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Isolation and characterization of an anti-microbial biflavonoid from the chloroform-soluble fraction of methanolic root extract of *Ochna schweinfurthiana* (Ochnaceae)

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The chromatographic investigation of the chloroform soluble fraction of methanolic root extract of *Ochna schweinfurthiana* using a combination of silica and sephadex LH-20 led to the isolation of tri-methoxy derivative of lophirone A. The structure of this new compound was determined using both 1 and 2D nuclear magnetic resonance (NMR). The antimicrobial activity of the isolated compound was also investigated using agar diffusion and broth dilution techniques. Clinical isolates obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria were used for the studies. The compound was found to show activity against *Staphylococcus aureus*, *Streptococcus pyrogenes, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Samonella typhi*, but was not active at the tested dose on methicillin resistant *S. aureus, Bacillus subtilis, Corynobacterium ulcerans, Escherichia coli* and the only fungi tested *Candida albicans.* The low concentration for minimum inhibitory concentration (MIC, 5 µg/ml) and minimum bactericidal concentration (MBC, 20 µg/ml) suggests the compound has a good antimicrobial activity against the susceptible organisms and validates the ethno medicinal use of the plant in the treatment of various bacterial infections, including infected wounds and typhoid fever.

Key words: *Ochna schweinfurthiana* extract, antimicrobial, tri-methoxy, lophirone A, nuclear magnetic resonance (NMR).

INTRODUCTION

Antimicrobial resistance (AMR) is not a recent phenomenon, but it is a critical health issue today that has evolved to become a worldwide public health threat (World Health Organization (WHO), 2012). AMR is driven by both appropriate and inappropriate use of anti-infective medicines for human, animal health and food production,

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production, together with inadequate measures to control the spread of infections (WHO, 2002; Goosens et al., 2005; Mathew et al., 2007; Orzech and Nichter, 2008).

At least 2 million people become infected with bacteria that are resistant to antibiotics each year in the United States out of which about 23,000 die as a direct result of these infections, while many more people die from other complications related to antibiotic resistance (Centers for Disease Control and Prevention (CDC), 2013). Most European countries similarly witness a seemingly unimpeded increase of antimicrobial resistance in the major Gram-negative pathogens which could unavoidably lead to loss of therapeutic treatment options (European Centre for Disease Prevention and Control (ECDC), 2012).

Natural products have been the most significant source of drugs and drug leads in history (Cragg and Newmann, 2005). The emergence of multidrug resistance in human and animal pathogenic bacteria as well as undesirable side-effects of certain antibiotics has triggered immense interest in the search for new antimicrobial drugs of plant origin (Ahmed and Beg, 2001). Theoretically, bacteria will continue to develop resistance once exposed to any antimicrobial agent, thereby imposing the need for a permanent search and development of new drugs (Silver and Bostian, 1993). Amongst the priority actions required to hold back the spread of AMR and curtail its potential catastrophic effect is the continuing research and development of newer effective antimicrobial agents (European Commission (EC), 2011) and higher plants represent a potential source of novel antibiotic prototypes (Meurer–Grimes et al., 1996).

Ochna schweinfurthiana F Hoffm is a shrub or small tree up to 4 m tall that belongs to the family Ochnaceae. The family has been reported to be a rich source of complex dimmers of biflavonoids and chalcones (Anuradha et al., 2006; Ichino et al., 2006; Jayaprakasam et al., 2000; Kaewamatawong et al., 2002). We have previously reported the anti-microbial effect of the methanol and acetone extracts of the stem bark and leaves extract of the O. *schweinfurthiana* (Abdullahi et al., 2010) and also isolated and investigated the anti-microbial activity of a flavonoid di-glycoside from the butanol soluble fraction of methanolic leaves extract of *O. schweinfurthiana (*Abdullahi et al., 2011*)*. In continuation of our search for bio-active compounds from the plant, we hereby report the isolation and antimicrobial effect of a new biflavonoid from the chloroform soluble fraction of the methanol extract of the root of O. *schweinfurthiana.*

MATERIALS AND METHODS

Collection, identification and preparation of plant

The whole plant material of *O. schweinfurthiana* was collected in Samaru-Zaria, Nigeria in June 2007. It was authenticated by Mr. Musa Muhammad of the herbarium section of Biological Sciences Department, Ahmadu Bello University, Zaria, and a voucher specimen (number 900229) was deposited. The leaves were removed, air-dried and powdered.

Extraction

The powdered root of *O. schweinfurthiana* (2.7 kg) was extracted three times each for 3 days with methanol (3.5 L) by cold maceration. The solvent was removed *in vacuo* to afford a reddish-brown product (336 g) referred to as *O. schweinfurthiana* root methanol extract coded (OSR). 100 g of the extract was suspended in distilled water and filtered. The filtrate was then successively partitioned with hexane, chloroform, ethylacetate and n-butanol to afford chloroform, ethylacetate, butanol and residual aqueous fractions.

Thin layer chromatography (TLC)

The chloroform-soluble portion of the methanol extract of *O. schweinfurthiana* (SRC) was subjected to TLC using pre-coated aluminium plate. The solvent systems used were chloroform and methanol (9:1 and 15:1). The spots on TLC were visualized under ultraviolet (UV), spraying with Gibbs reagent followed by exposure to ammonia solution or spraying with 10% sulphuric acid followed by heating at a temperature of 110°C for 5 to 10 min.

Chromatographic separation

Separation of chloroform-soluble fraction of O. schweinfurthiana root extract

Column chromatography, silica gel: Chloroform soluble fraction (5 g) was mounted over a glass column (75 × 3.5 cm) packed with silica gel (60 to 230 mesh). The column was eluted continuously using n-hexane, dichloromethane and methanol mixture by gradient elution technique; 50 ml each was collected as eluates and the progress of elution was monitored using TLC. A total of 10 fractions (A1-A10) from 110 collections were made; fractions A7 and A8 (coded SR-8A) were combined and further purified by Sephadex LH-20 gel filtration column chromatography. In both cases, the progress of separation was monitored using TLC.

Gel filtration: Further purification of SR-8A was undertaken using sephadex LH-20 eluted with methanol. The progress of separation was monitored using TLC. Repeated gel filtration led to the isolation of a compound coded 8A.

Phytochemical analysis

The isolated compound was subjected to shinoda and ferric chloride tests (Silva et al., 1998).

Spectral analysis

UV spectra were obtained in methanol on a Helios-zeta, UV-VIS Spectrophotometer. IR spectra was recorded (KBr) on Shimadzu FTIR8 400S Fourier Transform Infrared Spectrophotometer. NMR spectra (both 1D and 2D) were obtained on a Bruker AVANCE (600 MHz for ^{1}H and 125 MHz) for ^{13}C spectrometer, using the residual solvent peaks as internal standard. Chemical shift values (δ) were reported in parts per million (ppm) relative to internal solvent standard and coupling constants (J values) were given in Hertz. Heteronuclear multiple bond correlation (HMBC) spectroscopy spectra were optimized for a long range $J_{H\text{-}C}$ of 7Hz ($d_6 = 0.07s$).

The solvent used was deutrated chloroform ($CDCl_3$)

Antimicrobial assay

The microorganisms tested include *Staphylococcus aureus,* methicillin resistant *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis, Corynobacterium ulcerans, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia, Salmonella typhi* and *Candida albicans*. All the organisms were clinical isolates obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria.

Reference drug

Sperfloxacin 5 mcg/disc made by Himedia Laboratories Pvt. Ltd. Mumbai, India was used as the reference drug.

Susceptibility studies

Preliminary antimicrobial activity of the compound was carried out using stock concentration of 20 µg/ml. The microorganisms were maintained on agar slant. The inocula were prepared by inoculating the test organisms in nutrient broth and incubating them for 24 h at 37°C for the bacteria, while for *C. albicans*, Sabouraud Dextrose broth was used and was incubated for 48 h. After incubation, the broth cultures were diluted to 1:1000 for the Gram-positive bacteria and 1:5000 for the Gram-negative bacteria. One milliliter of the diluted cultures was inoculated into a sterile molten nutrient agar at 45°C and poured into sterile petri-dish. Similarly, 1 ml of the diluted fungal suspension was poured into sterile Sabouraud dextrose agar plates and the excess sucked up with Pasteur pipette. These were swirled gently and allowed to solidify. Wells were bored into the solidified inoculated nutrient agar plates using cork borer of 6 mm diameter. The wells were filled with 0.1 ml of the compound. Sparfloxacin standard disc was also placed on the agar plate. 1 h was allowed for the compound to diffuse into the agar after which the plates were incubated overnight at 37 and 25°C for bacteria and fungi, respectively. At the end of incubation period, diameter of inhibition zone was measured using transparent ruler and recorded. The compound and standard antibiotic were tested in duplicate and mean zones of inhibition were calculated.

Minimum inhibitory concentration (MIC)

MIC was determined using broth dilution method (Volleková et al., 2001). Two fold serial dilutions of the compound were made to obtain concentrations of 20, 10, 5, 2.5 and 1.25 µg/ml. 0.2 ml suspension of standard inoculum of each organism was inoculated to the different concentrations of the compound. The test tubes were then incubated at 37°C for 24 h after which they were observed for growth. Inhibition of growth was indicated by a clear solution. The MIC was defined as the lowest concentration of the compound inhibiting the visible growth of each microorganism.

Minimum bactericidal concentration (MBC)

The contents of the MIC tubes and the preceding tubes in the serial dilution were sub-cultured into appropriately labeled nutrient agar plates by dipping a sterile wire loop into each test tube and streaking the surface of the labeled nutrient agar plates. The plates were then incubated at 37°C for 24 h after which they were observed for colony growth. The lowest concentration of the subculture with no growth was considered as minimum bactericidal concentration (Volleková et al., 2001).

RESULTS AND DISCUSSION

Compound 8A was obtained as a colorless amorphous powder (15 mg) from the chloroform-soluble fraction of *O. schweinfurthiana* root. It gave pink-reddish colour upon spraying with 10% H_2SO_4 in methanol and heating over hot plate at 110°C plate for 5 to 10 min. It also gave a purple colour on spraying with GIBBS reagent signifying the presence of phenolic ring.

The UV in methanol showed absorptions at 243, 247 and 277nm suggestive of an isoflavonoid nucleus (Enas et al., 2012). The IR spectrum (KBr), Vmax cm^{-1} comprised of absorption bands at 3061 (C-H aromatic stretching), 1574 and (aromatic C=C). Absence of absorption band at 1620 to 1670 suggests compound as an isoflavonoid or a chalconoid (Mabry et al., 1970; Peng et al., 2006).

The ^{1}H-NMR spectrum exhibited a characteristic proton signal downfield at δ_H 12.62 corresponding to a free H-bonded OH group. It also displayed signals typical of a 1, 2, 4-trisubstituted benzene ring indicated by a set of meta-coupled proton at (δ_H 6.70 (d, *J*=2.2 Hz, H-3B_1), δ_H 6.77 (dd, *J*=2.2 Hz, 8.58 Hz, H-5B_1) and an ortho-coupled proton downfield at δ_H 7.93, (d, *J*=8.94), representing H-6B_1 (Mabry et al., 1970). The proton resonance which appeared as a singlet further downfield at δ_H 8.06 presumably due to the influence of a keto group suggests a H-β_1 proton in an isoflavone system (Mabry et al., 1970; Pegnyemb et al., 2003).

Another set of 1, 2, 4-trisubstituted benzene ring system is represented by ^{1}H NMR resonances at (δ_H 6.24 (d, *J*=2.5 Hz, H-3'), δ_H 6.39 (dd, *J*=2.5 Hz, 9.1 Hz, H-5') and an ortho-coupled proton downfield at δ_H 8.17 (d, *J*=9.1, H-6')) (Pegnyemb et al., 2003). This ring sub structure is in close proximity with a carbonyl group as confirmed by the HMBC spectroscopy.

The ^{1}H NMR spectrum also exhibited two closely overlapping 1, 4-disubstituted benzene rings which was adequately visualized with Bruker Topsins Software. The protons integrated for 8 hydrogens and these 2 AA'BB' systems comprise of ortho-coupled protons at δ_H7.22, (d, *J*=8.7, 2H) assignable to H-2''/6'' and δ_H6.64 (d, *J*=8.7, 2H) assignable to H-3''/5'' in Ring A_1 as well as δ_H7.19 (d, *J*=8.52, 2H) assignable to H-2'''/6''' and δ_H6.68 (d, *J*=8.52, 2H) assignable to H-3'''/5''' of Ring A_2. The above assignment was corroborated by the Homonuclear Correlation Spectroscopy (^{1}H-^{1}H COSY) and the Heteronuclear Correlation Spectroscopy (HSQC).

The ^{13}C-NMR spectrum exhibited signals for 33 carbons, including the intensely overlapping (double) signals at δ_c129.12 (C-2''/6''), 128.43 (C-2'''/6'''), 113.87 (C-3''/5'') and 113.92 (C-3'''/5''') (Markham et al., 1978). The carbon chemical shift values showed 8 of the carbons are oxygenated. The distortion-less enhancement

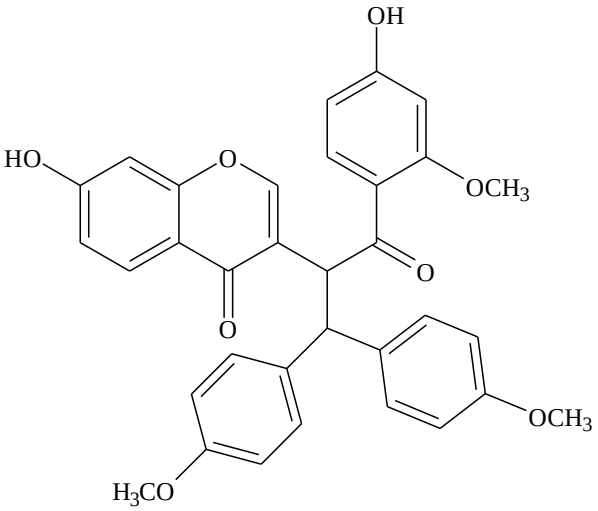

Figure 1. Tri-methoxy lophirone A.

by polarization transfer (DEPT) experiments established the nature and chemical environments of the carbon atoms and allowed the identification of 13 quaternary carbon atoms including 2 carbonyl groups at δ_c 203.47 and 175.28, and 15 aromatic sp^2 hybridized carbon atoms suggesting the presence of about 4 benzene rings in the molecule. The 3 singlet signals at δ_c 55.53, 55.13 and 55.06 are due to methoxy (OCH_3) group attached to aromatic ring system. The ^{13}C-NMR spectrum and the DEPT experiments also showed 2 aliphatic sp^3 carbon resonances at δ_c 52.97 and 43.04 corresponding to ^{1}H NMR aliphatic resonances at δ_H 4.69 (d, J=12.3) and δ_H 6.07 (d, J=12.3) due to α- and β protons, respectively. The cross peaks observed in the ^{1}H-^{1}H COSY further confirmed these aliphatic resonances as an ethinyl chain.

Heteronuclear multiple bond correlations between protons and carbons in the molecule (up to 3 bonds) facilitated the assignments of the H- and C-signals not captured in the HSQC, the quaternary carbons, the three methoxy groups as well as established the connectivity between the ethinyl chain to the three aforementioned substructures.

The summary of the 1 and 2D results of compound 8A established and characterized the structure of 8A as tri-methoxy lophirone A (Figure 1), which is, to our knowledge, a new compound. Tri-methoxy lophirone A is a derivative of known compounds: lophirone A (Ghogomu et al., 1987; Messanga et al., 2001; Pegnyemb, 2003; Anuradha et al., 2006), calodenone (Messanga et al., 1992; Pegnyemb et al., 2003; Likhitwiyawuid et al., 2005; Anuradha et al., 2006) and afzelone D (Pegnyemb et al., 2003). The difference between Compound 8A (tri-methoxy lophirone A) and the three known compounds, being the presence of 3-methoxy groups in 8A instead of none, one and two methoxy groups in lophirone A, calodenone and afzelone D, respectively (Table 1).

The results of antimicrobial investigations suggest the compound has activity against *S. aureus*, *S. pyrogenes, P. aeruginosa, K. pneumonia* and *S. typhi*, but were not active, at the tested dose, on methicillin resistant *S. aureus, B. subtilis, C. ulcerans, E. coli* and the only fungi tested, *C. albicans* (Table 2).

The low concentration for the MIC (5 µg/ml) and the MBC (20 µg/ml) as reflected in Table 3 suggests the compound has a good antimicrobial activity against the susceptible organisms considering that compounds with MICs of less than 100 µg/ml are regarded as having strong antimicrobial potential (Tang et al., 2003).

S. pyogenes has been implicated in many important human diseases including skin infections, pharyngitis and rheumatic fever, and certain strains have already developed resistance to macrolides, tetracyclines and clindamycins (Malhotra-Kumar et al., 2009).

P. aeruginosa and *K. pneumonia* have also been implicated along with *E. coli* amongst the causative agents in urinary tract infection (UTI) which is the most common infectious diseases at the community level (Linhares et al., 2013).

S. typhii bacteria is the causative agent for typhoid fever and this work has further confirmed the ethno-medicinal use of various parts of *O. schweinfurthiana* in treating typhoid fever, skin and other bacterial infections.

Conclusion

Conclusively, the results of the study showed the root of *O. schweinfurthiana* contains an antimicrobial biflavonoid (tri-methoxy lophirone A) as one of its constituents: its antimicrobial activity is significant and may serve as a lead towards the development of more potent, safe and cost effective antimicrobial agents. The result also validates

Table 1. ^{1}H NMR, ^{13}C NMR, DEPT, HSQC and HMBC Summary on 8A in ($CDCl_3$).

Position	DEPT	δC	δH, *J* = Hz	HMBC
B_1-1	C	117.1	-	-
2	C	157.7	-	-
3	CH	114.02	6.70 (1H, d, *J* = 2.22 Hz)	C-1, 5
4	C	161.1	-	-
5	CH	115.06	6.77 (1H, dd, *J* = 2.22, 8.6 Hz)	C-1, 3
6	CH	127.94	7.93 (1H, d, *J* = 8.94 Hz)	C-2, 4, c_{-1}
c_{-1}	C	175.28	-	-
α_1	C	121.19	-	-
β_1	CH	155.26	8.06 (1H, s)	C-2, c_{-1} , α_1, α_2
B_2-1’	C	113.7	-	-
2′	C	166.8		
3′	CH	100.83	6.24 (1H, d, *J* = 2.34 Hz	C-1’, 2’, 4’, 5’
4′	C	165.8	-	-
5′	CH	107.96	6.39 (1H, dd, *J* = 2.34, 9.1 Hz)	C-1’, 3′
6′	CH	132.67	8.17 (1H, d, *J* = 9.1 Hz)	C-, 2′, 4′ and c_{-2}
c_{-2}	C	203.49	-	-
α_2	C	43.04	6.07 (H, d, *J* = 12.12 Hz)	C- β_1, β_2, α_1, c_{-1}, c_{-2},1″, 1‴
β_2	C	52.97	4.69 (H, d, *J* = 12.12 Hz)	C- 1″, 2″, 1‴, 2‴ c_{-2}
A_1- 1″	C	135.28	-	-
2″	CH	129.12	7.22 (2H, d, *J* = 8.7 Hz)	C- 2″, 3″, 4″, β_2
3″	CH	113.87	6.64 (2H, d, *J* = 8.7 Hz)	C-1″ 3″, 4″
4″	C	157.99		
5″	CH	113.87	6.64 (2H, d, *J* = 8.7 Hz)	C-1″ 3″, 4″
6″	CH	129.12	7.22 (2H, d, *J* = 8.7 Hz)	C- 2″, 3″, 4″, β_2
A_2- 1‴	C	134.16	-	-
2‴	CH	128.43	7.19 (2H, d, *J* = 8.52 Hz)	C- 2‴ 3‴, 4‴, β_2
3‴	CH	113.92	6.68 (2H, d, *J* = 8.52 Hz)	C-1‴ 3‴, 4‴
4‴	C	157.91	-	-
5‴	CH	113.92	6.68 (2H, d, *J* = 8.52 Hz)	C-1‴ 3‴, 4‴
6‴	CH	128.43	7.19 (2H, d, *J* = 8.52 Hz)	C- 2‴ 3‴, 4‴, β_2 ,
OH	-	-	12.62 s	-
2-OCH_3	CH_3	55.53	3.72 s	-
4”-OCH_3	CH_3	55.13	3.66 s	-
4”-OCH_3	CH_3	55.06	3.60 s	-

Table 2. Susceptibility of 8A to various pathogenic test organisms.

Test organism	Mean zone of inhibition (mm)	
	8A (20 µg/ml)	Sparfloxacin (5 µg/ml)
Staphylococcus aureus	19	22
Methicillin resistant *Staph. aureus*	0	19
Streptococcus pyogenes	17	24
Bacillus subtilis	0	27
Corynbacterium ulcerans	0	19
Escherichia coli	0	14
Klebsiella pneumoniae	19	14
Salmonella typhi	17	27
Pseudomonas aeruginosa	16	17
Candida albicans	0	0

0: No activity.

Table 3. Minimum inhibitory and minimum bactericidal concentrations of 8A against the test organisms.

Test organism	Concentration (µg/ml)	
	MIC	MBC
Staphylococcus aureus	5	20
Methicillin resistant Staphylococcus aureus	R	R
Streptococcus pyogenes	5	20
Bacillus subtilis	R	R
Corynbacterium ulcerans	R	R
Escherichia coli	R	R
Klebsiella pneumoniae	5	20
Salmonella typhi	5	20
Pseudomonas aeruginosa	5	20
Candida albicans	R	R

R: Resistant, no activity against organisms.

the ethno medicinal use of the plant in the treatment of various bacterial infections, including infected wounds and typhoid fever. This is the first report of isolation of this compound, to the best of our search.

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