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Antimicrobial screening and chemical investigation of the root wood ethanolic extract of *Dalbergia saxatilis* (Fabaceae)

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Abstract
The ethno-medicinal uses of *Dalbergia saxatilis* have made it a useful plant for both chemical and biological investigations. The dried root wood was extracted with 95 % ethanol to obtain a crude extract, part of which was partitioned into acidic, basic, polar and non-polar neutral fractions. The crude extract was subjected to preliminary phytochemical screening and both the crude extract and fractions were screened for antimicrobial activity. The qualitative phytochemical investigation showed the presence of cardenolides, tannins, alkaloids, saponins, steroids, terpenoids, and resins. The antimicrobial activity of the crude and the different fractions was determined against some bacteria, including *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa* and fungi, including *Candida albicans*, and *Aspergillis niger*, using different culture media for sensitivity test, Minimum Inhibitory Concentration and Minimum Bacteriocidal Concentration determinations. The crude extract and fractions exhibited strong concentration-dependent activity against the test organisms at various concentrations. Column chromatographic fractionation of the antimicrobial neutral polar fraction, followed by GC-MS analysis of column fractions revealed the presence of metabolites, including oleic acid, oleamide, 4-phenylbut-3-ene-1-yne and homopterocarpin, known to have bioactivities that are of medicinal significance. Their presence therefore supports the ethno-medicinal uses of the plant in some Nigerian communities.

Keywords: *Dalbergia saxatilis*, root wood, polar-neutral fraction, antimicrobial activity, GC-MS analysis, phtoconstituents

Introduction
The use of plants as healing remedies dates as far back as 2000 years ago and different local communities have folkloric accounts of what particular plant species are used for [1]. Over 248,000 species of higher plants have been identified and from these, 12,000 plants are known to have medicinal properties. However, less than 10% of all known plants have been investigated from a phytochemical and/or pharmacological point of view [1]. Nevertheless, from this small percentage, uncountable bioactive organic compounds have been isolated such as alkaloids, terpenes and terpenoids, flavonoids, examples are sterols of which the sex hormones have been particularly important in the production of contraceptives, the fever-reducing quinine from the bark of the Peruvian tree, *Chincona officinalis*, the narcotic paregoric morphine from the poppy capsule, and the various heart stimulating glycosides produced from *Digitalis spp* applied widely in treating cardiac issues, plants vitamins and a large range of antibiotics used in the treatment and management of various bacterial infections [2].
Awareness of the medicinal potentials of plants is as a result of years of struggles against diseases with debilitating effects, compelling the search for drugs from unusual sources, particularly natural products from plant parts such as the bark, seeds, fruits, roots, leaves and wood[3]. Contemporary science has acknowledged their active nature and has included in modern pharmacotherapy a range of drugs of plant origin [4].
The subfamily (*fabiodeae*) includes plants that are mostly herbs, shrubs or trees and the leaves are pinnate or palmate to tri-foliolate or apparently simple leaves [5]. The genus *Dalbergia* has about 300 known species out of which nearly 25 species occur in India [6]. The *Dalbergia* species are widely distributed around the globe, but predominantly found in most parts of Africa, Madagascar, Europe, Asia, Central and South America [7].
*D. saxatilis*is is usually a vigorous climbing shrub, producing stems up to 20 meters long, though sometimes it remains smaller, growing as a shrub only 2-3 meters tall [8]. It occurs in the humid and dry deciduous forest from Senegal to Western Cameroons and extending southwards to Angola. The flowers are white or pink, borne in loose axillary or terminal panicle 5-15 cm long. The foliage is graceful and the young fruits are wine-red in colour [9].

In these respects, it can be cultivated as an ornamental plant. The flowers are strongly scented and were used in Ghana to produce shea butter as skin pomade [10]. Tender young leaves are cooked and eaten as vegetable. The plant is used as an anthelmintic, an abortifacient and in the treatment of leprosy [11].

Dalbergia saxatilis (*fabaceae*), an African shrub widely distributed in Southern Nigeria, is noted for producing hard and durable ornamental woods used as implement handles, grain mortars, and drumsticks by the natives in West Africa [12]. The durability of the wood and the resistance of the wood to termite and fungal attack are attributed to the presence of natural phenols and quinones, mainly neoflavonoids, isoflavonoids and flavonoids [13]. Other parts of the plant are thought to possess compounds of some medicinal values and are used by African natives in the treatment of some common diseases and ailments [14].

In Southern Nigeria, decoctions from the roots and leaves of *Dalbergia saxatilis* are claimed to cure topical skin lesions, smallpox, toothache, and asthmatic symptoms [15]. Fresh leaves of *Dalbergia saxatilis*, mixed with red tree ants and native salt, are used as a remedy for coughs in Liberia [12].

Previous studies on the plant had found the dry powdered leaves to be insecticidal and protectant against the cowpea bruchid as well as showed anti-oxidant activity while the bark ethanolic crude extract showed antimicrobial activity against some human pathogens as well as insecticidal activity against mosquitoes using cage experiment [16, 17]. In a recent investigation, the root wood showed strong antimicrobial activity and from the polar neutral fraction of the alcoholic crude extract a pentacyclic triterpenoid, betulinic acid, known to possess anti-HIV and anti-malarial activities was isolated [18]. In this work the root wood was further investigated for antimicrobial activity and chemical constitution by increasing the spectrum of organisms and subjecting the column chromatographic fractions of the polar neutral fractions to GC-MS analysis.

Materials and Methods

Materials

The root wood of *Dalbergia saxatilis* was collected in January 2015, during the Harmattan dry season, from the forest of Suleja, Suleja Local Government area of Niger State and was authenticated at the National Institute for Pharmaceutical Research and Development (NIPRD) by a botanist. The voucher specimen was deposited at the Herbarium of the same institute with voucher number NIPRD/H/6647. The samples were collected in nylon bags, chopped into smaller pieces. The bark of the root was thereafter separated from the root wood and was allowed to dry. The dried pieces were pulverized using a milling machine at the National Institute for Pharmaceutical Research and Development (NIPRD) with a sieve diameter of 1 mm, and was stored in a well-ventilated environment under ambient temperature.

All solvents and reagents used were of standard grades and produced by May and Baker, and BDH, Poole, England. The solvents were redistilled before use.

The medium used in the antimicrobial screening was the molten nutrient agar using the filter paper disc method against the following microorganisms: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillis niger*. The test organisms used for the analysis were clinical isolates of bacteria and fungi obtained from the Department of Microbiology Laboratory, Ahmadu Bello University, Zaria, Nigeria.

Methods

Extraction and fractionation

The dried, pulverized root wood of *Dalbergia saxatilis* (DS) was extracted using cold maceration in 95% ethanol. In two separate operations, the root wood (500g) was extracted with 1.25 litres of 95% absolute ethanol for 48 hours with regular shaking at intervals to ensure proper extraction after which it was filtered using a suction pump. The extracts obtained from the two bottles (1000g in 2.5 litres of 95% ethanol) were combined and evaporated to dryness using a rotary evaporator at 78^{o}C to yield a dark brown semi-solid (20.24 g). Prior to evaporation to dryness, thin layer chromatography (TLC) was run using a silica gel precoated on an aluminium plate and 1:1 ethyl acetate/hexane as the solvent system.

The crude extract was then fractionated according to the procedure of Mitscher *et al* [19].

The ethanolic crude extract (5g) was then dissolved in dichloromethane (DCM) (100 ml), filtered and the filtrate was taken in a separatory funnel (250 ml). The DCM solution was extracted with 5 % HCl and this gave an upper aqueous layer of HCl and the basic components and the lower DCM layer of the acid and neutral fractions. The upper aqueous layer was basified with 5 % NaOH and extracted with DCM, (50 ml) to give the bases (4.08 g) on evaporation to dryness. The lower DCM layer was extracted with 5 % NaOH and the aqueous layer was acidified and extracted with DCM which gave the acids (0.79 g).

The DCM layer which contained the neutrals was evaporated to dryness. The residue was re-dissolved in aqueous methanol and then taken in a separatory funnel, and extracted with hexane to yield the polar neutrals (methanol-soluble fraction) (3.34 g) and the non-polar neutrals (hexane-soluble fraction) (0.12 g).

Phytochemical screening of the crude ethanol extract of *Dalbergia saxatils*

The phytochemical screening was based on standard procedures [20-22]. A total of fourteen (14) metabolites were screened for on the crude extract of *Dalbergia saxatilis* root wood.

Antimicrobial Screening of Fractionation Extracts

Antimicrobial screenings of the crude ethanolic extract, alkaloid, acids, the polar neutral (methanol) and the non-polar neutral (hexane) fractions were carried out using culture media and based on a standard procedure [19]. The culture media used for the analyses included Mueller Hinton agar (MHA), Mueller Hinton broth (MHB), potatoes dextrose agar (PDA) and nutrient agar (NA). The mentioned media were used for sensitivity test, determination of minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC). All media were prepared according to manufacturer's instruction and sterilized by autoclaving at 15 lbs pressure (121^{o}C) for 15 minutes and allowed to cool [23].

Determination of Inhibitory Activity (sensitivity test)

The procedure was based on the agar well diffusion method. The standardized inocula of both the bacterial and fungal isolates were steaked on sterilized Muller Hinton and potatoes dextrose agar plates respectively with the aid of a sterile swab stick. Four wells were punched on each inoculated agar plate with a sterile cork borer. The wells were properly labelled

according to different concentrations of the extract prepared which were 100, 50, 25, and 12.5 µg/ml respectively. Each well was filled with approximately 0.2 ml of the extract.
The inoculated plates with the extract were allowed to stay on the bench for about an hour. This was to enable the extract to diffuse on the agar. The plates of Muller Hinton agar were then incubated at 37ºC for 24 hours while the plates of potato dextrose agar were incubated at room temperature for about 3-5 days.
At the end of the incubation period, the plates were observed for any evidence of inhibition which will appear as a clear zone that was completely devoid of growth around the wells (zone of inhibition) [23]. The diameter of the zones was measured using a transparent ruler calibrated in millimetre and the results are shown in Table 3.

Determination of minimum inhibitory concentration (MIC)
The minimum inhibitory concentration of the extract was determined using tube dilution method with the Mueller Hinton broth used as a diluent. The lowest concentration of the extract showing inhibition for each organism when the extract was tested during sensitivity test was serially diluted in the test tube containing Mueller Hinton broth. The organisms were inoculated into each tube containing the broth and the extract. The inoculated tubes were then incubated at 37ºC for 24 hours [23].
At the end of the incubation period, the tubes 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 results recorded are shown in Table 4.

Determination of Minimum Bateriocidal Concentration (MBC)
The result from the minimum inhibitory concentration (MIC) was used to determine the minimum bacteriocidal concentration (MBC) of the extract. A sterilized wire loop was dipped into the test tubes that did not show turbidity (clear) in the MIC test and a loop full was taken and streaked on sterile nutrient agar plates. The plates were incubated at 37ºC for 24 hours [23].
At the end of the incubation period, the plates were observed for the presence or absence of growth. This was done to determine whether the antimicrobial effect of the extract is bacteriostatic or bactericidal.

Column chromatographic fractionation, FTIR and GC-MS analyses
The antimicrobial polar neutral fraction (1.85g) was chromatographed on a column of silica gel and eluted with mixtures of ethyl acetate and hexane as solvent. It gave 4 sub-fractions, DS1, DS2, DS3 and DS4 which were subjected to FTIR and GC-MS analyses.

Results and Discussion
Extraction and Fractionation
The root wood of the *Dalbergia saxatilis* (DS) (1000 g) was extracted with ethanol to give 2.02% of a dark brown gum. The crude ethanol extract was partitioned using the bioassay-guided fractionation protocol [19] to yield the bases, acids and the neutrals (Table 1).

Table 1: Physical characteristics and yields of crude and fractions based on dry weight of the root wood of *Dalbergia saxatilis*

Extractives	Yield in (g) (%)	Colour and consistency
Crude	20.24 (2.02)	Dark brown gum like semi-solid
Acids	0.79 (0.079)	Light brown Viscous oil
Bases	4.08 (0.408)	Black solid
Polar neutrals	3.34 (0.334)	Dark brown gum
Non-polar neutrals	0.12 (0.012)	Brownish yellow oil

Phytochemical Screening
The results of the phytochemical screening of the crude ethanolic extract of *Dalbergia saxatilis* are shown in Table 2.

Table 2: Phytochemical screening results of the crude extract of the root wood of *Dalbergia saxatilis*

Metabolites	Result
Cardenolides	+
Tannins	+
Alkaloids	+
Glycosides	-
Saponins	+
Steroids	+
Flavonoids	-
Cardiac glycosides	-
Volatile oils	-
Phlobotannins	-
Terpenoids	+
Balsam	-
Triterpenoids	-
Resin	+

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

Antimicrobial screening of crude extract and fractions
The results of antimicrobial screening are shown in Tables 3 and 4.

Table 3: Results of Inhibitory activity (sensitivity test) of crude extract and fractions of DS at concentrations of 100, 50, 25 and 12.5µg/ml.

Organisms	Acids 100	50	25	12.5	Bases 100	50	25	12.5	Polar- neutrals 100	50	25	12.5	Non-polar neutrals 100	50	25	12.5	Crude Extract 100	50	25	12.5
S. aureus	15	10	-	-	14	-	-	-	19	16	13	11	15	-	-	-	22	20	15	11
B. subtilis	18	15	12	-	15	12	-	-	20	16	14	12	15	10	-	-	19	16	14	11
E. coli	27	18	15	-	20	15	12	-	27	22	18	-	30	25	19	13	26	20	11	-
S. typhi	16	-	-	-	14	10	-	-	17	13	12	10	-	-	-	-	-	-	-	-
P. aeruginosa	15	-	-	-	16	12	-	-	15	-	-	-	20	15	12	-	17	12	-	-
C. albicans	-	-	-	-	13	10	-	-	14	-	-	-	-	-	-	-	12	-	-	-
A. niger	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Key: (-) = No Activity

Table 4: Results of Minimum Inhibitory Concentration (MIC) and Minimum bacteriocidal Concentration (MBC) of the extracts and fractions

Test Organisms	MIC					MBC				
	Polar			Polar Non		Polar			Polar Non	
	Acids	Bases	Neutrals	Neutrals	Crude	Acids	Bases	Neutrals	Neutrals	Crude
Staphylococcus aureus	100	100	12.5	100	12.5	-	-	50	-	25
Bacillus subtilis	25	50	12.5	100	12.5	50	100	25	-	25
Escherichia coli	12.5	25	12.5	6.25	12.5	25	50	25	12.5	25
Salmonella typhi	50	25	25	ND	ND	100	100	50	ND	ND
Pseudomonas aeruginosa	50	25	100	12.5	50	100	100	-	25	100
Candida albicans	ND	100	100	ND	100	ND	-	-	ND	-
Aspergillus niger	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

KEY: ND = Not determined, (-) = No MBC

GC-MS analysis of column chromatographic fractions: The column chromatographic fractions were subjected to GC-MS analysis. The GC-MS data obtained are shown in Figures 1-4 and Table 5.

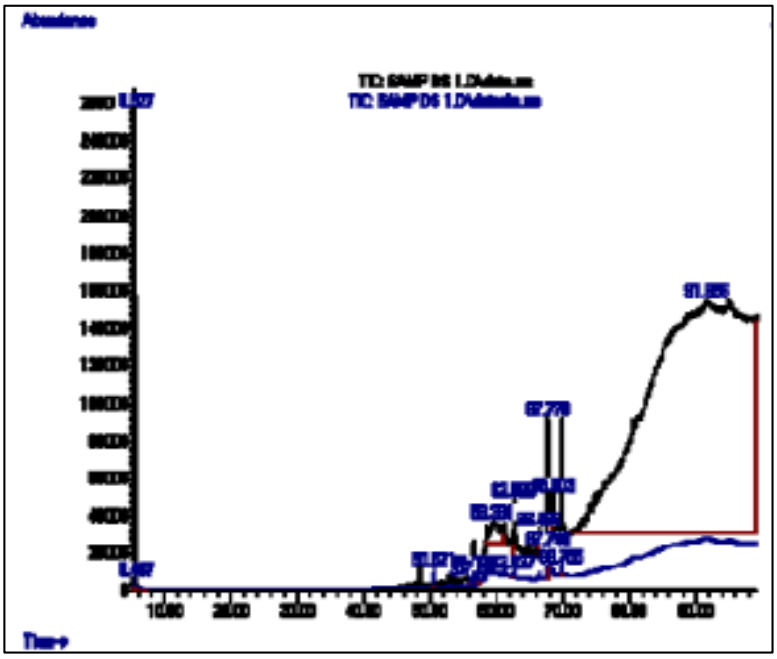

Fig 1: GC of fraction DS1

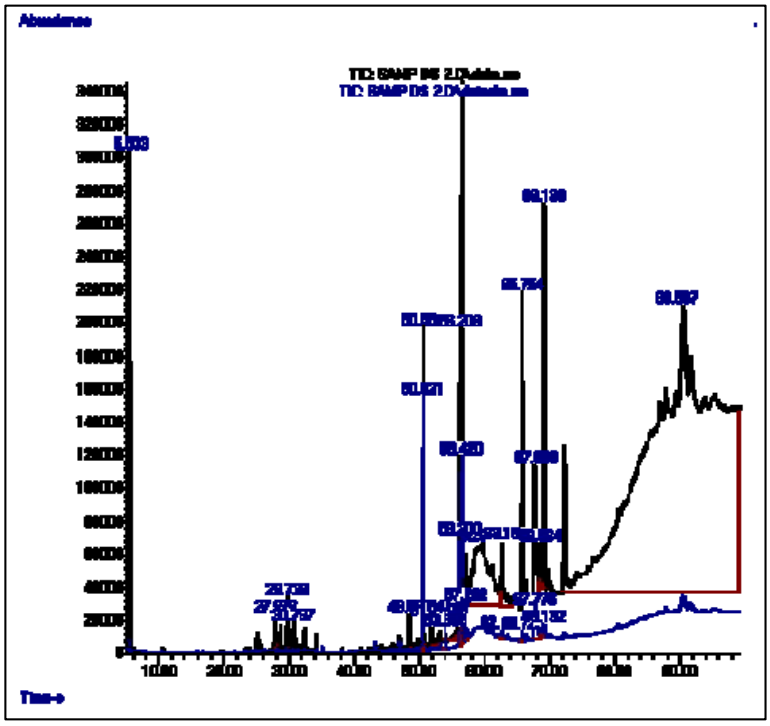

Fig 2: GC of fraction DS2

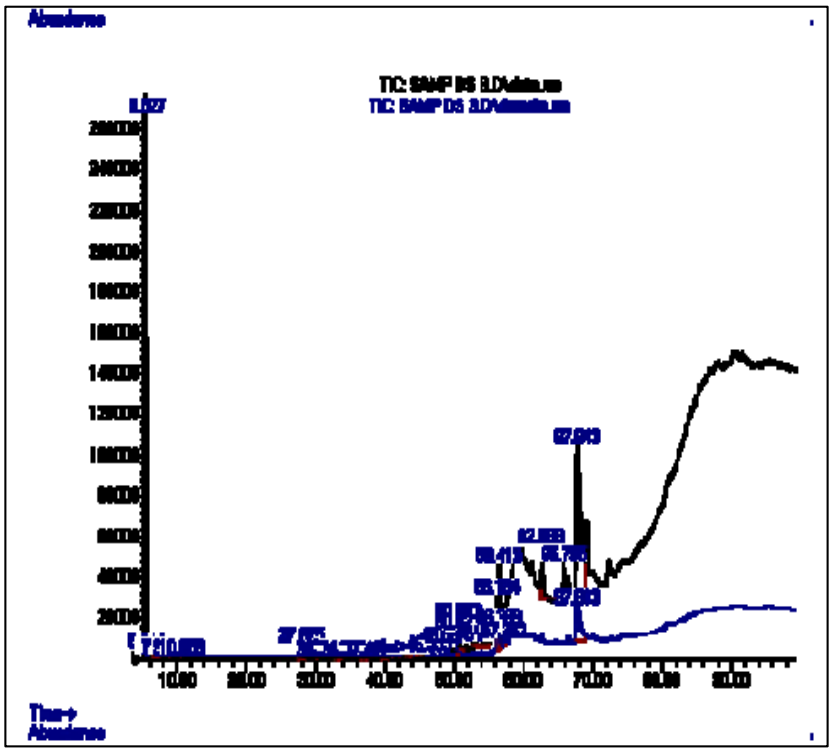

Fig 3: GC of fraction DS3

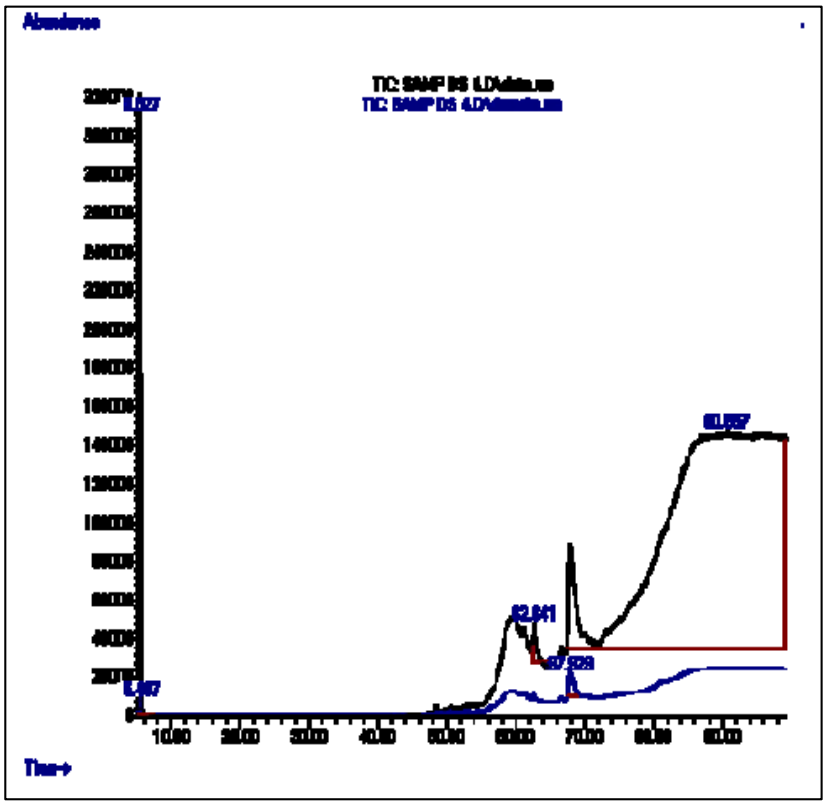

Fig 4: GC of fraction DS4

Table 5: Summary of GC-MS analyses data on chromatographic fractions DS1-DS4

S/N	Components	Retention Time	Molecular Weight	Molecular Formula
1	9,17-Octadecadienal	66.49	264.45	$C_{18}H_{32}O$
2	Hexadecanoic acid, methyl ester	50.55	270.46	$C_{17}H_{34}O_2$
3	9,12-Octadecadienoic acid, methyl ester	56.21	294.48	$C_{19}H_{34}O_2$
4	9-Octadecadienoic acid, methyl ester	56.42	296.46	$C_{19}H_{36}O_2$
5	Homopterocarpin	69.13	284.31	$C_{17}H_{34}O$
6	13-Octadecenal	62.57	266.47	$C_{18}H_{34}O$
7	4-Hydroxy-4-methylpentan-2-one	5. 527	116.16	$C_6H_{12}O_2$

Discussion

The crude ethanol extract of the root wood of *Dalbergia saxatilis* was partitioned into acids, bases, polar neutral (methanol), non-polar neutral (hexane) fractions to yield a light brown viscous oil, black solid, dark brown gum, and brownish yellow oil, respectively. The fractionation showed that the root wood had the bases in the highest yield (0.0408%) as shown in Table 1. Phytochemical screening of the crude ethanolic extract of the root wood showed that cardenolides, tannins, bases, saponins, steroids, terpenoids, and resins are present while glycosides, flavonoids, cardiac glycosides, volatile oils, phlobotannins, balsam, and triterpenoids were absent as shown in Table 2.

The results of the antimicrobial screening on the five (5) extractives showed that the plant has a broad antimicrobial spectrum activity against known Gram-positive and Gram-negative bacteria and fungi. From the results, the crude ethanolic extract was active against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* at concentrations ranging from 100-12.5 µg/ml. It however showed minimal activity against *Candida albicans* at the concentration of 100 µg/ml and inactive against the pathogens, *Salmonella typhi* and *Aspergillus niger* at the same concentrations.

The polar neutral fraction showed the highest sensitivity against a wide range of bacteria at concentrations ranging from 100-12.5 µg/ml. It showed sensitivity towards six (6) pathogens it was tested against but did not show any sensitivity towards the fungus *Aspergillus niger*.

The non-polar neutral fraction showed activity towards all but three (3) pathogens, *Salmonella typhi*, *Candida albicans and Aspergillus niger*. The basic fraction also exhibited a wide range of activity towards all the bacteria it was tested against except the fungus *Aspergillus niger*. The acid fraction was active against all pathogens but had no activity against *Candida albicans and Aspergillus niger* at 100 µg/ml. The results of the antimicrobial screening clearly showed that the root wood of *Dalbergia saxatilis* possess both anti-bacterial and anti-fungal activities as demonstrated by the crude extract, the polar neutral fraction and the basic fraction against most of the test organisms, including the fungus, *Candida albicans*.

From the results of the antimicrobial screening in Tables 3-4, it can be vividly seen that the root wood of *Dalbergia saxatilis*, as previously reported for the leaves and bark has great potentials as a source of new anti-infective agents [16]. Previous studies on *Dalbergia saxatilis* leaves had suggested that the biological activities could be as a result of the presence of sterols, aliphatic esters and phenolic compounds [16].

Chromatographic fractionation of the antimicrobial polar neutral fraction gave fractions DS1-DS4 which were subjected to GC-MS analysis. The summary of components identified from the chromatograms is recorded in Table 5 and indicated the presence of long-chain aliphatic aldehydes and esters as well as homopterocarpin and 4-hydroxy-4-methylpentan-2-one. These findings are in agreement with the biological and chemical characteristics of species of the genus Dalbergia and support the traditional medicinal uses of the plant [7, 24].

Conclusion

In relation to the results obtained from this study, it is obvious that the woody root extract of *Dalbergia saxatilis* contains a wide range of phytochemicals and exhibits a broad spectrum of pharmacological activities. It contains cardenolides, tannins, alkaloids, saponins, steroids, terpenoids, and resins which are generally known to have protective effects against several diseases. Particularly, the work has confirmed that the root wood of *Dalbergia saxatilis* possesses a broad spectrum antimicrobial activity against six (6) of the test pathogens namely: *Staphylococcus aureus, Bacillus substilis, Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa*, and *Candida albicans* and with varying level of sensitivity.

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