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RESEARCH ARTICLE

TWO AZULENE-TYPE SESQUITERPENES FROM ANTIMICROBIAL ROOT-BARK OF *DALBERGIA SAXATILIS* (FABACEAE)

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ABSTRACT

The root-bark of *Dalbergia saxatilis* (Fabaceae), a plant used traditionally in ethnomedicine in Nigeria, was extracted with 95% ethanol to give the crude extract. The crude extract was partitioned into acidic, basic and neutral polar and non-polar fractions. Preliminary phytochemical screening of *D. saxatilis* root bark crude extract revealed the presence of alkaloids, tannins, phenols, flavonoids, terpenoids, and glycosides. The antimicrobial screening was carried out on the crude extract and the fractions and all the samples were active against the test organisms: *Staphylococcus aureus, Salmonella .typhi, Escherichia coli, Bacillus substilis a*nd *Candida albicans* .The basic fraction was the least active against *S. typhi* while the neutral non-polar (hexane) fraction was the most active against *B. substilis.* Column chromatographic separation of the biologically active hexane fraction gave fractions which on GC-MS analysis and by comparison with the standard library computer MS data led to the identification of two azulene-type sesquiterpenes,1, 2,3,5,6, 7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)azulene,and1H-3a,7-methanoazulene,2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-.The presence of these constituents in the plant extract may account for the antimicrobial properties of the plant. Thus, the root bark of *D. saxatilis* can be harnessed for anti-infective purposes.

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INTRODUCTION

Dalbergia saxatilis is usually a climbing shrub, the branches being very tortuous, with some of them modified as woody spine hooks. The plant is harvested from the wild, mainly for local medicinal use and for its wood (Dalziel, 1937). The leaves are used medicinally to treat stomach disorders (Ayensu, 1978).The leaves decoctions are used as beverages and vapour bath for treating bronchial trouble in Cote d'Ivoire. The root is therapeutically employed as febrifuge analgesic and as anti-dysentery. In Southern Nigeria, the aqueous root extract is used by the natives to accelerate birth and expel the placenta in human subjects (Oliver, 1960). For over a decade researchers have investigated the bioactivities of different species of the genus, *Dalbergia* in an effort to validate the folkloric claims as well as identify some of their chemical constituents (Cheng *et al.,* 1998; Hajare *et al.,* 2001; Barragan-Huerta *et al.,* 2004; Mujumdar *et al.,* 2008; Okwute *et al.,* 2009, Okwute and Fatokun, 2014, Okwute and Isyaka, 2014, Ogwuda and Okwute, 2020).

In continuation of our studies on the species, *Dalbergia saxatilis*, we recently studied the root bark for its antimicrobial activity and chemical constituents. This work reports on the anti-infective properties of the crude methanol extract and fractions, and the identification of two azulene-type sesquiterpenes from the neutral non-polar fraction of the root bark using GC-MS analysis.

MATERIALS AND METHODS

The plant material, *Dalbergia saxatilis* was collected from Suleja Forest in Suleja Local Government area of Niger state, Nigeria. It was authenticated at the National Institute for Pharmaceutical Research and Development, Idu, Abuja and a voucher specimen was stored at the Herbarium (Number NIPRD/H/6647).The solvents used were of analytical grade or redistilled before use. Thin layer chromatography (TLC) was carried out on pre-coated Merck Kieselgel 60F 254 with thickness of 0.25mm. The spots were visualized by spraying with 10% sulphuric acid in methanol and heating on a hotplate for five minutes to view the spots.

The organisms used for antimicrobial activity screening were clinical isolates of bacteria and a fungus obtained from the Department of Microbiology, Ahmadu Bello University, Zaria. The isolates included *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonela typhi and Candida albicans* The culture media used in the antimicrobial screening included Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), Potato Dextrose Agar (PDA) and Nutrient Agar (NA). The media were used for sensitivity test, determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). All media were prepared according to manufacturer's instructions and sterilized by autoclaving at 121^{o}C for 15 minutes. The GC analysis was performed using Fison 8035 with a split injector and split ratio of 1:10. The column has Restek TRX-20 coating. It is a 30-metre long x 0.25 mm Id with a 0.25 micrometer thick coating. MS analysis was done using Fisons Trio with electron impact ionization. IR spectral analysis was done using Nicolet iS5 FTIR and NMR spectra were obtained with Jeol 400 MHz FT-NMR with multinuclear probe, Gradient Amplifier and Robotic Sample changer (2000) at Felician University in New Jersey and Lafayette College, Pennsylvania.

Extraction and Fractionation of Crude Extract: The air-dried root back of *D.saxatilis* was ground into powder and 1000g of the ground material was divided into two portions of 500g each. Each portion was then macerated in a round-bottomed flask with 1.5 litres of 95% ethanol and left to stand for 48 hours. The extract obtained was filtered and concentrated with a Rotary evaporator to give 24.25g of residue. The crude extract was subjected to a fractionation protocol (Mitscher *et al*., 1972). The crude extract (17.42g) was taken in 300cm^3 of dichloromethane and the solution was transferred into a separating funnel and extracted twice with an equal volume of 5% HCl. The upper aqueous HCl layer was combined and basified with NaOH to precipitate the bases which were extracted twice with dichloromethane. The dichloromethane extract was evaporated to dryness to give the basic fraction(1.00g). The original dichloromethane layer was extracted with an equal volume of 10% NaOH twice and combined. The aqueous layer containing the sodium salts was removed and acidified with 2M HCl to litmus red and extracted with dichloromethane twice. The dichloromethane layer containing the acids was then evaporated to dryness to give the acidic fraction (7.34g). The remaining dichloromethane layer was evaporated to dryness and the residue was dissolved in 10% aqueous methanol and extracted with hexane twice. Both the hexane and aqueous methanol layers were evaporated to dryness separately to give the neutral non-polar hexane fraction (0.99g) and the polar methanol fraction(4.04g), respectively.

Phytochemical screening of the crude ethanol extract: The phytochemical screening was done using standard procedures (Harborne, 1984; Sofowora, 1993; Trease and Evans, 2002).

Antimicrobial sensitivity screening of extracts: The inhibitory activity (sensitivity test) was determined using Agar Diffusion method as reported previously by Levenson (2010).

The standardized inocula of both the bacterial and the fungal isolates were streamed on sterilized Mueller Hinton and potato dextrose Agar plates, respectively, with the aid of a sterile swab stick. Folar wells were placed on each inoculated agar plate, respectively, with a sterile cork borer. The wells were properly labeled according to different concentrations of the extract prepared which were 100, 50, 25, and 12.5µg/ml, respectively. Each well was filled up with approximately 0.2ml of the extract. The inoculated plates with the extract were allowed to stay on the bench for about one hour to enable the extract diffuse on the agar. The plates were then incubated at 37^{0}C for 24 hours for Mueller Hinton Agar (MHA) plates, while the plates of potatoes dextrose Agar(PDA) were incubated at room temperature for about 3 days. At the end of incubation period, the plates were observed for any evidence of inhibition which will appear as a clear zone, completely devoid of growth around the wells (zone of inhibition).The diameters of the zones were measured using a transparent ruler calibrated in millimeter and the results were recorded.

The Minimum Inhibition Concentration (MIC) of the extract for each organism was determined using tube defiant method (Chikezie, 2017) with the Mueller Hinton broth used as defiant. The lowest concentration of the extract showing inhibition for each organism when the extract was tested during sensitivity test was severally diluted in the test tubes containing Mueller Hinton Broth. The organisms were inoculated into each tube containing the broth and the extract. The inoculated tubes were then incubated at 37^{0}C for 24 hours. They were observed for the presence or absence of growth using turbidity as a criterion. The lowest concentration in the series without visible sign or growth (turbidity) was considered to be the minimum inhibitory concentration (MIC).The result from the minimum inhibitory concentration (MIC) was used to determine the minimum bactericidal concentration (MBC) of the extract.

A sterilized wire loop was dropped into the test tube that did not show turbidity (clear) in the MIC test and a loopful was taken and streaked on a sterile nutrient agar plate. The plates were incubated at 37^{0}C for 24hrs. At the end of the incubation period, the plates were observed for the presence or absence of growth. This was to determine whether the antimicrobial effect was bacteriostatic or bactericidal.

Antimicrobial susceptibility test: Antibiotic discs of ciprofloxan (910µg) were placed at the centre of the inoculated Mueller Hinton Agar plates for bacteria whereas Econazole (10µg) were placed at the centre of potato dextrose agar plates for fungi. All plates of MHA were incubated at 37^{0}C for 24 hrs while the PDA plates were incubated at room temperature for 24hrs. At the end of the incubation period, the plates were observed for any evidence of inhibition which appeared as a clear zone. The zones of inhibition were then measured and the results were recorded.

Column chromatographic purification of the antimicrobial non-polar neutral fraction: The biologically active non-polar (hexane) neutral fraction obtained from the partitioning of 95% ethanol

crude extract on TLC showed more than one component. Consequently, it was subjected to purification by chromatography. The sample (5g) was then subjected to column chromatography on silica gel and eluted using mixtures of hexane and ethyl acetate, 100% ethyl acetate and then finally washed down with methanol and collecting 20cm^3 portions each. The purity of each collection was monitored using TLC. Those containing common components based on Rf values were combined and evaporated to dryness. Repeated column chromatography eventually gave a partially pure colorless solid (38mg), coded B.

Gas Chromatography-Mass Spectroscopy of column chromatographic fraction B: The partially pure sample **B** from the column chromatography of the non-polar neutral fraction was subjected to GC-MS analysis to identify the components.

RESULTS AND DISCUSSION

The results of the phytochemical screening of the 95% ethanol crude extract of the dry root bark of *D.saxatilis* are shown in Table 1 which showed the presence of alkaloids, flavonoids, carbohydrates, saponins, terpenoids and tannins (Table 1).

Table 1. Phytochemical screening of crude ethanol extract of *D. saxatilis* root bark

Phytochemicals	Remarks
Alkaloids	+
Tannins	+
Phenols	+
Flavonoids	+
Resins	-
Sterols	-
Terpenoids	+
Cardiac glycosides	+
Saponins	+
Carbohydrates	+
Volatiles	+

Key: (-)=Absent, (+)=Present

These phytochemicals have been reported to possess pharmacological activities (*Okwu*, 200, Kosalec *et al.,*2005). Terpenoids have been classified as active insecticidal and antimicrobial compounds (Wang *et al.,* 2000). The antimicrobial screening results for the crude ethanol extracts and fractions are presented in Tables 2 and 3.The fractions of D.saxatilis crude extract showed concentration-dependent activities against all the test organisms with zones of inhibition ranging from 10-26mm at the following MIC values: S. aureus: (6.25, 6.25, 12.5, 6.75, 6.25) µg/ml; B. substilis: (12.5, 12.5, 25, 25, 25) µg/ml, E. coli (12.5, 12.5, 25, 12.5, 12.5), S. typhi: (25, 12.5, 25, 12.5, 12.5) µg/ml, C. albicans: (12.5, 25, 12.5, 25,25) µg/ml for polar neutral, non-polar neutral, basic and acidic fractions, respectively. From the results the stem-bark contains important phytochemicals and has demonstrated antimicrobial activities similar to those previously reported for the leaves and root wood (Okwute and Fatokun,2014, Okwute and Isyaka, 2014, Ogwuda and Okwute, 2020). The column chromatographic purification of the antimicrobial non-polar neutral fraction gave a partially pure sample 5B,

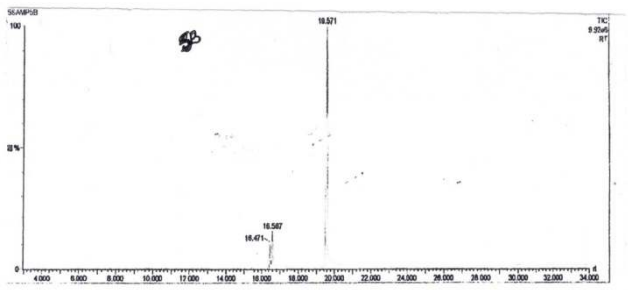
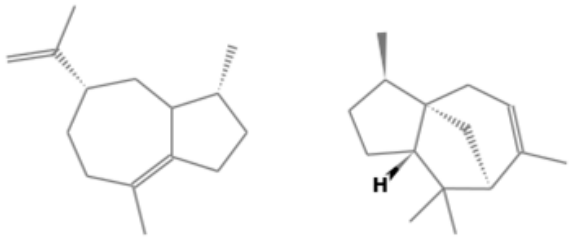


Figure 1. Gas chromatogram of column *chromatographicfraction* 5B



1 2

which was subjected to GC-MS analysis. The gas chromatogram (Figure 1) showed a medium peak for a compound 5B1 with a retention time (RT) of 16.5873 and a major peak for compound 5B2 with a retention time(RT) of 19.571 minutes. The MS of compound 5B1showed a base peak of 161.4 and other significant fragmentation peaks at m/z 204.5(M^+), 189.5, 162.4, 147.4, 133.4, 120.3, 119.3, 105.3, 93.3, 79.3 and 69.3. The compound has a Reverse Fit Factor of 692 and based on the Standard Computer Mass Spectral Data (NIST MS, 2014) is suggestive of azulene-1,2,3,5,6,7,8,8a-octahydro-1,4- dimethyl-7-(1-methylethenyl)-, 1. It is therefore a member of the guaienes which are sesquiterpenes and have been isolated from a variety of plant sources.

For example, δ-guaiene is a major component (4.41%) of the essential oil of Caryopteris incana. The essential oil of Caryopterisincana aerial parts was found to possess strong insecticidal activities against the maize weevil, Sitophilus zeamais (ShaSha *et al*, 2011). The MS of the major component, 5B2 showed a molecular ion peak at m/z (M^+) 204.5 and a base peak at 119.3. The other fragment ions included 189.5, 162.4 161.4, 133.4, 120.4, 105.3,93.3,91.3, 79.3, 69.3 and 55.3. It also has a reverse fit factor of 821 which is in agreement with 1H-3a,7-methanoazulene, 2,3,4,7,8,8a-hexahydro- 3,6,8,8-tetramethyl-,[3R-(3α,3aβ,7β,8aα)]-, 2, which is cedrene. Being a sesquiterpene, cedrene is a highly fragrant constituent of the essential oils of cedar. It falls under the category of fragrance agents and is predominantly used in perfumery (Lee *et al* 1998). Cedrene has been found to exhibit powerful trypanocidal activities when the essential oils and their individual components were studied for their structure-activity relationships between trypanosomes and human leukemia cells (Nibret, 2010). Cedrene has also been previously obtained from Juniperus macropoda and Juniperu .communis and was found to possess mild antiseptic properties (University of Virginia, 1964).

Table 2. Determination of inhibitory activity (sensitivity test) of crude 95% ethanol extract and fractions

Test organism	Zone of inhibition (mm)/ µg/ml of the extract				Standard antibiotics Zone of Inhibition(mm)				Econazole 10ug
	P 100 50 25 12.5	N P 100 50 25 12.5	AK 100 50 25 12.5	A 1005025 12.5	C 100 50 25 12.5	CP 10 µg	AM 10 µg	EC 10 µg	
S.aureus	21 18 16 14	17 15 14 12	13 11 10 0	22 20 19 17	20 18 17 14	35	28	-	
B.substilis	22 20 18 16	26 24 20 18	20 18 15 13	24 21 19 17	23 20 19 17	30	22	-	
E.coli	23 22 19 17	21 20 18 16	21 19 17 14	22 20 18 16	22 20 18 17	35	15	-	
S.typhi	18 17 15 12	18 16 14 12	14 12 11 10	15 13 12 10	17 13 12 0	38	20	-	
C.albicans	18 16 14 12	17 15 13 11	18 16 14 10	15 12 10 0	16 14 12 10	-	-	38	

Key: P= Polar, NP=non polar, AK=Alkaloid, A=Acid, C=Crude, CP=Ciprofloxacin , AM=Amoxicillin EC=Econazole

Table 3. Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC) against the test organisms

Test organism	MIC (µg/ml)					MBC(µg/ml)				
	P	NP	B	A	C	P	NP	B	A	C
S.aureus	6.25	6.25	12.5	6.75	6.25	12.5	12.5	25.0	12.5	12.5
B.substilis	12.5	12.5	25.0	25.0	25.0	25.0	25.0	50.0	50.0	50.0
E.coli	12.5	12.5	25.0	12.5	12.5	25.0	25.0	50.0	25.0	25.0
S.typhi	25	12.5	25.0	12.5	12.5	50.0	25.0	50.0	25.0	25.0
C.albicans	12.5	25.0	12.5	25.0	25.0	25.0	50.0	25.0	50.0	50.0

Key: P= Polar neutral, NP=Non-polar neutral, B=Basic, A=Acidic, C=Crude

It has been established that anulene derivatives such as cedrene, guaine and cissampelone constitute the antimicrobial agents in some plants including, Zingiber officinale, Cissampelos owariensis and Azadirachta indica (Al-Rahmah *et al*, 2013, Efiom *et al*., 2009, Swapna *et al*., 2018). Thus, both compounds 5B1 and 5B2 have the same molecular formula, $C_{15}H_{24}$ and are therefore sesquiterpenoids. They possess very close mass spectral fragmentation patterns in terms of the molecular ion peak of m/z 204(M^+) and other fragment ions, but possess different base peaks of m/z 161 and 119, respectively. Their presence in the extracts, particularly cedrene may account for the antimicrobial activity of the root-bark of *Dalbergia saxatilis*. This is probably the first report of the presence of these anulene-type sesquiterpenes in the genus *Dalbergia.*

Conclusion

Phytochemical screening of *D. saxatilis* root-bark revealed the presence of alkaloids, tannins, phenols, flavonoids, terpenoids and glycosides. This work has also identified two azulene-type sesquiterpenes that may account for the antimicrobial and insecticidal activity of the plant which is traditionally used to manage a number of infections. The compounds identified belong to classes of natural products known to possess insecticidal, anti-cancer, trypanocidal, antiseptic, antifungal and antibacterial properties which support the claim for the traditional medicinal uses of the plant. Although guaiene and cedrene have been reported previously in some plants the present work reports for the first time their presence in *D. saxatilis* and indeed in the genus, *Dalbergia.*

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