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Betulinic Acid from Antimicrobial Root Wood Extract of *Dalbergia saxatilis* Hook f.(Fabaceae)

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Authors' contributions

The corresponding author OSK designed, supervised and drafted the manuscript based on the thesis by the author IMS who worked directly on the bench and obtained the spectral data as a postgraduate student.

Original Research Article

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ABSTRACT

Aims: The root wood of *Dalbergia saxatilis* was studied to assess its antimicrobial activity and chemical constituents and confirm its traditional medicinal uses.
Study Design: The source of the plant was identified and the plant material was collected at a particular time of the year and authenticated. The crude 95% ethanol extract of the dry root wood was obtained and screened for phytochemicals and antimicrobial activity against pathogens of economic interests. A process for isolation and identification of bioactive components was then developed using standard procedures.
Place and Duration of Study: The study was undertaken between October 2011 and November 2012 at the Department of Chemistry, University of Abuja, Nigeria.
Methodology: The 95% ethanol crude extract was obtained by percolation and then fractionated into acidic, basic, polar and non-polar neutral fractions. The crude extract and fractions were subjected to antimicrobial screening against *Staphylococcus aureus, Escherichia coli, Bacillus subtillis* and *Streptococcus viridans.* The crude extract was also subjected to phytochemical screening. The active non-polar neutral fraction was purified using flash column chromatography. The isolates were characterized by spectral analyses.
Results: The extracts, with the exception of the acidic fraction, were found to possess antimicrobial activity against some of the test organisms, particularly *E. coli* and *B. subtilis.* Phytochemical screening of the crude 95% ethanol extract showed the presence

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of phenols, flavonoids, sterols, terpenoids, carbohydrates and saponins. Column chromatography of the antimicrobial non-polar neutral fraction gave a partially pure compound which on spectral analyses led to the identification of the previously known bioactive pentacyclic triterpenoid, betulinic acid.
Conclusion: This is the first report of the presence of betulinic acid in the genus, *Dalbergia.* Based on this a crude management drug for some globally important infections such as HIV/AIDS, malaria and cancer may be formulated using betulinic acid as a biomarker.

Keywords: Dalbergia saxatilis; root wood; non-polar neutral fraction; antimicrobial activity; betulinic acid; spectral analyses.

1. INTRODUCTION

The genus Dalbergia belongs to the family Fabaceae which is one of the largest among the flowering plants, consisting of 300 species [1]. A large number of these species are commonly found in both the tropical and subtropical regions of the world, including Central and Southern America, Africa and Southern Asia, where they are used for the management of a wide range of disorders such as diarrhoea, dysentery, stomach ache, gonorrhoea, syphilis, scabies, leprosy and pains [2,3]. In Southern Nigeria, the aqueous root extract of D. saxatilis is used by the natives to accelerate birth and expel the placenta in human subjects [4]. Previous pharmacological studies have also confirmed the folkloric use of D. saxatilis as analgesic, anthelmintic, antibiotic, anti-inflammatory, antipyretic, pesticidal, anti-oxidant, and contraceptive agents [5-14].

A number of phytochemicals including cinnamyl phenols, quinine, furans, steroids, benzophene, styrene, terpenoids, flavonoids, isoflavonoids, neo-flavonoids and polyphenols have been isolated from various species of *Dalbergia* [15-20]. However, only very few studies have been carried on *Dalbergia saxatilis* and the few reports have been on the biological activities [8,11,21,22]. In fact, no work on the chemical constituents of any part of *Dalbergia saxatilis* has previously been reported. In continuation with our studies on Nigerian medicinal plants, we wish to report on the antimicrobial activity of the root wood extracts of *D. saxatilis* and the identification of the previously known medicinally important pentacyclic triterpenoid, betulinic acid, in the non-polar neutral fraction.

2. MATERIALS AND METHODS

2.1 General

All solvents and reagents used were of standard grade and the solvents were redistilled before use. Thin Layer Chromatography (TLC) was run on pre-coated aluminum sheets (0.2-0.5mm mesh silica gel) manufactured by Kommanditgesellschaft auf Aktien (KGaA), Germany. The plates were activated at 35°C in an oven before use. The spots were visualized using UV light from a UV lamp model UV-21 long wavelength (UV-366mm) manufactured by United Visual Products (UVP), Inc. USA and also by the use of iodine vapour. Flash column chromatography was carried out using silica gel 0.2-0.5mm mesh reagent grade made by Aldrich Chemical Company, Limited.

The medium used in the antimicrobial screening was the molten nutrient agar, using the filter paper disc method against the following organisms: *Escherichia coli* (Ec) (Gram-negative), *Streptococcus viridans* (Sv) (Gram-positive), *Bacillus subtilis* (Bs) (Gram-negative), and *Staphylococcus aureus* (Sa) (Gram-positive).The organisms were American Type Culture Collection (ATCC) and clinical isolates obtained from the Microbiology Laboratory of the University of Abuja Teaching Hospital, Abuja, Nigeria.

Proton Nuclear Magnetic Resonance (^{1}H-NMR) and Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR) spectra were run on JEOL NMR AS400 (400MHz) spectrometer using $CDCl_3$ as solvent and TMS as internal standard. The values are recorded in δ (ppm). HRESI-MS was run on LTQ Orbitrap (Thermo) Mass Spectrometer at a capillary temperature of 200°C and capillary voltage of 46.00V for the positive mode and capillary voltage of -48.00V for the negative mode.

2.2 Plant Material

The roots of *Dalbergia saxatilis* were collected from the forest of Gumau, Toro Local Government area of Bauchi State, Nigeria, in October, 2011. The plant was authenticated at the Bauchi State Ministry of Forest Resources and a voucher specimen (No.6572) was deposited in the herbarium at the National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Nigeria. The collected root wood was cut into small pieces and air-dried. The dried pieces were pulverized in a size 8-inch laboratory hammer mill with a sieve diameter of 1mm, and stored in a well ventilated environment.

2.3 Extraction and Fractionation

The dry pulverized root wood (500g) was extracted twice using cold extraction in an aspirator bottle with 2.5L of 95% ethanol (48hr) after which it was filtered. The two extracts were combined and evaporated to dryness in vacuo to yield a dark brown gum (27.51g, 5.52%). The crude dry extract (20g) was dissolved in chloroform (300ml) and the solution was transferred into a separating funnel (1L) and fractionated according to a standard procedure [23]. This gave the alkaloidal fraction (0.47g), the acidic fraction (0.37g) and the non-polar and polar neutral fractions (1.85g and 0.35g, respectively).

2.4 Phytochemical and Antimicrobial Screening of Extractives

The crude 95% ethanol extract was subjected to phytochemical screening based on standard procedures [24-26] to determine the classes of phytochemicals present.

The crude extract and the fractions were screened for activity against some micro-organisms using the agar-disc diffusion method [23] to authenticate the traditional medicinal uses of the plant in the management of some infections.

2.5 Isolation and Spectral Analyses of DSD1

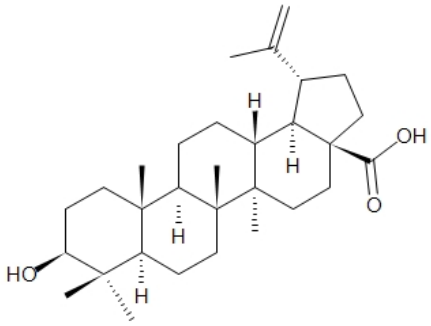
The antimicrobial non- polar fraction (1.85g) was chromatographed on a column of silica gel (20g) using mixtures of hexane and ethyl acetate as eluents. 100% hexane eluted a partially pure yellow viscous oil, DSD1 (9.0mg). The sample was analysed for NMR and MS characteristics.^{1}H NMR(400 MHZ) $CDCl_3$ δ(ppm): 4.72(1H, singlet), 4.67(1H, singlet) both for H-29, 2.26(m), 2.02(m), 1.61(s,3H,H-30) and 0.86-1.04 attributable to rest of the protons; ^{13}C NMR(400MHZ) $CDCl_3$ δ(ppm)(C-1 to C-30):39.15, 28.12, 78.45, 41.28, 46.98, 18.41, 34.17, 41.55, 52.22, 36.30, 22.72, 25.44, 36.30, 43.68, 31.32, 32.98, 62.16, 46.92, 46.98, 156.87,

29.80, 36.30, 27.25, 17.27,14.16,14.02, 14.48, 173.75, 106.29, 19.44; ESIMS m/z: 455.40[M-H]$^{-}$, 457.27[M+1]$^{+\cdot}$

3. RESULTS AND DISCUSSION

Phytochemical screening of the crude ethanol extract of the root wood showed that flavonoids, sterols, phenols, terpenoids, carbohydrates and saponins were present Table 1. The results of the antimicrobial screening of the crude extract and the four fractions showed that the plant has reasonable antimicrobial activity against the test organisms at 1000µg/ml Table 2. The 1000µg/ml concentration is the minimum acceptable activity for crude extracts in our laboratories [23]. From the results the crude ethanol extract was active against Escherichia coli and Staphylococcus aureus at 1000µg/ml, but inactive against *Streptococcus viridans* and *Bacillus subtilis* at the same concentration. The results also showed that the non-polar fraction was active against both *Escherichia coli* and *Bacillus substilis* but inactive against *Staphylococcus aureus* and *Streptococcus varidan*, while the polar neutral was active against the above three organisms at 1000µg/ml. Thus, from Table 2 it is suggestive that the root wood of *Dalbergia saxatilis* has reasonable bioactivity which is in good agreement with previous report on the leaves and bark [11]. Although the active components were not identified previous reports had suggested the presence of sterols, fatty acid esters, terpenoids and phenolics as being responsible for some of these activities [27].

Column chromatography of the antimicrobial non-polar neutral fraction on silica gel gave a viscous yellow oil, DSD1, which was subjected to spectral analyses, including ^{1}H-NMR, ^{13}C-NMR and MS. The ^{1}H-NMR, ^{13}C-NMR and MS spectra were typical of a pentacyclic triterpenoid of the lupane series [28-30] and comparison with literature 13C NMR chemical shifts Table 3.[31-34] suggested it was betulinic acid, 1. Of great significance in the spectra of DSD1 are carbon resonances at 78.4, 156.7, 173.7 and 106.0 for C-3, C-20, C-28, and C-29, respectively, as well as the MS mass ions m/z 455[M-H]$^{-}$ and m/z 457[M+H]$^{+}$ Fig. 1. in the negative and positive modes, respectively. The molecular ion (M^{+}) was hardly observed at m/z 456. These spectral characteristics have been well documented for betulinic acid and its derivatives by previous workers [35,36,] Thus, the above spectral characteristics support structure 1, betulinic acid, for DSD1.



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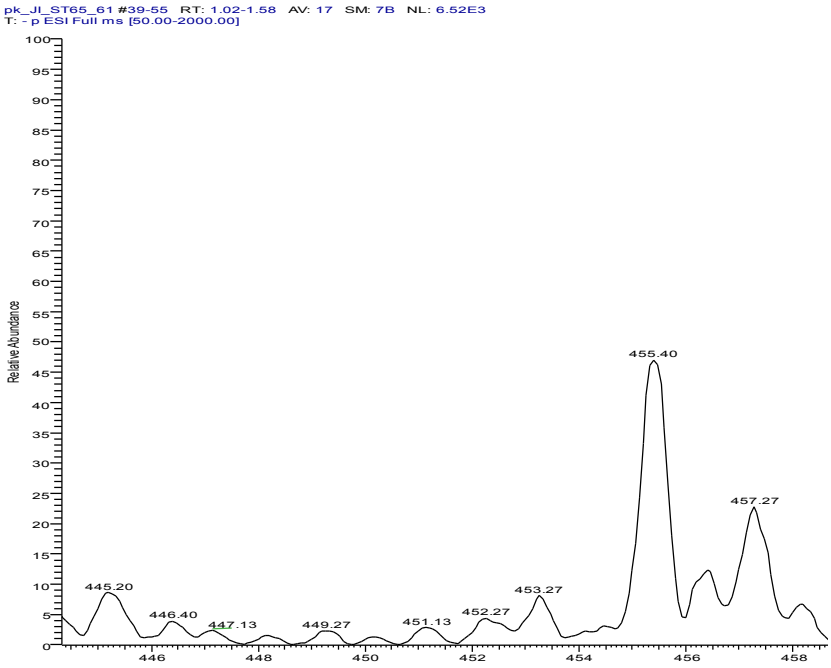

Fig.1. Expanded mass spectrum (MS) of isolate, DSD1

Table 1. Phytochemical sceening results for crude 95% ethanol extract of Root Wood of *Dalbergia saxatilis*

Phytochemicals	Results
Tannins	-
Flavonoids	+
Sterols	+
Phlobatannins	-
Glycosides	-
Alkaloids	-
Cardiac glycosides	-
Phenols	+
Terpenoids	+
Balsams	-
Resins	-
Volatile oils	-
Cabohydrates	+
Saponnins	+

Key: (+) = present; (-) = absent

Betulinic acid, 1**,** is a naturally occurring pentacyclic triterpenoid which has been reported to have potentials as an immunomodulatory, anti-malarial, anti-inflammatory, and anti-cancer agent (anti-melanoma) [37-41]. The activity has been associated with the carboxylic acid group [31]. It was previously isolated from *Betula pubescens*, *Tiziphas mavritian*, *Prunella vulgaris* and *Triphyophyllum pellatum* [40].

Table 2. Antimicrobial assay results for the crude extract and fractions of the root wood of *Dalbergia saxatilis* at 1000µg/ml

Extract	Ec	Sv	Bs	Sa
Crude extract	-	+	+	-
Acidic fraction	+	-	+	+
Basic fraction	+	-	+	-
Non-polar neutral fraction	-	+	-	+
olar neutral fraction	-	-	-	+

Key: (+) =Growth; (-) =No growth; Ec=Escherichia coli; Sv=Streptococcus viridans; Bs=Bacillus subtilis; Sa= Staphylococcus aureus.

Table 3. Comparison of literature ^{13}C-NMR chemical shifts for betulinic acid with those of DSD1

Carbon	Chemical Shifts (ppm)		
1	39.1 (2)* (a)	39.3(b)	39.15(c)
2	28.1 (2)	28.3	28.12
3	78.4 (1)	78.2	78.45
4	39.4 (0)	39.6	41.28
5	55.7 (1)	56.0	46.98
6	18.6 (2)	18.8	18.41
7	34.7 (2)	34.9	34.17
8	40.9 (0)	41.2	41.55
9	50.8 (1)	51.0	52.22
10	37.3 (0)	37.6	36.30
11	21.1 (2)	21.2	22.72
12	25.9 (2)	26.2	25.44
13	38.4 (1)	38.7	36.30
14	42,4 (0)	42.9	43.68
15	31.1 (2)	31.3	31.32
16	32.7 (2)	32.9	32.98
17	56.3 (0)	56.7	62.16
18	47.6 (1)	47.8	46.92
19	49.5 (1)	49.8	46.98
20	150.7(0)	151.4	156.87
21	30.1(2)	30.3	29.80
22	37.5 (2)	37.6	36.30
23	28.5 (3)	28.7	27.25
24	16.3 (3)	16.4	17.27
25	16.2 (3)	16.5	14.16
26	16.2 (3)	16.5	14.02
27	14.8 (3)	14.9	14.48
28	178.7(0)	178.9	173.75
29	110.3(2)	110.0	106.29
30	19.4 (3)	19.5	19.44

*Key: * Number of protons attached to carbon; (a) Chatterjee and Kouli [31]; (b) Nan et. al.[34], (c) chemical shifts for DSD1.*

4. CONCLUSION

This work has reported the antimicrobial and phytochemical characteristics of *D. saxatilis*. A known bioactive pentacyclic triterpenoid, betulinic acid, has been identified in the non-polar neutral fraction. The presence of betulinic acid and other unidentified phytochemicals may explain the many traditional medicinal uses of the root of *Dalbergia saxatilis* . This is the first identification of a natural product from the species *Dalbergia saxatilis*, and the first report of betulinic acid from the genus *Dalbergia*. Further work will examine other active fractions as well as investigate the possibility of developing a crude management drug based on the root of *D. saxatilis* and using betulinic acid as a marker.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

The authors have declared that there are no competing interests associated with this work.

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