



Isolation Identification and Characterization of Diterpenes from the Stem bark of *Detarium Microcarpum*

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Abstract

The antibacterial activity of the stem bark of *Detarium microcarpum* was investigated with the aim of isolating the most active ingredient. Extraction solvents used to prepare the crude extracts of the stem bark were, n-hexane, ethyl acetate, chloroform, methanol. Test bacteria included *Escherichia coli*, *Salmonella enteritidis*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Vibro cholerae*. The extracts demonstrated a wide range of antibacterial activity on the microorganisms, with the ethyl acetate crude extract being the most active. Using liquid vacuum chromatography over silica gel, the extract was eluted with n-hexane, ethyl acetate and methanol and fifty-five fractions labeled G1 - G55 were obtained. Further antibacterial assays of the fractions led to the identification of fraction G34 as the most active fraction. This fraction was subjected to spectroscopic analysis using NMR, [¹H - ¹H NMR, ¹³C-NMR) and 2D-NMR (COSY and HMBC)]. The results showed good agreement with reported data on Copalic acid, a diterpene with chemical formula C₂₀H₃₄O₂. This compound is the most active ingredients of this plant and is likely to be responsible for its antibacterial activities.



Introduction

Human society has had from its beginning a strong dependence on plants and its products, for their health care needs particularly, and for various other purposes.

This almost total dependence continued until the introduction of chemotherapeutic agents, powerful chemical preparations that offered almost immediate and direct cure to ailments, and also easy to carry about. With these agents the dependence on herbal medicines waned.

The challenge of multidrug resistant microbial strains with their reduced susceptibility to antibiotics has awakened interest in the search for alternative sources of drugs (Aibunu *et al.*, 2007). Researchers are now focusing on plants as the main source of health care or a compulsory alternative (VanWhyk, 2002; and Teklehaymano, *et.al.* 2007). Recent study on chemical diversity of plants reveals the presence in them of powerful healing agents' the-phytochemicals.

According to WHO (2001), 80% of people depends on plant for most of their health care need. Most of the information about the efficacy of medical plants is from the mouth of the herbalists. Information on the efficacy and the chemical diversity of the most medicinal plant based on scientific investigation is lacking. Since there is a thin line between the hyaollunogenic and toxic dosages, there is need to investigate plants not only to defend efficacy but up to the assessment of their active ingredients, which is the aim of this study.

Materials and Methods.

Plants collection: The plants were collected at the new layout near owner occupier housing unit, Makurdi and the department of Forestry and Wild life help in the identification.

Preparation of Crude Extracts

Stem bark of *Detarium microcarpum* were dried under shade and ground into fine powder using sterilized laboratory mortars. N-hexane (500 mL) was added to 100 g of the powdered sample in a 1000 mL conical flask. The mixture was stirred and allowed to stand for 72 h, and then filtered using Whatman filter paper No. 2. The filtrates were kept in sterilized containers and the residue was dried under shade. The same procedure was repeated sequentially using ethyl acetate, chloroform, and methanol in that order. At each point of extraction the sample was dried before another solvent was introduced. The extracts were then concentrated using rotary evaporator at 50 °C. All the extracts were cooled and stored in the refrigerator at 2 °C.

Test Microorganisms

Strains of *Samonella enteritidis*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli*, and *Vibro chorelae*; were obtained from Veterinary Research Institute, Vom, nd Plateau State. All the strains were subjected to Gram staining and biochemical tests, for proper identification.

Phytochemical Screening

Presence of bioactive compounds or secondary metabolites like free anthraquinones, anthraquinones glycosides, saponins, tannins, alkaloids, flavonoids and cardiac glycosides were determined using recommended procedures as described by Trease and Evans (2009).

Determination of Antimicrobial Activity

The antibacterial assay procedures as described by Cheesbrough (2009) and Okeke *et-al*; (2001) were slightly modified and used. Mueller Hinton agar was used as growth medium for the bacterial species. The medium was autoclaved and sterilized at 121 °C for 15 minutes at 15 lb pressure, and poured into sterile Petri dishes, and allowed to cool to 45 °C and solidify. The sterilized medium was seeded with 0.1 mL of pure cultures of test organisms. Each extract (0.4 g) was dissolved in 2.0 mL of sterile distilled water to make up an initial concentration of 200 mg/mL. A sterile cork borer was used to make holes 6 mm in diameter and 2 mm deep and evenly distributed in the inoculated plates. Each hole was filled with 0.1 mL of extract solution. 200 mg/mL of ofloxacin solution was used as positive control while sterilised water served as negative control. The inhibition zones diameter of different extracts was observed after 24 h of incubation at 37 °C.

Ethyl acetate extract showed the highest activity, it was further purified / separated and fractions collected subjected to spectroscopic analysis by the NMR.

Fractionation of Crude Extract.

Crude ethyl acetate extract (5 g) was pre adsorbed on Celite and ground to a fine powder on drying. The fine powder was subjected to Liquid Vacuum Chromatography (LVC) according to the methods described by Hostettmann *et al.* (1998). The column was run thus: Silica gel GOG (35 g) was introduced under suction into a sintered glass funnel, mounted on a separatory funnel and connected to a vacuum pump. The silica gel (5 cm) was compacted and tested with n-hexane to check column bleed. The pre adsorbed extract was introduced as a thin layer (0.6 cm) atop the silica gel layer. The column was run gradient using n-

hexane and increasing aliquots of ethyl acetate until 100 % ethyl acetate before finally washing with methanol.

Fifty five fractions of (20 mL) each was collected in sterile bottles labeled (G1-- G55). These fractions were again subjected to antimicrobial assay using Kirby- Bauer method of antibacterial screening. The antibacterial assays yielded three fractions (G22, G34, &G48). In order to arrive at the fraction with the highest antibacterial activity, the procedure was repeated on the three fractions and they were further separated using thin layer chromatography (TLC). Fraction (G34), with the highest antibacterial activity and showing a single Spot on TLC (R_f 0.5; 1:1, N-hexane/ethyl acetate) was selected for NMR spectroscopic analysis.

Results.

NMR (Nuclear magnetic resonance) Analysis

NMR spectral analysis was carried out at University of Strathclyde Glasgow, using JEOL-400 MHz Spectrophotometers and these include proton-proton correlation spectroscopy (^1H - ^1H cosy), and Proton - carbon (^1H - ^{13}C), Hetero nuclear multiple bond connectivity (HMBC). The experiments were carried out using 0.01 mg of fraction (G34). The fraction was dissolved in 0.5 mL of CDCl_3 . This solution was put into a long thin glass tube and placed within a powerful magnetic field. The sample solution was irradiated with a short pulse of radio frequency. The energy given out was detected using radio receiver detector amplifier. Results were displayed in form of intensity (that is number of absorption) against frequency, and characteristic spectral data was generated.

Table 1: Antibacterial activity of the stem bark extracts of *Detarium microcapum*.

Extracts	Bacterial spp. and zones of inhibition in (mm).					
	<i>E.coli</i>	<i>Staph.</i>	<i>Salmonella</i>	<i>Shigella</i>	<i>V.cholerae</i>	
n-hexane		15	20	18	16	6
Ethylacetate		28	27	22	25	9
Chloroform		24	25	24	24	8
Methanol		23	25	22	21	7
Control		30	32	30	27	20

Table 2: Sensitivity test of the three fractions from the ethyl acetate extract.

Fractions	(Tested Microorganisms).				
	<i>S.aereus</i>	<i>E.coli</i>	<i>Salmonella.</i>	<i>Shigella</i>	<i>V.cholerae</i>
G22	24	16	10	9	7
G34	27	22	20	18	11
G48	15	12	14	10	8

Table 3: Thin layer Chromatography of Fractions G 34, G22 and G48, in (1:1 n-hexane /ethyl- acetate).

Extracts	number of spots	distance moved in (cm)	solvent front (cm)	R_f value
Fraction 22	2	2.8, 4.2	6.5	0.43, 0.65
Fraction 34	1	3.3	6.5	0.5
Fraction 48	2	3.5, 2.7	6.5	0.41, 0.4

Table 4: Proton and (^{13}C -NMR) Spectrum data for the separated fraction. NMR spectrum (^1H and ^{13}C) data of fraction G34 in CDCl_3

Position of H/C	^1H	Isolated compound	^{13}C
1	0.99,1.71		39.1
2	1.49,1.56		19.4
3	1.15,1.33		42.3
4	--		33.6
5	1.06		55.5
6	1.31,1.57		24.5
7	1.94,2.35		38.3
8	--		148.3
9	1.55		56.2
10	--		39.7
11	1.48,1.66		21.6
12	1.99,2.28		40.1
13	--		164.3
14	5.14		115.1
15	--		171.9
16	2.13		19.1
17	4.47		106.8
18	0.85		33.6
19	0.78		21.8
20	0.66		14.5

The result of proton NMR and carbon 13 NMR analysis of fraction G34 compared with literature report was as shown in Table 5.

Table 5: NMR spectral data of fraction G34 in CDCl₃ compared with literature reports.

Position of ¹ H/ ¹³ C	¹ H	(K)	¹³ C	¹ H	(M)	¹³ C
1	0.99,1.71		39.1	1.0,1.73		39.1
2	1.49,1.56		19.4	1.5,1.57		19.4
3	1.15,1.33		42.3	1.17,1.39		42.1
4	--		33.6	--		33.6
5	1.06		55.5	1.08		55.5
6	1.31,1.57		24.5	1.30,1.55		24.5
7	1.94,2.35		38.3	1.98,2.38		38.3
8	--		148.3	--		148.3
9	1.55		56.2	1.57		56.2
10	--		39.7	--		39.7
11	1.48,1.66		21.6	1.50,1.67		21.5
12	1.99,2.28		40.1	1.98,2.31		40.1
13	--		164.3	--		164.3
14	5.14		115.1	5.67		114.6
15	--		171.9	--		171.8
16	2.13		19.1	2.16		19.2
17	4.47		106.	4.49,4.55		106.4
18	0.85		33.6	0.80		33.6
19	0.78		21.8	0.80		21.7
20	0.66		14.5	0.68		14.5

Key: K=Isolated compound; M=literature reports, (Cavin, *et al*;2006).

The bioactivity of the isolated compound against test bacteria showed a considerable antibacterial activity with zones of inhibition wider than the crude extract. *S.aureus*, was most susceptible with the widest zone of inhibition

followed by *Shigella*. The isolated compound showed competitive inhibition with the control (ofloxacin) at 200 mg/mL (Table 6). The HMBC correlations showing the proton –Carbon relations over long range is shown in Table 7

Table 6: Sensitivity test of Isolated Compound.

Microorganisms	Zones of inhibition (mm). Control (Ofloxacin)	Fraction (G34)
<i>E.coli</i>	29	30
<i>S. aureus.</i>	31	31
<i>Salmonella</i>	28	29
<i>Shigella</i>	30	31
<i>V.cholerae</i>	11	21

Table.7: HMBC Correlations of the Isolated Compound.

Proton ¹ H	Carbon13 (¹³ C)
4.82(H-17b)	38.3 (C-7), 56.2 (C-9)
4.46(H-17a)	38.3 (C-7), 56.2 (C-9)
0.66(H-20)	56.2 (C-9), 55.6 (C-5), 39 (C-10),40 (C-10)
0.78(H-19)	55.6(C-5), 42.1(C-3), 34.29 (C-4)
0.85(H-18)	55.6(C-5), 42.2(C-3), 34.25(C-4), 22.8(C-19)
2.14(H-16)	164.8 (C-13), 114.8 (C-14),40 (C-12), 34.1(C-18)
5.14(H-14b)	19.1(C-18),40.1(C-12),172(C-15)

Discussion.

The result of the NMR analysis of Fraction (G34), is as in Table 4. A preliminary examination of the ¹H and ¹³C NMR spectrum of fraction G34 suggest that the compound could be a diterpene. The carbon spectra showed that fraction G34 is a twenty carbon atom compound, made up of one carboxylic, four olefinic, four methyl carbons, others are, seven methylene, two methane, and two quaternary carbons. Further assessment by 2--D NMR showed that in proton-proton correlation spectroscopy (¹H—¹H COSY), the absence of a

long range ¹H—¹H correlation between H-14 (5.64) and H-16 (2.13) suggest that the double bond is in the E configuration. A NOE between H-20 (0.66) and H-11 (1.66) showed that the methyl group (C-20) has an axial orientation and the side chain is equatorial. In its heteronuclear multiple bond coherence spectrums (HMBC) the long range coupling between H-20 and C-19 methyl protons (0.78) indicates that the methyl group C19 has an equatorial position, implying that the C-18 methyl group is axial. As H-18 (0.85) showed a NOE with H-15 (1.06), the presence of a transe -

decalin ring is evident, since there was no coupling between H-5 and H-20 (Cavin *et al.*, 2006, Urzua *et al.*, 2008).

In summary the correlation between COSY and HMBC indicates 2 olefinic protons attached to the same carbon atom (4.47, 4.48) indicating an oxocyclic double bond typical of a labdane diterpene. Thus the NMR spectral data 1-D (^1H and ^{13}C) and 2-D (COSY and HMBC) showed agreement with reported data of a typical diterpene (Cavin, *et al.*, 2006). Fraction G34 was identified as Copalic acid, (chemical formula $\text{C}_{20}\text{H}_{32}\text{O}_2$ and molecular weight 304.5 g/mol.) Several authors have reported on the isolation of diterpenes from *D. microcarpum*. For instance, Lajide *et al.*; (1995) and Cavin *et al.*; (2006) have reported the isolation of diterpenes from the leaves of *D. microcarpum*.

Among all classes of plant metabolites, diterpenes are considered to be more active and displays a wide spectrum of biological activities, including antibacterial activity (Kuzma *et al.*, 2007). Although, the mechanism(s) responsible for their exceptional biological activity have not yet been very well elucidated, Urzua *et al.* (2008) have reported that these metabolites promote bacterial lysis and disruption of the cell membrane.

According to these authors, the structural features that promote the efficient antibacterial activity include a lipophilic structure, capable of insertion into the cell membrane, and one strategically positioned hydrogen-bond-donor group (HBD; hydrophilic group), which interacts with the phosphorylated groups on the membrane. These structural features, (the lipophilic structure and the HBD; hydrophilic group) present in copalic acid are the likely ones that promote cell lysis and the disruption of cell membrane.

Copalic acid has been shown to demonstrate considerable antibacterial activity against several oral pathogens that cause periodontal disease. It is an important and selective metabolite that should be considered for the control of periodontitis. This diterpene is also very active against *Streptococcus mutans*, another key oral pathogen associated with dental caries. These data denote that future oral care products containing this compound could be of great value for application in the treatment and control of oral pathologies.

The fact that the compound is nontoxic (Souza *et al.*, 2011), supports its use in traditional medicine for the management of diarrheal and related infections. The antibacterial sensitivity testing carried out on the isolated compound showed that the compound, has demonstrated high antibacterial activity on the test organisms with zones of inhibition (30 mm), higher than the crude extracts. The higher activity may be as a result of the presence of the lipophilic structure and the

hydrogen bond donor which unite to cause cell lysis.

It safe to state at this point that the screening and scientific evaluation of plants extracts against microbes may provide new antimicrobial substances. Generally the data generated in this study have provided scientific backing needed for wide use of this plants and the others documented as therapeutic agents for the treatment gastrointestinal infections.

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