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Isolation, Characterization and Antibacterial Activity of Lanostane Triterpenoid from the Leaves of *Stachyterpheta jamaicensis* Linn Vahl

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Abstract

Chemical investigation of the bioactive constituents from the leaves of *Stachytarpheta jamaicensis* Linn Vahl (Gervao, Brazilian tea or Bastard vervain) resulted in the isolation of a new lanostane phenylacetate (1,3,16β-yl-phenypropylacetate-lanostan-5,11,14,16,23,25-hexen-22-one). The structure was elucidated using NMR spectroscopy in combination with IR and MS spectral data. The isolated compound inhibited *Streptococcus fecalis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. This result supports the use of *Stachyterpheta jamaicensis* in phytomedicine for the treatment of cough, burns, gonorrhea, asthma, boils, sores, burns and healing of wounds in herbal medicine in Nigeria.

Keywords: *Stachyterpheta jamaicensis*, lanostane phenylpropylacetate, antibacterial agent, phytomedicine.

Introduction

There has been a tremendous pressure on medicinal plants for their extensive utilization as source of raw materials for the pharmaceutical industries. Demands for medicinal plants are rapidly increasing not only in developing countries but also in developed ones. Medicinal plants are proven source of bioactive compounds with therapeutic indications against a wide spectrum of diseases and infections[1-4]. In particular, *Stachytarpheta jamaicensis* Linn Vahl has been shown to produce secondary metabolites that display anti-inflammatory, antioxidant, neuroprotective, oxytocic, antifungal, antibacterial, antiviral, liver protective, cardioactive and antitumorous effects [4-7].

In our search for therapeutic agents from natural sources, *Stachyterpheta jamaicensis* Linn Vahl (Verbenaceae) commonly known as (Gervao, Brazilian tea, Verbena cimarrona,

Bastard varvain, Blue flower or Rooter comb) was selected for studies because of its applications in the treatment of diverse diseases and ailments. In herbal medicine, *S. jamaicensis* is used to treat various ailments such as inflammation, pain, fever, hepatic and renal disorder, helminthiasis, constipation, hypertension, stress, rheumatism, malaria and diabetes[8]. The leaves and stem bark extracts are used extensively in traditional medicine in the preparation of drugs used by Nigerian herbalists and practitioners as stomach tonic to stimulate the function of the gastrointestinal tract, for dyspepsia, allergies, asthma and fever as well as for the treatment of cirrhosis and hepatitis (inflammation of the liver) [9-12]. The leaves are used to cure cough, dysentery or chest colds in herbal medicine[8]. Extracts from the leaves prevent malaria, colic disorder, cure head or chest colds [13], suppressed cough[14] and is often used in the treatment of cirrhosis and hepatitis [11]. Externally, it is used to treat ulcers, sores, cuts, and wounds [9]. In some countries, it is used for abortive, childbirth, lactation stimulation and for the treatment of menstrual disorders[9]. Several studies[15-18] have documented the scientific basis for the efficacy of plants in phytomedicine. This study seeks to ascertain the usefulness of *S. jamaicensis* in the treatment of infections conditions caused by common pathogens. The study involves the isolation, structural elucidation and characterization of the bioactive constituents in the plant and consequently evaluates the antibacterial activity against some pathogenic bacteria for possible development of new drugs for the prevention and treatment of infections.

Results and Discussion

Compound **1** was obtained as black crystal needles. Its molecular composition was $C_{57}H_{64}O_7$ as determined from combined analysis of its HREIMS (m/z 860.89) and ^1H and ^{13}C NMR spectra data. The IR spectrum of compound **1** exhibited absorptions at Vmax 2922.23 cm^{-1} and 2853.26 cm^{-1} both peaks representing the aliphatic -CH_2- group. Other functional groups identified include the carbonyl (C=O) (Vmax 1741.02 cm^{-1}), the aromatic (C=C) (Vmax 1463.44 cm^{-1}) and the ether (C-O) group which absorption peak appeared at Vmax 1072.52 cm^{-1}).

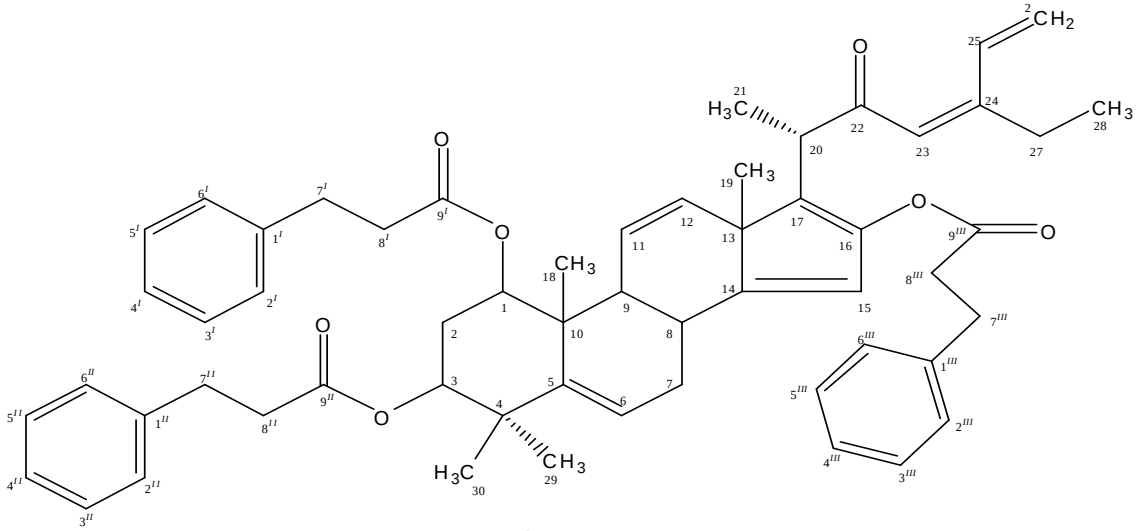

Figure **1** Compound **1:** $C_{57}H_{64}O_7$

The ^1H NMR spectrum produce signals due to four tertiary methyl peaks at δH 0.8291, 0.8525, 0.9200 and 0.9494 respectively, one secondary methyl (δH 1.0057) and one primary

methyl (δH 1.5615). The olefinic protons were observed at δH 5.2667, 5.3208, 5.3428, 5.3428, 5.3581 and 5.3829 respectively. The presence of the olefinic was confirmed by the ^{13}C NMR spectrum which showed olefinic carbon peaks at δC 109.6010, 110.2210, 113.4200 and 114.2010 respectively while the tertiary methyl carbons appeared at δC 20.2410, 20.1001, 23.2012 and 23.5010 respectively.

The aromatic protons appeared as a singlet peaks at δH 7.4103, 7.4272 and 7.5984. Analysis of the ^{13}C NMR spectrum confirmed the presence of a lanostane skeleton with 30 carbon atoms as well as the presence of aromatic and carbonyl carbons which were easily observed and identified. The spectrum gave the resonance of the aromatic chemical shifts at δC 127.268 – 132.107. The chemical shifts for carbonyl carbon occurred at δC 177.3010 and δC 173.202 respectively. The data suggests that **1** is tetracyclic triterpenoid with acetate linkages at C_1, C_3, and C_{16} respectively attached to the lanostane skeleton.

Table 1: ^{1}H NMR and ^{13}C NMR Chemical shifts of Compound 1 ($CDCl_3$)

Position	**δH**		**δC**	
1	1.4152	1Ht	31.5434	CH
2	1.3845	2Hm	20.6905	CH_2
3	1.4272	1Hm	32.9459	CH
4			22.8289	C
5			130.0671	C
6	5.2667	1Hm	133.6000	CH
7	1.2537	2Hm	25.6755	CH_2
8	1.5984	1Hm	34.0671	CH
9	1.4152	1Ht	32.9459	CH
10			34.0671	C
11	5.3208	1Ht	130.3900	CH
12	5.3428	1Ht	130.3901	CH
13			38.0435	C
14			134.1010	C
15	5.3581	1Hs	135.1100	CH
16			134.4014	C
17			135.0620	C
18	0.8291	3Hs	12.2028	CH_3
19	0.8525	3Hs	12.5060	CH_3
20	1.5984	1Hs	49.0240	CH
21	1.0057	3Hd	20.6905	CH_3
22			177.3010	C=O
23	5.3428	1Hs	132.1070	CH
24			132.107	CH
25	5.3581	1Ht	132.200	CH
26	5.3829	2Hd	131.109	CH_2
27	1.4154	2Hd	29.218	CH_2
28	1.5615	3Hd	15.4000	CH_3

29	0.9200	3Hs	14.4044	CH_3
30	0.9494	3Hs	14.2505	CH_3
1^{I}			130.390	C
2^{I}	7.2613	1Hd	127.205	CH
3^{I}	7.5267	1Ht	127.902	CH
4^{I}	7.5361	1Ht	128.434	CH
5^{I}	7.5267	1Ht	127.902	CH
6^{I}	7.2613	1Hd	127.205	CH
7^{I}	2.0227	2Ht	25.5755	CH_2
8^{I}	2.0344	2Ht	25.7535	CH_2
9^{I}			173.2020	C=O
1^{II}			130.3900	C
2^{II}	7.5135	1Hd	127.9020	CH
3^{II}	7.5267	1Ht	127.2050	CH
4^{II}	7.76763	1Ht	128.4340	CH
5^{II}	7.5267	1Ht	127.2050	CH
6^{II}	7.5135	1Hd	127.9020	CH
7^{II}	2.0227	2Ht	25.5755	CH_2
8^{II}	2.0344	2Ht	25.7535	CH_2
9^{II}			173.2020	C=O
1^{III}			130.3900	C
2^{III}	7.7042	1Ht	127.9020	CH
3^{III}	7.7140	1Ht	127.2050	CH
4^{III}	7.7273	1Ht	128.4340	CH
5^{III}	7.7140	1Ht	127.2050	CH
6^{III}	7.7041	1Hd	127.9020	CH
7^{III}	2.3113	2Ht	25.5755	CH_2
8^{III}	2.0344	2Ht	25.7535	CH_2
9^{III}			173.2020	C=O

S = singlet, d = doublet, m = multiplet, t = triplet

Compound **1** was assigned the molecular mass m/z 860.89 (M^+) calculated for $C_{57}H_{64}O_7$ (m/z 860) with base peak at m/z 413.20 calculated for $C_{30}H_{36}O_7$. The base peak occurred due to the detachment of the phenylacetate portions. The other prominent peaks at m/z 414.22 and 415.25 were observed and they occurred as a result of proton migration and re-arrangement. The pattern of fragmentation of compound **1** is shown in Figure **2**. Combining the MS, NMR and IR spectra data, compound **1** is identified as a lanostane phenylacetate that is 1,3,16β-yl-henypropylacetate-lanostan-5,11,14,16,23,25-hexen-22-One.

The compound exhibited anti-bacterial activity *in vitvo* against some pathogenic microorganisms (Table 2). The compound successfully inhibited *S. fecalis, S. aureus, E. coli* and *P. aeruginosa*. This compound exhibited highest anti-bacterial activity against *S. fecalis, E. coli,* and *S. aureus*. In general, the order of activity of compound **1** against the bacteria was *S. fecalis>E. coli>S. aureus>P. aeruginosa* at 50 mg/ml concentration (Table 2). Many of these organisms are natural flora of the skin and also known etiologic agents of several skin

and mucous membranes infections of man[21] and these microorganisms are infections of wounds, sores and boils[21].

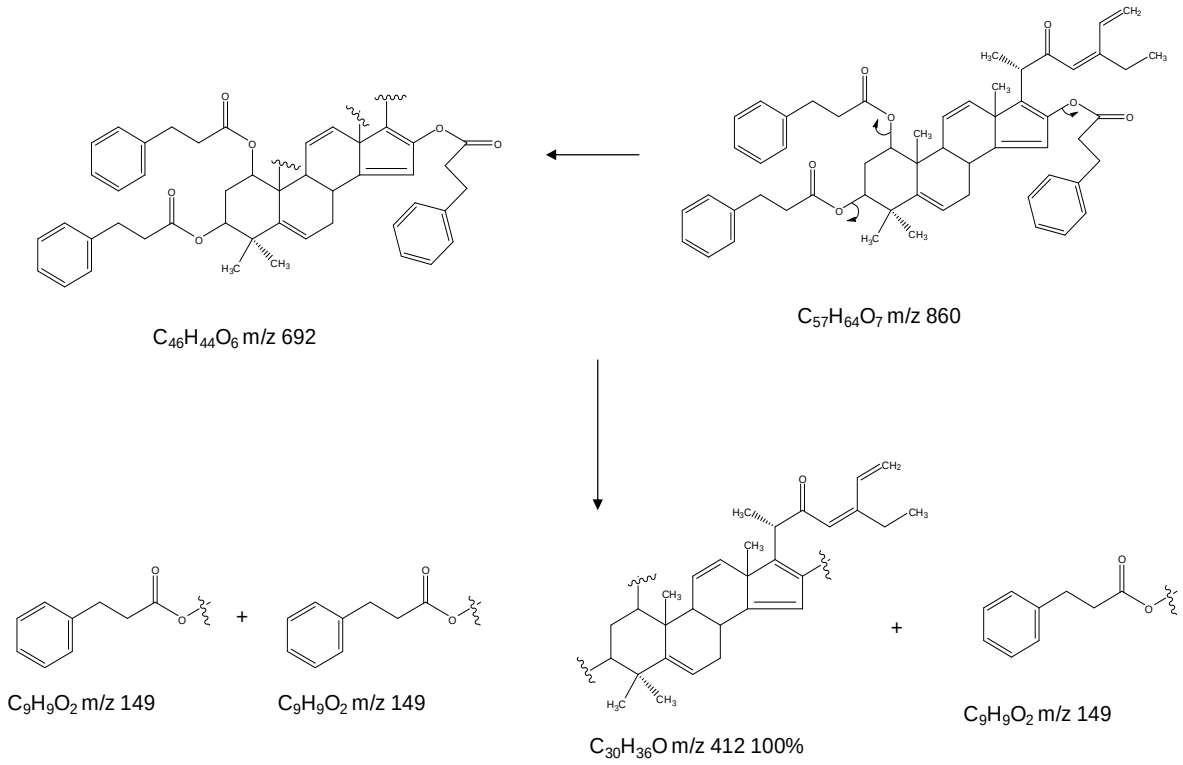

Figure 2: Fragmentation pattern of compound 1

The observed inhibitory role on these micro-organisms explains the reason behind the utilization of *S. jamaicensis* extract in traditional medicine as cough suppressant, anti-tumor agent, wound healing and in the treatment of boils and sores[4,6,8]. The minimum inhibitory concentration (mic) of the compound was 6.5 – 12.5 mg/ml (Table 2). *S. fecalis, S. aureus* and *E. coli* are the common cause of urinary track infections[18]. *S. fecalis*, and *S. aureus* cause inflammation of the genital organs, damaged sperm cells and skin infections[22-23]. Compound **1** cause varying degrees on inhibition of the growth of these pathogens. This finding supports the use of the leaves of *S. jamaicensis* in the treatment of urogenital infections in herbal medicine[8]. Compound **1** showed inhibition against *E. coli*, *S. aureus* and *P. aerguinosa*. This finding also supported the use of *S. jamaicensis* for the treatment of wounds for which these pathogens are associated[8]. The leaves of *S. jamaicensis* possess phyto-constituents capable of inhibiting the growth of microbial wound contaminants; accelerate wound healing and consequently resulting to cell proliferation. This study demonstrates that *S. jamaicensis* posses antibacterial activities. These however, justify the traditional use of *S. jamaicensis* in herbal medicine. The isolated compound from the leaves of *S. jamaicensis* can be used by pharmaceutical firms for drug formulation.

Table 2: Diameter of Zones of inhibition (mm) of compound 1 on the Pathogens (mg/ml)

Pathogens	Concentration of compound **1** (mg/ml)				mic mg/ml
	50	25	12.5	6.5	
	Zone diameter of inhibition (mm)				
S. fecalis	18.0	12	5	-	12.5
P. aeruginosa	14.0	12	8	5	6.5
E. coli	17.0	12	7	5	6.5
S. aureus	16.0	5.0	5	-	12.5

Data are means of triplicate determinations
- No inhibition

Material and Methods

Experimental: General Experimental Procedure

IR spectrum was determined on a Thermo Nicolet Nexus 470 FT- IR spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 FT NMR spectrometer using Tetramethylsilane as internal standard. Chemical shifts are expressed in δ-values. LC-ESIMS spectra were determined in the positive ion mode on a PE- Biosystem API 165 single quadruple instrument. HRESIMS (positive ion mode) spectra were recorded on a Thermo Finniga MAT 95 XL mass spectrometer. Column chromatography was carried out with silica gel (200-300 mesh) and to monitor the preparative separations, analytical thin layer chromatography (TLC) was performed at room temperature on pre-coated 0.25 mm thick silica gel 60 F_{254} aluminum plates (20 x 20 cm) Merck, Darmstadt, Germany. Reagents and solvents like ethanol, chloroform, diethyl ether and hexane, were all of analytical grade and were procured from Merck, Darmstadt, Germany. The nutrient agar was purchased from Scharian Chemie (APHA) Spain.

Plant Materials

Fresh leaves and stems of *S. jamaicensis* were harvested from Edibe-Edibe, Calabar, Cross River State, Nigeria on 10th January, 2008. Plant samples (flowers, stems and leaves) were identified by Dr. A. Nmeregini of the Taxonomy Section, Forestry Department, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. Voucher Specimen No. SJ/3348 has been deposited at the Forestry Department of the University.

Extraction and Isolation of Plant Materials

Plant materials were treated and analyzed at the Chemistry laboratory, Michael Okpara University of Agriculture, Umudike, Nigeria. The leaves (1Kg) were dried on the laboratory bench for 10 days. The dried samples were milled and ground into powder (850g) using a Thomas Wiley Machine (Model 5 USA). The powdered plant sample (500g) was packed into Soxhlet apparatus (2L) and extracted exhaustively with 1000 ml ethanol for 24 hrs. The ethanol extract was concentrated using a rotary evaporator at 45^{0}C and left on the laboratory bench for 2 days to obtain a dark gummy residues (22.64 g).

The column was packed with silica gel and 20.0 g of the gummy residue was placed on top of the silica gel and eluted with chloroform, petroleum ether, methanol (50:30:20) respectively to afford three fractions (L_1, L_2 and L_3). The third fraction (L_3) was crystallized with hexane to produce black crystals lanostane phenylacetate (1,3,16β-yl-phenypropylacetate-lanostan-5,11,14,16,23,25-hexen-22-one) compound **1** yield 1.46 g R_f 0.54, IR Vmax 2922.23 (-CH_2-),

2853.26 (-CH_2-), 1741.02 (C=O), 1463.44 (C=C), 1072.52 (C-O). HREIMS 860.89 [M^+] calculated for $C_{57}H_{64}O_7$ (m/z 860). Base peak m/z 413.20. ^{1}H NMR and ^{13}C NMR were presented in Table **1**.

Bioassay

The *in vitro* antibacterial activity of compound **1** was carried out for 24h culture of four selected bacteria. The bacteria used were two Gram-negative organisms (*Escherichi coli* and *Pseudomonas aeruginosa*) and two Gram-positive strains (*Staphylococcus aureus* and *Streptococcus fecalis*). All the test organisms' are resistant clinical isolates of human pathogens obtained from the Federal Medical Centre (FMC) Umuahia, Nigeria. Cultures were brought to laboratory conditions by resuscitating the organisms in buffered peptone broth (Scharian chemie) and thereafter nutrient agar (peptone 5g/l and meat extract 3g/l) and incubated at 30^{o}C for 24hrs[19]. The antibacterial activity was performed by a filter paper disc diffusion technique[19]. The medium (7g nutrient agar) in 250ml distilled water, autoclaved at 115^{o}C for 15mins was cooled to 50^{o}C. 20ml of the medium was poured into sterile Petri dish and allowed to stay for 8h and observed for contamination. Compound **1** (1g) was dissolved in 1ml of absolute ethanol and made up to 10ml with distilled water to give a concentration of 100 mg/ml (10% dilution), 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.5 mg/ml respectively. A colony of each test organism was sub-cultured on nutrient broth which contained peptone (5 g/l) and meat extract (3 g/l) and incubated aerobically at 37^{o}C for 8h. 30 ml of the nutrients broth was used to flood the agar plates. A single sheet of sterilized Whatman No. 1 filter paper disc soaked in compound **1** (0.02 ml) was used to test for the sensitivity or anti-microbial effect of compound **1**. The plates were incubated at 37^{o}C for 24h. After incubation, plates were observed for zones of inhibition (in mm diameter). The minimum inhibitory concentration was determined.

Statistical Analysis

All measurements were replicated three times and standard deviation determined. The student t-test at $P<0.05$ was applied to assess the difference between the means[20].

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