. albicans* and the results suggests weak to moderate activity.

INTRODUCTION

The genus *Albizia* comprises of approximately 150 species, mostly trees and shrubs native to tropical and sub-tropical regions of Asia and Africa. The species *Albizia adianthifolia* (Schumach) (Leguminosae-Mimosoideae) is a traditional herb largely used in African traditional medicine reported to possess analgesic, purgative, anti-inflammatory, anti-malarial, anti-oxidant, anti-microbial properties and also used as a psychotic principle for memory-enhancement among others [1-3]. Previous phytochemical investigations on the genus and particularly on the species *Albizia adianthifolia* have revealed the presence of secondary metabolites mostly triterpene saponins [4].

As part of our ongoing interest to find novel natural products and biologically active components from plant species, the present study was undertaken in order to evaluate the chloroform and methanolic extracts of the heartwood of *A. adianthifolia*. Perhaps, a more comprehensive search for phytochemicals embedded within the species could possibly serve as chemotaxonomic markers and/or new chemical entities (NCE's) as lead to the species identification as well as substantiate the ethnomedicinal claims amplified by traditional practitioners.

MATERIALS and METHODS

Solvents

Analytical reagents (AR) and general purpose reagents (GPR) solvents were used. GPR solvents were distilled using simple distillation before use.

Collection of plant material

The heartwood of *Albizia adianthifolia* was collected in Sokoto, North western Nigeri, in January, 2011 and was identified

by Mal. Auwal Umar of Botany Unit, Biological Sciences, Usmanu Danfodiyo University, Sokoto. The voucher specimen (UDUH/ANS/0029) was deposited in the herbarium of the University.

Extraction and isolation

The freshly milled *A. adianthifolia* heartwood (657.51 g) was extracted successively with *n*-hexane (*n*-hex); chloroform ($CHCl_3$); methanol (MeOH) and 20% aqueous methanol (3.0 L x 3 each for 72 hrs).The solvent was removed using a rotary evaporator to yield 1.2 g, 10.2 g, 146.5 g and 20.2 g of the *n*-hexane, chloroform, methanol and aqueous methanol extracts respectively Part of the $CHCl_3$ extract (9.20 g) was subjected to vacuum liquid chromatography (VLC) over silica gel eluted with a variety of solvent systems in increasing polarity [i.e from 100% pure *n*-hexane to chloroform to ethyl acetate to methanol]. Fractions of about 200 mL were collected and these were further pooled together due to similarity of TLC profiles to give seventeen fractions A-Q. Fraction G (351 mg) was further fractionated by CC on silica gel eluted with *n*-hex-$CHCl_3$ (1:1); $CHCl_3$, $CHCl_3$-EtOAc (1:1) and EtOAc as solvent systems to afford four sub-fractions G1-G4. Sub-fraction G1 was purified using preparative thin layer chromatography (PTLC) using acetone/*n*-hex (7:3) to yield **4** (7.5 mg). Fraction K (1.60 g) was run on a Sephadex column using $CHCl_3$/MeOH (1:1) to give four sub-fractions (K1-K4). Sub-fraction K3 was further purified on a smaller size Sephadex column using $CHCl_3$/MeOH (1:1) followed by PTLC using acetone/*n*-hex (1:1) to give **1** (6.8 mg). Compound **3** (9.4 mg) was obtained from sub-fraction K4 using PTLC (acetone/*n*-hex 7:3; x2 developments). Fraction M (2.3 g) was fractionated on Sephadex column using $CHCl_3$/MeOH (1:1) to give four sub-fractions (M1–M4). Sub-fraction M4 was purified using PTLC (acetone/$CHCl_3$ 3:7 with few drops of glacial acetic acid to give **2** (2.7 mg). Compound **5** (68.1 mg) precipitated from fractions **M, N, P** and **Q**. The methanol crude extract (146.5 g; dark brown paste) was reconstituted in water and then partitioned with $CHCl_3$ and EtOAc and upon solvent evaporation yilded the chloroform fraction (0.4 g) and the ethyl acetate (70.6 g) fractions. The $CHCl_3$ fraction, due to small sample quantity was not investigated further while the EtOAc fraction was loaded onto a Sephadex column with $CHCl_3$/MeOH (1:1). Four sub-fractions coded **Et1, Et2, Et3** and **Et4** were obtained. Sub-fraction **Et3** (dark brown) was subjected to repeated purification processes using PTLC [acetone/dichloromethane/n-hex (3:5:2)] to give compound **6** (1.5 mg).

Instrumentation

The UV was recorded using a Shimadzu UV-2101PC UV-Vis scanning spectrophotometer and FTIR spectra on a Perkin-Elmer spectrum 100 spectrophotometer (Spectrum Two) respectively. The ^{1}H (300 MHz) and ^{13}C (75.5 MHz) spectra were all recorded on Bruker Avance DPX 300 spectrometer and referenced to residual solvent signals. The signals in the ^{1}H and ^{13}C NMR spectra were assigned using 2D (COSY, NOESY, HMQC and HMBC) NMR techniques. HR-TOF-EI-MS spectra were measured using a Waters GCT Premier mass spectrometer. Column chromatography (CC) was performed using silica gel 60 (Merck; 0.040-0.63 mm) and gel-filtration using Sephadex LH-20 while preparative thin layer chromatography (PTLC) was carried out using silica gel 60 (Merck; $PF_{254+366}$). Vacuum liquid chromatography (VLC) was carried out using silica gel 60 (Merck; 0.040-0.63 mm). Analytical TLC were performed on pre-coated silica gel (Merck; 0.25 mm; 60 $HF_{254+366\ nm}$); spots and bands on analytical TLC and PTLC respectively were visualized using UV and/or by spraying with 1% vanillin-sulphuric acid followed by heating in an oven at 110°C for 5 minutes. Concentration of the extracts was achieved using Buchi® rotary evaporator (model R-210) with vacuum pump (V-700) and water chiller under reduced pressure and at 40°C. Vertical type steam sterilizer ~ Autoclave HL340 at 121°C/15 mins/2.5 bars was used for sterilization of the broth/agar. Nutrient agar (Hi Media Laboratories, Ltd, India, pH 7.1^{+}/-) and nutrient broth (Hi Media Laboratories, Ltd, India) were used. Sterile Biohazard cabinet (class II Type A at 76 psi) was used for agar overlaying and cooling. New Bruns-Wick scientific Incubator shaker (Innova 4000 at 30°C; 150 rpm; 24 hrs) was used to homogenize the pathogens. Thomas Wiley mill model 4 was used to grind the plant material into fine powder.

Antimicrobial susceptibility testing

The antimicrobial potencies for compounds 1-6 was evaluated using modified agar overlay method [5-7]. The nutrient broth was prepared by dissolving 13 g nutrient broth in one litre of distilled water and heated on a hotplate equipped with magnetic stirrer until a homogenous mixture was obtained. Nutrient broth (50 mL) was then transferred into a 250 mL (x5 for each organism) Erlenmeyer flask and then stoppered with cotton wool and aluminum foil. The nutrient agar was prepared by dissolving 28 g of nutrient broth in one litre of distilled water in similar manner as nutrient broth. The nutrient broth and nutrient agar were separately autoclaved for 15 min at 121°C. The nutrient broth was cooled in a Biohazard cabinet while the nutrient agar was kept in an oven set at 45°C until ready to use.

10 mL suspensions of the activated pathogens were separately introduced into labeled 250 mL (x5) Erlenmeyer flasks containing 100 mL warm nutrient agar. Using sterile graduated pipettes, the pathogens were administered and spread as evenly as possible onto the pre-coated silica gel TLC plates already loaded with the different compounds in various loadings i.e 100, 50, 10, 5, 1 µg. The nutrient agar containing the pathogens administered was allowed to solidify before being incubated for 24 h at 37°C and 28°C for the bacterial and fungal strains respectively. The minimum inhibition quantities (MIQ) after 24 h were determined after staining of the plates with methylthiazolyltetrazolium chloride [MTT: 2 mg/mL)]. Chloramphenicol and miconazole were used as control for the bacterial and fungal strains respectively. The entire microbial assay was conducted under strict aseptic conditions.

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Antimicrobial assay

The isolated compounds 1-6 were tested for antibacterial activity against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27833, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and antifungal activity against *Candida albicans* ATCC 10231. The pathogens were obtained from the cell bank of microbiology unit, University of Botswana. Chloroamphenicol and miconazole were used as control for the bacterial strains and the fungus respectively. Methylthiazolyltetrazolium chloride (MTT) was used to aid visualization of activity whereby zones of inhibition are shown as white/yellow spots against a purple background.

RESULTS AND DISCUSSION

Structure elucidation

The structural elucidation for compounds **1-6** were achieved using UV, IR, MS, 1D and 2D NMR spectral analysis and in some cases by comparison with the literature data.

Compound **1** was obtained for the first time from the chloroform extract of the heartwood of *Albizia adianthifolia*. The as a faint red colored paste. HR-TOF-EI-MS showed $[M]^+$ ion peak at 300.0644, corresponding to the molecular formula $C_{16}H_{12}O_6$ (calcd. for 300.0634) from which eleven double bond equivalents were deduced. The UV λ_{max} (nm) (203), 338, 360(s) [+$AlCl_3$, 337, 360(s)] supports the presence of flavonoid system. The IR spectrum revealed a strong and broad stretching absorption at u_{max} 1634 cm^{-1} indicative of a conjugated carbonyl (C=O) and (C=C) stretching at u_{max} 1603 and 1566) cm^{-1}, which further supports the presence of an aromatic system. Furthermore, a broad absorption at u_{max} 3211 cm^{-1} suggestive of a hydroxyl group (OH) was also observed.

The ^{13}C NMR revealed a total of sixteen carbons and DEPT-135 showed that seven were protonated suggesting that nine of these were non-protonated. The ^1J (C-H) correlations of the protonated carbons were confirmed by the HMQC experiments. Of these protonated carbons, six were methine (sp^2) carbons resonating at δ_C [118.5 (C-2′), 115.4 (C-5′), 112.5 (C-6′), 105.1 (C-3), 96.4 (C-6), 95.1 (C-8)] and the last being the methoxy (OMe) carbon at δ_C 55.0. Some HMBC correlations were observed between the methoxy protons at δ_H 3.91 with carbon at δ_C 161.0 (^3J, -OC<u>H</u>$_3$ to C-5) and another between δ_H 6.50 with carbon at δ_C 162.3 (^2J, H-3 to C-2) **(Figure 1 and Table 1)**.

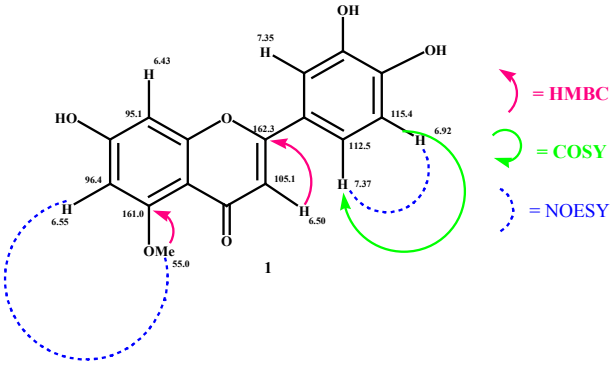

Figure 1. Proposed structure for compound **1.**

Table 1: ^1H (300 MHz) and ^{13}C (75.5 MHz) NMR spectra of compound **1** in CD_3OD.

Position	δ_H(ppm)	δ_C (ppm)
1′	-	122.3
2′	7.35, 1H, d, (2.0 Hz)*	118.5
3′	-	145.6
4′	-	149.2
5′	6.92, 1H, d, (8.9 Hz)	115.4
6′	7.37, 1H, dd, (8.9, 2.0 Hz)*	112.5
1	-	-
2	-	162.3
3	6.50, 1H, s	105.1
4	-	178.9
5	-	161.0
6	6.55, 1H, d, (1.7 Hz)	96.4
7	-	164.2
8	6.43, 1H, d, (1.7 Hz)	95.1
9	-	159.9
10	-	106.6

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OMe	3.91, 3H, s	55.0
*appeared as super imposed.		

Further analysis of ^{1}H NMR and COSY spectral data revealed two sets of spin systems for compound **1**. An ABX spin system [involving δ_H 7.37, dd, *J*=8.9, 2.0 Hz; δ_H 7.35 d, *J*=2.0; δ_H 6.92 d, *J*=8.9 Hz] on B ring and an AX system [δ_H 6.55 d, *J*=1.7 Hz; δ_H 6.43 d, *J*=1.7 Hz] on A ring. The close chemical shifts between the protons resonating at δ_H 7.37 (H-6′) and δ_H 7.35 (H-2′) made it difficult to completely resolve signals H-6 and H-2 as they appeared almost as superimposed on each other and hence their coupling constants could not easily be determined, thus were averaged out (300 MHz NMR spectrum). The NOESY data showed correlation between the methoxy protons at δ_H 3.91 (3H, OMe) with the proton at δ_H 6.55 (1H, H-6) - being the only correlation the methoxy protons were involved in (shown on A ring) suggests that the methoxy group wouldn't have been attached at any position apart from C-5. Furthermore no chelated hydroxyl was observed, as would have been the case if a hydroxyl group was at C-5 position. The presence of the methoxy at C-7 was ruled out on the basis that a NOESY correlation would have been observed between methoxy protons and both C-6 and C-8 protons. On the other hand, no correlation was shown between the methoxy protons with either of the protons at (H-6′ and H-2′~ δ_H [7.37 and 7.35) on the B ring. Thus the B ring could only have hydroxyl substitutions in an *ortho* arrangement due to carbon resonances observed at δ_H 149.2 and 145.6 (C-4′ and C-3′) respectively. The substitution pattern in B ring is confirmed by the observed ABX spin system and also the fact that the UV spectrum did not show any significant shift in both band I and band II when the spectrum was run in methanol followed by $AlCl_3$ and then $AlCl_3$ with HCl (UV λ_{max} (nm) (203), 338, 360(s); +$AlCl_3$, 337, 360(s); +$AlCl_3$/HCl, 338, 362] these data compare with literature (UV (MeOH, λmax, nm): 340 +$AlCl_3$ 274, 355; +$AlCl_3$/ HCl 273, 353) [5]. Some fragmentation (including retro-Diels Alder) patterns observed from the HR-TOF-EI-MS spectrum for compound 1 is shown **(Figure 2)**.

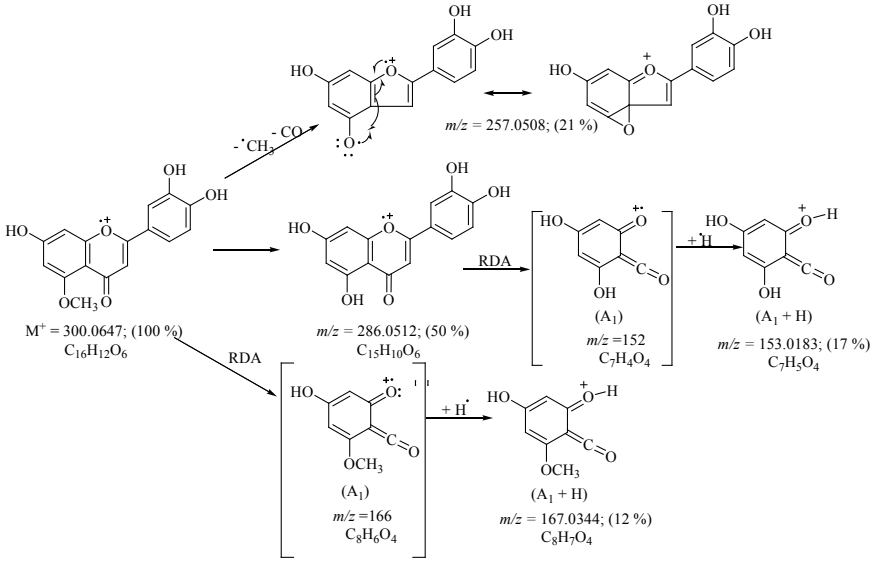

Figure 2. Suggested mass fragmentation pattern for compound 1.

The mass fragmentation data in **(Figure 2)** supports the proposed structure which enabled compound 1 to be identified as 3′,4′,7-trihydroxy-5-methoxyflavone which is trivially known as luteolin-5-methyl ether or 5-methoxyluteolin. 5-methoxyluteolin **1** has been reported occurring naturally in several plant species [5,6], but to the best of our knowledge this is the first time it was isolated from *A. adianthifolia.* Other known compounds but isolated for the first time from the $CHCl_3$ and MeOH extracts of the heartwood of *A. adianthifolia* were 3′,4′,3,4,7,8-hexahydroxyflavanol (melacacidine) **2** [7,8]; 3′,4′,7,8-tetrahydroxyflavanone (isookinin) **3** [9]; 2,3-epoxy-3-[4-hydroxy-3,4-dimethoxyphenyl]propan-1-ol whose other suggested names included 4-[3′-(hydroxymethyl)oxirane-2′-yl]-2,6-dimethyoxyphenol **4** [10]; and D-pinitol **5** [11], and protocatechuic acid **6** [12] **(Figure 3)**.

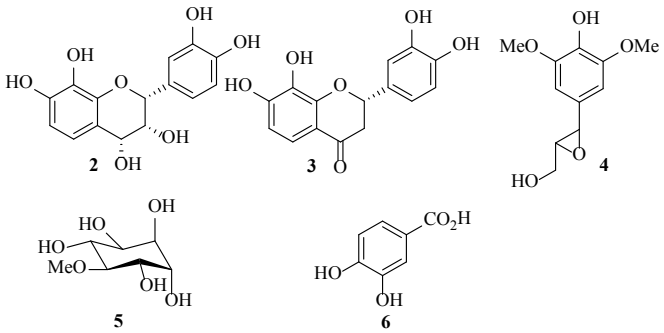

Figure 3. Structures of other constituents from heartwood of *A. adianthifolia.*

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Antimicrobial activity

The results for the antimicrobial activity demonstrated by the compounds isolated from the heartwood of *A. adianthifolia* against Gram negative (*E. coli* and *P. aeruginosa*), Gram positive (*B. subtilis* and *S. aureus*) bacterial strains and a fungus (*C. albicans*) using agar overlay [13-15] is presented. The minimum inhibitory quantities (MIQ) or minimum loading of the individual isolates are shown in **Table 2**.

Table 2. Antimicrobial activity of the isolates from the heartwood of *A. adianthifolia.*

Sample	Microbial strains and MIQ (µg)
	Gram (–ve) bacteria Gram (+ve) bacteria Fungus
	E. coli P. aeruginosa B. subtilis S. aureus C. albicans
Compound 1	100 50 100 100 50
Compound 2	>100 50 10>100 10
Compound 3	>100>100>100>100 50
Compound 4	100>100 50 50>100
Compound 5	>100>100>100>100>100
Compound 6	50>100 50 50>100
Chloramphenicol	0.01 0.01 0.01 0.01 NT
Miconazole	NT NT NT NT 0.01
NT: not tested; >100 not active at a loading of 100 µg	

Compound **1** showed a broad spectrum of activity against all the test organisms. Most potent activity of **1** was observed against *P. aeruginosa* and *C. albicans* [both with MIQ values at 50 µg]. However, **1** showed poor activity against *E. coli*, *B. subtilis*, and *S. aureus* [both with MIQ values of 100 µg]. Compound **2** showed moderate activity against *B. subtilis* and *C. albicans* [MIQ 10 µg]. It also displayed moderate activity against *P. aeruginosa* and with no activity against *E. coli* and *S. aureus*. Compound **3** only exhibited weak activity [MIQ 50 µg] against *C. albicans* while compound **4** showed poor antibacterial response against *E. coli* [MIQ 100 µg] and moderate responses against *B. subtilis* and *S. aureus* [MIQ 50 µg]. Compound **5** did not show any activity against all the test organisms. Lastly, compound **6** showed weak activity [MIQ 50 µg each] against *E. coli, B. subtilis* and *S. aureus.* The agar overlay antimicrobial activity revealed sporadic/varied weak to moderate activity against the test organisms. To some extent, the compounds activity perhaps explains the ethnomedicinal potency of the species.

CONCLUSION

The mid polar extract ($CHCl_3$) of *A. adianthifolia* is mainly composed of several classes of secondary metabolites. Three flavonoids from the heartwood of *A. adianthifolia* **1-3**, an epoxide **4**, and a sugar **5**, were obtained from the $CHCl_3$ extract while a benzenoid **6** was isolated from the EtOAc fractions of the partitioned MeOH extract. All these constituents isolated and reported herein **(1-6)** are to the best of our knowledge reported from *A. adianthifolia* species for the first time. Thus, contributed to the diversity of secondary metabolites in the species. Secondly, the antimicrobial activity demonstrated against some of the selected Gram negative and Gram positive and fungal strains by these constituents may be said to supports some of the acclaimed traditional uses. The physiological action of the species could be ascribed to the individual potencies of the constituents or due to synergistic effects.

EXPERIMENTAL

3′,4′,7-trihydroxy-5-methoxyflavone 1

R_f: 0.37 (acetone-*n*-hexane, 7:3).

IR (KBr): 1634, 1603, 1566 cm^{-1}.

UV/Vis λ_{max} (MeOH) nm: (203), 338, 360(s), [+$AlCl_3$, $AlCl_3$/HCl 337, 360(s)].

^{1}H NMR (300 MHz, CD_3OD): 7.37 (1H, dd, *J*=8.9, 2.0 Hz, H-6′), 7.35 (1H, d, *J*=2.0 Hz, H-2′), 6.92 (1H, d, *J*=8.9 Hz, H-5′), 6.55 (1H, d, *J*=1.7 Hz, H-6), 6.50 (1H, s, H-3), 6.43 (1H, d, *J*=1.7 Hz, H-8), 3.91 (3H, s, OMe). **^{13}C NMR** (75.5 MHz CD_3OD): 178.9(C), 164.2 (C), 162.3 (C), 161.0 (C), 159.9 (C), 149.2 (C), 145.6 (C), 122.3 (C), 118.5 (CH), 115.4 (CH), 112.5 (CH), 106.6 (C), 105.1 (CH), 96.4 (CH), 95.1 (CH), 55.0 (OMe).

MS (EI, 70 eV): *m/z* (%)=300.1 [M^+] (100), 286.1 (50), 257.1 (21), 167. 0 (12), 153.0 (17), 137. 0 (12), 124. 0 (9). HREI-MS: *m/z* [M^+] calcd for $C_{16}H_{12}O_6$: 300.0644; found: 300.064.

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