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ISOLATION AND CHARACTERISATION OF A NOVEL XANTHONE WITH BROARD-SPECTRUM ANTIBACTERIAL ACTIVITY FROM THE ROOTS OF *ALLANBLACKIA FLORIBUNDA* OLIVER (GUTTIFERAE)

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ABSTRACT

The increasing antimicrobial resistance of pathogens to existing therapeutic agents is currently a global health challenge, which led us to a bioassay-guided investigation of *Allanblackia floribunda* Oliver (Guttiferae) roots for new antimicrobial constituents. The roots were extracted successively in Soxhlet with hexane, ethyl acetate and methanol. Isolation from the ethyl acetate extract was done using an open Column Chromatography, and Preparative Thin Layer Chromatography for purification. Structural elucidation was done using extensive 1-D and 2-D NMR, IR, MS data and physico-chemical properties. Antimicrobial activity and Minimum Inhibitory Concentration (MIC) were determined using agar-well diffusion and broth dilution methods, respectively. The root extracts, pooled fractions and isolated compound were tested on bacteria including *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633 and clinical isolates of *Salmonella typhi*. The root extracts and pooled fractions (Al_1, Al_2A, F_{19}, A_{18} and A_{100}) were active on the test bacteria with zones of inhibition ranging from 12 to 20 mm. Fraction Al_2A showed highest consistent activity comparable to gentamycin standard and yielded compound **Al_2**, a novel xanthone: **1, 2, 3, 4, 5, 8-hexahydroxy-9H-xanthen-9-one**. The MICs in mg/mL of Al_2 on the test organisms are: *S. aureus* (0.75), *E. coli* (1.25), *P. aeruginosa* (1.13), *B. subtilis* (0.15) *and S. typhi* (0.08). These results justify the ethnomedicinal use of the root bark of *Allanblackia floribunda* in the treatment of diseases and the isolated xanthone has a high potential to become a chemotherapeutic agent for bacterial infections.

INTRODUCTION

Antimicrobial resistance has contributed a lot to morbidity and mortality of the populace globally. The prevailing increase in resistance of bacterial pathogens to most commonly used antibiotics and chemotherapeutic agents necessitates the search for new and effective antimicrobial agents. Recent researchers are giving attention to exploration into the plant, animal and marine kingdoms looking for better antibacterial, antifungal and antiviral principles to combat the menace of antimicrobial resistance. Medicinal plants and their constituents are broadly used in traditional medicine and have led to the development of new pharmaceutical remedies [1-3].

→*Allanblackia floribunda* Oliver, belonging to the family Guttiferae (subfamily Clusiodeae), is traditionally useful in Nigeria and Cameroun for the treatment of various diseases such as typhoid, gastrointestinal infections, among others [4]. Various traditional and pharmacological uses of the plants have been reported. The decoction of the leaves and fruits of *A. floribunda* have been reported to be useful in the treatment of malaria and toothache. The root, stem bark and leaves are used locally in the treatment of viral infections such as measles, chickenpox and smallpox, while the fatty component in the seeds is a mild purgative [4, 5]. Many bioactive compounds have been isolated from the leaves, stem bark and roots. The root bark and hearthwood have been reported to contain xanthones, benzophenones and biflavonoids, some of which possess wide range of pharmacological activities such as cytotoxicity, anti-inflammatory, antihypertensive, antioxidant and antimicrobial [6-8]. Kuete et al. reported antitumour, antioxidant and antimicrobial activities of the extract and isolated compounds [9]. Phytochemical screening conducted on the leaves, stem bark and root bark of A. floribunda indicated the presence of tannins, alkaloids, anthraquinones, phenols, anthocyanines, cardiac glycosides, flavonoids, terpenes and saponins [10-11]. Other bioactivities of the plant reported include aphrodisiac, ejaculatory and vaso-relaxant properties [12, 13]

A myriad of compounds belonging to different classes of secondary metabolites have been previously isolated from *A. floribunda.* These include: two xanthones with two rotameric (3→8) bioflavonoids [14], a new prenylated xanthones (1,5-dihydroxyxanthone) from the stem bark [15] and some other known compounds [16]. Other xanthone derivatives isolated include Allanxanthone-A, 1,5-dihydroxyxanthone, 1,3,6,7-tetrahydroxy-2-(-3-methylbut-2-enyl) xanthone, Forbexanthone and Guttiferone-F [17-19]. The present work was therefore aimed at evaluating the root extracts *A. floribunda* for antibacterial activity and using bioassay-guided method to isolate the antimicrobial compound(s) from the chromatographic fractions.

RESULTS AND DISCUSSION

Isolation of compound from *A. floribunda*: The results of the ^{1}H-NMR and ^{13}C-NMR in (DMSO-d_6) signals are presented in Table 1, while the structure of the isolated compound (Al_2) is

shown in Figure 1.

Antimicrobial Activity of extracts, fractions and isolated compound from the root of *A. floribunda*: The antimicrobial assay of the root fractions and antimicrobial activity of chromatographic fractions *A. floribunda* as presented in Tables 2 and Table 3, respectively. The minimum inhibitory concentration (MIC) of isolated xanthone Al_2 in (mg/mL) was presented in Table 4.

Characterisation and Structural Elucidation

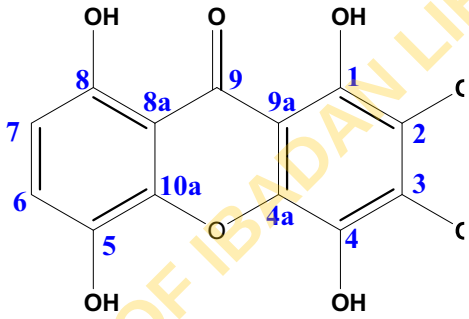
1, 2, 3, 4, 5, 8-hexahydroxy-9H-xanthen-9-one; [Al_2]

Crystalline yellow powder; 29 mg, R_f 0.7 (Methanol: Acetone; 2:5), composition: $C_{13}H_8O_8$. M.p: 188-191^{O}C. Molecular weight: 292 g/mol. IR (KBr) *Vmax*: 3415, 1641, 1516, 1448, 1368, 1262, 1262, 1111, 1089, 1051 cm^{-1}; ESI-MS: m/z (relative abundance, %): 91 [45] 127 [55], 291 [M^+-H, 100], 292 [M^+, 2], 293 [M^++H, 2]. ^{13}C NMR (DMSO-d_6, 150 MHz) δ 151.6 (C-1), 150.2 (C-2), 150.1 (C-3), 138.4 (C-4), 146.1 (C-4a), 150.0 (C-5), 101.9 (C-6), 128.6 (C-7), 151.6 (C-8), 108.6 (C-8a), 182.1 (C-9), 114.8 (C-9a), 157.2 (C-10a). ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.13 (d, 1H, J = 6.0 Hz, H-6), 6.39 (d, 1H, J = 6.0 Hz, H-7).

Table 1: ^{1}H-NMR and ^{13}C-NMR (DMSO-d_6, 300 and 150 MHz) of 1, 2, 3, 4, 5, 8-hexahydroxy-9H-xanthen-9-one

S/N	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)	Carbon type
1	151.6	-	Qc
2	150.2	-	Qc
3	150.1	-	Qc
4	138.4	-	Qc
4a	146.1	-	Qc
5	150.0	-	Qc
6	101.9	7.13 (d, 1H, J = 6.0 Hz)	CH
7	128.6	6.39 (d, 1H, J = 6.0 Hz)	CH
8	151.6	-	Qc
8a	108.6	-	Qc
9	182.1	-	Qc
9a	114.8	-	Qc
10a	157.2	-	Qc

Note: Qc **represents quaternary carbon**



Al_2

Figure 1: Structure of Isolated compound; Al_2

Table 2: Antimicrobial Activity of Hexane, Ethyl acetate and methanol extracts of *A. floribunda* root

	Diameter Zone of Inhibition (mm) of Extracts at 20mg/mL						
Extracts	***S.aureus***	***E.coli***	***B.subtilis***	***P.aeruginosa***	***S.typhi* 16**	***S.typhi* 17**	***S.typhi* 18**
Hexane	16	15	20	14	-	12	14
Ethylacetate	6	15	20	14	15	-	12
Methanol	12	12	12	12	15	12	12
Gentamycin	25	30	25	20	20	20	20

NB: Gentamycin at10 µg/mL, - means no activity

Table 3: Antimicrobial activity of chromatographic fractions

Mean Zone of inhibition (mm) at 10.0 mg/Ml, Gentamycin at 10 µg/Ml

Fractions	***S. aureus*1**	***S. aureus*2**	***E. coli***	***P. aeruginosa***	***B. subtilis***	***S. typhi***
AL_1	13	20	-	13	20	-
Al_2	24	24	19	13	20	15
F_{19}	10	10	12	-	13	15
A_{18}	-	26	15	13	13	13
Al_{100}	-	14	-	12	-	-
Gentamycin	20	20	20	20	20	20

Key:- No activity

Table 4: Minimum Inhibitory Concentration (MIC) of isolated xanthone Al_2 in (mg/mL).

Compound	*S. aureus*	*E. coli*	*P. aeruginosa*	*B. subtilis*	*S. typhi 18*
Al_2	0.75	1.25	1.13	0.15	0.08

Compound Al_2 showed a blue color under UV light (253 nm), and a brown color when heated with 10% H_2SO_4 on TLC plate. A dark green colouration was observed with methanolic Ferric Chloride suggesting a phenolic compound. The presence of phenolic hydroxyl group and carbonyl group was apparent from the absorption bands at 3415 (OH) and 1641 (C=O) respectively. Other absorption bands at 1111, 1089, 1051 (C-O stretch) and 1448 (aromatic) cm^{-1} in the **IR spectrum**, the compound with **molecular mass** of 292 g/mol confirmed by the presence of *m/z* 292; being the molecular ion which also corresponds to the **molecular formula** $C_{13}H_8O_8$. The presence of a peak at *m/z* 127 indicates $C_6H_7O_3^+$. **The ^{13}C-NMR** spectrum indicates a total of thirteen carbon signals in the molecule of compound Al_2. Eleven of which are aromatic quaternary carbons (δ_C 182.1, 157.2, 151.6, 150.2, 150.0, 150.1, 146.1, 138.4, 114.8 and 108.6, alongside with two methine sp-hybridised carbon signals (δ_C 128.6 and 101.9). **The ^{1}H NMR** spectrum of compound Al_2 showed the presence of two aromatic methine proton signals as follows: a doublet methine peak at δ_H 7.13; integrating for one proton and a doublet proton signals at δ_H 6.39 both with (J = 6.0 Hz), were assigned to H-6 and H-7, respectively. The assignments were confirmed by **^{1}H-^{1}H COSY** spectrum of Al_2. **The HMBC** spectrum of 1, 2, 3, 4, 5, 8-hexahydroxy-9H-xanthen-9-one; Al_2, shows correlation between H-7 (δ_H 6.39) and C-6 (δ_C 101.9); this suggests the presence of the methine carbons at C-6 and 7 respectively. Also, a correlation of H-6 (δ_H 7.13) with C-5a (δ_C 157.2) was observed; this suggests an oxo-bridged bond linked to C-5a. **The novel compound** Al_2 is an isomer of the previously identified compound 1, 2, 3, 4, 6, 8-hexahydroxy-9H-xanthen-9-one [23]. The methoxylated derivative of Al_2; 1, 2, 3, 4, 6, 7-hexamethoxyxanthone was previously isolated, characterised and derivatised in *Polygala macradenia* [24].

Antimicrobial resistance (AMR) has been on the increase globally, leading to the prevalence of infectious diseases. Consequently, there is constant search for new drugs with improved antimicrobial actions from plant origin [25]. Hence, *A. floribunda* root extracts were tested on five human pathogenic bacteria; *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Salmonella typhi*. The result in Table 2.0 showed that the crude extract of the root of *A. floribunda* inhibited the growth of *S. aureus*, *E. coli*, *B. subtilis*, *P. aeruginosa* and *S. typhi* strains with the higher mean diameter inhibiting zones (MDZ) ranging from 12 to 20 ± 0.5 mm. The antimicrobial activity of the ethyl acetate extracts is comparable to that of the control drug (Gentamycin). This is in agreement with the antimicrobial activity of crude extract of the plant parts reported by Azuonwu and Somba [26]. This justifies the previous report [17], supporting the ethnomedicinal use of the roots of the plant for treatment of certain microbial infections, and also the usage of the leaves traditionally to treat stomach ache and stomach disorder [4,5].

The result from the assay of the **pooled fractions** (Al_1, Al_2A, F_{19}, A_{18} and A_{100}) revealed that all the test organisms (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Salmonella typhi*) showed considerable susceptibility to the isolated fractions comparable to gentamycin used as control. However, Al_2 showed a more consistent activity (than other fractions) which is also comparable to the standard drug. The fact that many fractions showed antibacterial activity is a proof that the roots of *A. floribunda* contain many chemical compounds with antibacterial action, though the compounds may be analogous in structure (related TLC patterns but differing R_f values). This corroborates the previous antibacterial reports on the plant parts [5, 7, 9]. Among the previously isolated compounds from the plant and allied species, morelloflavone and allanxanthones have showed good antimicrobial activity [9, 17]. Generally, many xanthones [14, 16] have demonstrated excellent antibacterial activity worthy of a potential drug candidate. The new compound isolated in this study is also a xanthone, showing that *A. floribunda* parts are rich in xanthones that have good antimicrobial property. The MIC of the isolated and characterized xanthone; Al_2 from the root of *A. floribunda* on the different test organisms are as follows: *S. aureus, E. coli, P. aeruginosa, B. subtilis and S. typhi* with MIC of 0.75, 1.25, 1.13, 0.15 and 0.08 mg/mL, respectively. This result shows that Al_2 has a broad spectrum antibacterial activity, having inhibitory effect on both gram positive and gram-negative bacteria.

CONCLUSIONS

The investigation of the root extract of *Allanblckia floribunda* has confirmed its potency against tested bacteria including *S. aureus, E. coli, P. aeruginosa, B. subtilis* and *S. typhi* 18 at all tested concentrations. These results justify the ethnomedicinal use of the root bark of *A. floribunda* in the treatment of microbial infections. Further, it is obvious that *A. floribunda* roots contain many antimicrobial compounds that can be developed, especially by molecular docking, as chemotherapeutic agents to treat bacterial infections. More research is necessary to isolate and characterize more compounds, and to investigate the mechanism of antimicrobial action of the Al_2 (**1, 2, 3, 4, 5, 8-hexahydroxy-9H-xanthen-9-one**).

MATERIALS AND METHODS

Plant material

The leaves and root of *A. floribunda* were collected from Olokemeji Forest Reserve (7.42 N, 3.55 E) located between the cities Abeokuta and Ibadan, South-West of Nigeria. The plant parts were authenticated at the herbarium section of Forestry Research Institute of Nigeria (FRIN) with the voucher specimen number FHI 52102. The roots were washed, air-dried, pulverized and stored for use.

General experimental procedure

The solvents used were analytical grade from SIGMA. The IR spectra were obtained in KBr PerkinElmer-983. Melting point results were determined with a Reichert Austria 281313 melting point apparatus. The NMR spectra were recorded in deuterated solvent, dimethyl sulfoxide (DMSO-d_6) with TMS as internal standard on 300 MHz for proton, and 150 MHz for ^{13}C respectively. GC-MS spectra were recorded on Varian,

Palo-Alto, CA-USA spectrometer. Thin-layer chromatography (TLC) was performed on precoated silica gel plates (Merck, PF-254, 20 cm by 20 cm, 0.25 mm) size while column chromatography was carried out on silica gel (Merck, 70–230 mesh). Spots on the plates were visualized using UV (254, and 366 nm) and using 10% H_2SO_4 as a staining reagent, heating to about 110 °C.

Extraction and purification

The air-dried roots of *A. floribunda*. was extracted successively in n-Hexane, ethyl acetate and methanol using Soxhlet apparatus. The ethyl acetate fraction (9.0 g) were subjected to open column chromatography using gradient elusion system. A total of 315 fractions of 100 mL each were collected. These fractions were concentrated and fractions pooled based on TLC characteristics. The ethyl acetate fraction gave Al_1, Al_2A, Al_3, Al_{18} and Al_{100}. Re-chromatography of the mother liquor (Al_2A) using preparative Thin Layer Chromatography (PTLC) with a mixture of methanol and acetone in the ratio (2:3) to obtain a xanthone; yellow powder upon crystallization produces a white crystalline solid with the code Al_2.

The diagrammatic steps of bioassay-guided isolation procedure used for *A. floribunda* roots is shown in the Figure 2.

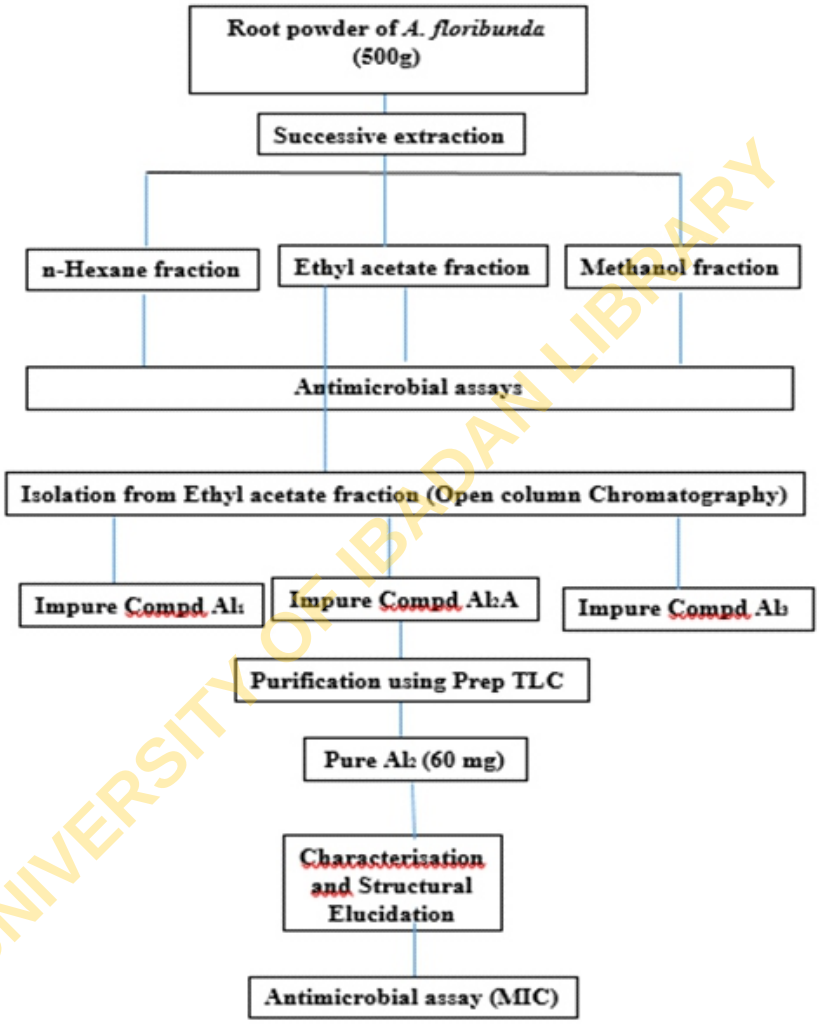


Figure 2: Diagrammatic steps of bioassay-guided isolation procedure used for *A. floribunda* roots.

Collection of Microorganisms Used

Bacteria used were typed strains and clinical isolates comprising *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Salmonella typhi* 16, *Salmonella typhi* 17, *Salmonella typhi* 18 (clinical isolates); they were obtained from the stocked culture in Pharmaceutical Microbiology Laboratory, University of Ibadan, Nigeria. The organisms were cultured on Nutrient Agar and Nutrient Broth pH 7.4 (Oxoid, England), maintained on agar slope at 4°C before use.

Determination of Antimicrobial Activity of Extracts, Fractions and Isolated Compound

The antibacterial activity of the extracts, chromatographic fractions and isolated compound were determined using agar-well diffusion method of Perez *et al*. [20] with slight modifications. Nutrient plates, containing 20mL of hot but cooled molten agar, were seeded with 200 µL of 10^{-2} overnight culture of each bacterial isolate (equivalent to 0.5 McFarland standard 1.0 x 10^8 CFU/mL). The seeded plates were gently mixed, allowed to set and a standard cork borer of 8.0 mm diameter was used to cut uniform, equidistant wells on the surface of the agar. The wells were then filled with 100 µL of

each extract at a concentration of 10-20 mg/ml in 40% methanol or DMSO (wells containing gentamicin at 10 µg/mL and 40% methanol or 2.0% DMSO were used as positive and negative controls, respectively. After a pre-incubation period of 60 mins at 4 °C, the plates were incubated at 37 °C for 24 hrs. Each test was carried out in triplicate. The zones of inhibition in mm were measured to determine antibacterial activity.

Determination of Minimum Inhibitory Concentration (MIC)

MIC of the isolated compound Al_2 was determined on *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Bacillus subtilis* using agar dilution method of Adeniyi *et al*. [21] and Andrews, [22] with slight modifications. The isolated compound AL2 at 10 mg/mL was serially diluted in a test tube to give concentrations 5.0, 2.5, 1.25, 0.625, 0.313, 0.157, 0.079 and 0.04 mg/mL. One milliliter of each dilution of the extract was mixed with 9 mL of Mueller Hinton agar, poured into 10 cm diameter petri dishes, allowed to set and dry for about 30 mins. Each plate was inoculated by streaking with 1:100 dilution of overnight broth cultures of each test organisms (containing 1.0×10^8 CFU/mL) and incubated for 24 h at 37 °C. Each test was carried out in triplicate. The least concentration that gave no visible colonies of the test organism was taken as the MIC of the compound.

Statistical analysis

Statistical analysis was performed using Student's t-test and significance of difference was accepted at $p < 0.05$. Data are presented as mean±SEM.

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