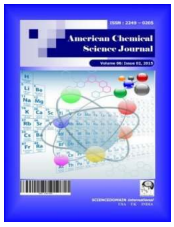

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Inhibition of the Developmental Stages of *Ascaris suum* and Antimicrobial Activity of 3β-Hydroxylolean-12,18-diene Isolated from the Aerial Parts of *Canarium schweinfurthii* (Engl)

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Authors' contributions

This work was carried out in collaboration between all authors. Author GIN is the Chairman supervisory committee of this research work. He was involved in the approval of all procedure. Author RGA was involved in the supervision of the antimicrobial analysis. Author JDH was involved in the testing of the isolated compounds on the developmental stages of Ascaris suum, confirmed the purity of the isolated compounds and provided us with a collaborating laboratory for the NMR analysis of the isolates at the University of Kwa-zulu natal, Durban, South Africa. Author BJO is the research student who carried out the isolation and testing of the isolates under the supervision of author GIN. All authors read and approved the final manuscript.

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ABSTRACT

Our search for anthelminthics from nature led to the isolation of 3β-hydroxylolean-12,18-diene, from the aerial Parts of *Canarium schweinfurthii* for the first time. A plant used in the treatment of parasitic worms in Nigeria, the structure was elucidated using spectral data and by comparison with literature data. Preliminary antimicrobial screening of the extract at 50 mg/mL showed 66.7% inhibitions against the test organisms. The isolated 3β-hydroxylolean-12,18-diene showed inhibitory activity at 6.25 µg/mL against *Staphylococcus aureus, MRSA, Eschoriahia coli, Shigella dysenteria, Bacillcis subtlis, Kebsiella pneumonia, Candida stellatoidea* while *Salmonella typhi, Candida albicans* and *Candida tropicalis* were inhibited at 12.5 µg/mL. The ovacidal and larvacidal activities of 3β-hydroxyl olean-12,18-diene against the pre-infective and infective stages of *Ascaris suum,*

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showed percentage inhibition between 62 to 65% and 76 to 86% as compared to the standard drug Albendazole which showed inhibition ranging between 96.1 to 97.5%. The results of this investigation clearly shows that the plant has potential that can be explore in the search for antiparasitic drug from nature.

Keywords: Canarium schweinfurthii; helminth; NMR spectral analysis; ovicidal and larvicidal activities.

1. INTRODUCTION

Helminth infections are among the most widespread infections in humans, distressing a huge population of the world. Majority of the infection due to helminthes is generally restricted to the tropical regions and cause enormous hazards to health and contribute to the prevalence of malnourishment, anemia, eosinophilia and pneumonia [1]. Gastrointestinal nematode infections of livestock, which are bred for the production of meat, milk, or wool all around the world, lead to enormous economic losses.

The control of these parasites has relied on the use of chemical anthelmintics, resulting in development of drug-resistant strains. Alternative control methods include vaccination and in developing countries the use of traditional medicinal plants, which have become the focus of examination all over the world today. The evidence of anthelmintic properties of plants is gained primarily from ethnoveterinary and ethnomedical knowledge [2]. Among bioactive compounds, an important group is that of triterpenes, which show anthelmintic properties against gastrointestinal nematode at low activity toward livestock [3].

Triterpenes are naturally occurring alkenes of vegetable, animal and also fungal [4-9] origin, classified among an extensive and structurally diverse group of natural substances, referred to as triterpenoids. Their structure includes 30 elements of carbon and they are constituted by isoprene units [10]. Taking into consideration the structure, triterpenes may be divided into linear ones—mainly derivatives of squalene, tetracyclic and pentacyclic, containing respectively four and five cycles, as well as two- and tricyclic ones [11]. Representatives of those show anti-cancer properties [12-13] as well as anti-inflammatory [14], anti-oxidative [15], anti-viral [16-17], anti-bacterial [18] and anti-fungal ones [19].

Canarium schweinfurthii which belongs to the Burceraceae family is a forest region plant, wide spread over all Africa, from Senegal to Democratic Republic of Congo, Sudan and Ethiopia. The stem bark decoction of *C. schweinfurthii* is used as a remedy for roundworms, colic, stomach pains, pain after child birth, gale, dysentery and gonorrhoea [20]. In this paper we report our findings on the evaluation of *C. schweinfurthii* for anthelminthic potential as claimed by ethnomedical practitioners.

2. MATERIALS AND METHODS

2.1 Plant Material

The Leaves and stem bark of *Canarium schweinfurthii,* were collected from Shendan Local Government Area of Plateau state, in the month of September, 2011. Identified and authenticated by the curator; Mallam Musa Mohammed of the Herbarium unit Department of Biological Sciences, Ahmadu Bello University, Zaria-Nigeria. A voucher specimen #7232 was deposited at the Herbarium.

2.2 Extraction

The pulverized plant material (1.5 Kg) was carefully weighed and transferred into a Soxhlet extractor and extracted successively with n-hexane (60 – 80℃), chloroform, ethyl acetate and methanol for 72 hours respectively. The various extracts were then concentrated *in vacuo* at 40 ℃ using rotary evaporator and subjected to air drying to give dried crude extracts.

3. BIOLOGICAL EVALUATION

3.1 Preparation of Inoculum

The test organism *Methicillin Resistant Staphylococcus aureus*(MRSA), *Vancomycin Resistant Enterococci* (VRE), *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillcus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Shigella dysenteria*, *Candida albicans*, *Candida Stellatoidea*, *Candida tropicalis* and *Candida krusei* were obtained from the Department of Medical Microbiology Ahmadu Bello University Teaching Hospital (ABUTH) Zaria, Nigeria. Stock culture was maintained at 5℃ on slants of

nutrient agar. Active stock culture was inoculated in fresh tubes of Muller–Hinton broth medium (MHB) and the bacteria and fungi were incubated for 24 h at 37℃ and 34℃ respectively.

3.2 Determination of the Zone of Inhibition

Spectrum of antibacterial activity was studied by using the technique described by Bauer et al. [21]. The leaf and stem bark extracts of the *C. schweinfurthii* were investigated for their antibacterial and antifungal activities against eleven bacteria and four candida strains. At the end of incubation, inhibition zones formed around the well were measured with transparent rule in millimetre. These tests were performed in triplicate and expressed as average mean ± SD.

3.3 Determination of the Minimum Inhibitory Concentration (MIC)

Minimum inhibition concentration (MIC) is the lowest concentration of drug that prevents the growth of a particular pathogen (22]. Different dilution concentrations of CB 47 (6.25–50 µg/ml) were prepared and inoculated with the suspension of the overnight grown bacteria and fungi inoculum. After 24 hrs at 37℃, MIC of each isolated compound was determined by measuring the optical density at 620 nm in UV–vis spectrophotometer-2402 (Shimadzu, Japan) [23].

3.4 Determination of the Minimum Bactericial/Fungicidal Concentration (MBC/MFC)

It was carried out to determine whether the test microbes were bactericidal or only bacteriostatic. The content of the MIC in the sterile dilution was subcultured into the prepared medium; incubation was made at 37℃ for 24 hrs, after which the plate of the medium was observed for colony growth, the MBC/ MFC over the plate lowest concentration of the extract without colony growth.

3.5 Egg Hatch Assay (EHA)

The egg hatch assay was conducted as published by McGaw et al. [24] and Bizimenyera et al. [25]. The counted eggs in a 0.5 mL of egg suspension were pipetted into test tubes. In addition, 0.5 mL of 3β-hydroxylolean-12,18-diene at 10 and 20 µg/mL were added. A commercial anthelmintic drug (Albendazole®) was used as the positive control at the same concentrations and DMSO as a negative control. All tests were carried out in triplicate.

The test tubes were incubated under humidified conditions at 25℃ temperature for 48 hours thereafter a drop of Lugol's iodine solution was added to each well to stop further hatching. All unhatched eggs and L_1 larvae were then counted. Inhibition percentages were calculated using the formula of Cala et al. [26]:

$$\mathrm{E}\,(\%) = \frac{(\mathrm{Eggs} + \mathrm{L_1}) - \mathrm{L_1}}{(\mathrm{Eggs} + \mathrm{L_1})} \times 100$$

3.6 Larval Development Assay (LDA)

The larval development assay was conducted as described by Bizimenyera et al. [25]. The counted number of eggs in a 0.5 mL of the egg suspension was put into each test tube with a 100 µl of lyophilized penicillin-streptomycin to combat fungal growth. The contents of the tubes were then mixed, and then placed in an incubator under humidified conditions at ambient temperature for 48 hours for incubation of the eggs. After 48 hours, 0.5 mL of 3β-hydroxylolean-12,18-diene as well as Albendazole® as a positive control at 10 and 20 µg/mL were added to respective plates. The negative control plates received 0.5 mL of DMSO. All experiments were replicated three times. Incubation of the tubes was continued for 21 days, after which all the tubes were examined to determine the survival of larvae at different concentrations. All the L_3 stage larvae in each well were counted and a percentage inhibition of larval development was calculated using the formula Cala et al. [26]:

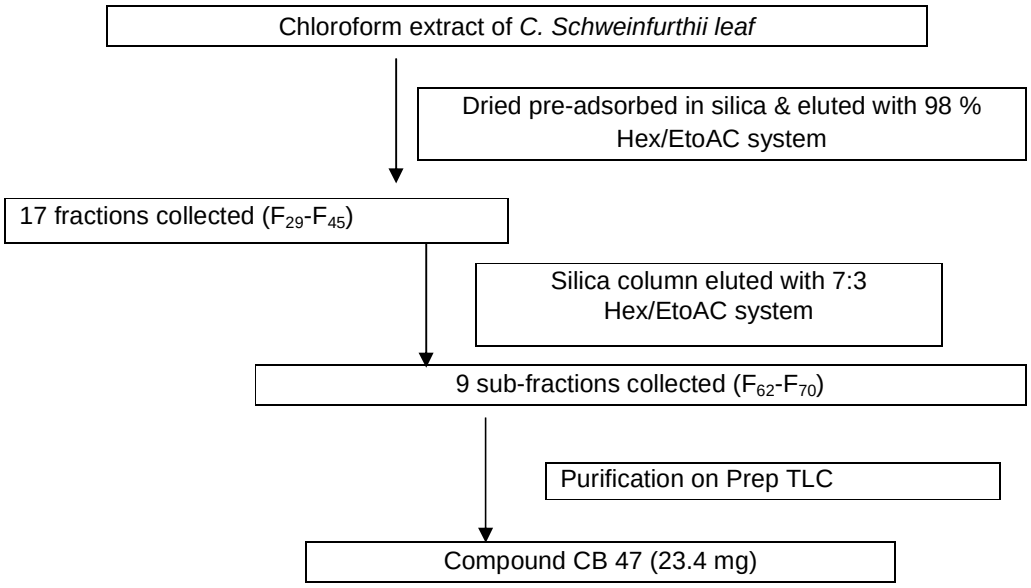
$$\mathrm{E}\,(\%) = \frac{(\mathrm{L_1} + \mathrm{L_2} + \mathrm{L_3}) - \mathrm{L_3}}{\mathrm{L_1} + \mathrm{L_2} + \mathrm{L_3}} \times 100$$

3.7 Phytochemical Screening

The crude chloroform extracts was qualitatively analyzed using standard methods [27-30] for presence of alkaloids (Meyer's and Drangedorffs test), tannins (Styassny's reagent), saponins (foaming test), flavonoids (Shibata's reaction), glycosides and phytosterols .

3.8 Isolation

The chloroform extract was the most sensitive extract from the antimicrobial and anthelmintic screening and was subjected to column chromatography (CC) for fractionation. The isolation scheme is presented below;



Scheme 1. Flow chart for the isolation of compound CB 47

4. RESULTS AND DISCUSSION

From the preliminary antimicrobial screening of the crude aerial plant parts extracts of *C. Schweinfurthii Engl*, chloroform extract exhibited the highest activity against the test microbes ,pre-infective and infective stages of *A. suum* via their zones of inhibition, percentage inhibition of egg and larval stages respectively. Therefore, activity guided isolation was undertaken.

The results of the phytochemical screening of the chloroform extracts of the aerial plant parts reviewed the presences of pytosterols and flavonoids (Table 1)

4.1 Antimicrobial Activity of the Isolated Compound

Compound CB 47 showed sensitive to three Gram-postive bacteria (*MRSA, S. aureus* and *B. subtilis*), four Gram-negative bacteria (*K. pneumonia, S. dysenteria, E. coli and S. typhi*) and three candidas (*C. albicans, C. tropicalis and C. Stellatoidea*). The zone of inhibition of Gram-positive bacteria are between 27-32 mm, Gram-negative 16-29 mm compared with positive control ciprofloxcian 35-40 mm while the inhibition zone of the candidas were measured at 24-26 mm compared to fluconazole 32-37 mm (Table 2). CB 47 possesses both antibacterial and antifungal properties and shows strong anti-candida activity.

It inhibited the growth of three Gram-postive bacteria (*MRSA, S, aureus* and *B. subtilis*) at 6.25 µg/mL, four Gram-negative bacteria (*K. pneumonia, S. dysenteria, E. coli and S. typhi*) between 6.25 -12.5 µg/mL and three candidas (*C. albicans, C. tropicalis and C. Stellatoidea*) at 6.25 -12.5 µg/mL (Table 3).

Table 1. Phytochemical screening of extracts

Phytochemical	Chloroform extract
Alkaloid	-
Cardiac glycoside	-
Saponins	-
Pytosterols	+
Phenols	-
Flavonoids	+
Tannins	-

***Key**: [+]-Presence and [-]- Absence*

The minimum concentration required to kill the microorganism various from one isolate the other depending on the transmembrane exclusion resistance of the compound. CB 47 was bactericidal towards three Gram-positive bacteria at 12.5 µg/mL, four Gram-negative bacteria between 12.5 - 25 µg/mL and fungicidal towards three candidas between 25-50 µg/mL (Table 3). CB 47 is bactericidal at very low concentration compared to their fungicidal activity.

Table 2. Zone of inhibition (mm) of the isolated compound against the test organisms

Test organism	CB 47	Control		
		Ciprofloxacin	Erythromycin	Fluconazole
Methrallin Rest staph aureas (MRSA)	29	35	-	-
Veancomyan Rest Entorococci (VRE)	-	-	32	-
Staphylococcus aureus	27	37	35	-
Streptococcus pyogenes	-	35	32	-
Bacillcis subtlis	32	40	37	-
Eschoriahia coli	27	35	35	-
Salmonella typhi	25	42	0	-
Kebsiella pneumonia	29	40	37	-
Pseudomonas aeruginosa	-	-	32	-
Proteus misrabilis	-	-	37	-
Shigella dysenteria	28	37	38	-
Candida albicans	26	-	-	35
Candida krusei	-	-	-	32
Candida tropicalis	24	-	-	37
Candida Stellatoidea	26	-	-	32

Table 3. Antimicrobial screening (μg/ml) of the isolated compound on test microorganism

Test organisms	CB47		
	MIC	MBC	MFC
Methicillin Rest staph aureus	6.25	12.5	-
Vancomycin Rest Enterococci	-	25	-
Staphylococcus aureus	6.25	12.5	-
Streptococcus pyogenes	-	-	-
Bacillcus subtilis	6.25	12.5	-
Escherichia coli	6.25		-
Salmonella typhi	12.5	50	-
Klebsiella pneumonia	6.25	25	-
Pseudomonas aeruginosa	-	-	-
Proteus mirabilis	-	-	-
Shigella dysenteria	6.25	25	-
Candida albicans	12.5	-	25
Candida krusei		-	
Candida tropicalis	12.5	-	50
Candida Stellatoidea	6.25	-	25

4.2 Anthelmintic Activity of the Isolated Compound

The eggs of *A. suum* are protected by a thick wall making it resistant to unfavourable conditions [31] which are made up of P-glycoproteins, which have a significant role in transmembrane exclusion of anthelmintics from the CNS, gut, and hepatobiliary tract of hosts, could account for reduced oral bioavailability of some anthelmintic compounds [32]. The present study is to measure the percentage eggs that metamorphose into the pre-infective stage and the pre-infective larva that metamorphose into the infective stage. The ovicidal activity of the extract was measured at 10 µg/mL and the percentage inhibition was 62% and at 20 µg/mL 68% for CB47 (Fig. 1). CB 47 is a potent ovicidal isolate relative to the control drug (Albendazole). The five carbocyclic rings of the triterpene will increase the lipophilic nature of the isolates and increase the diffusion of the isolates into the egg shell, which will interfere with the osmotic balance and hence leading to death (33]. The mechanism of action of Benzimidazole drug is by blocking glucose uptake in larval and adult stages of susceptible parasites, and also depleting their glycogen reserves, thus decreasing ATP formation. Clearly, the isolated compound does not have a benzimidazole frame work hence, another mechanism of action may be proposed. A natural product anthelmintic compound; having a receptor-mediated effect on glutamate-gated chloride (GluCl) ion channels,

which has been directly correlated to nematocidal activity and which is now considered as their major mode of action [34] maybe the mechanism of action of this isolate.

The larvicidal activities of the isolates are high compared to the ovicidal activity. The larvicidal activities ranges (Fig. 1); which is an indication that the P-glycoproteins does not play any significant role in transmembrane exclusion of the isolates. The high larvacidal activities may also be as a result of the free-feeding stage of the larva which allows the isolates to penetrate the gut and access the CNS and hepatobiliary tract of the host. Saponins a closely related member of the triterpene have also been reported to have nematicidal activities and are said to interact with the cell membrane causing changes in cell wall permeability [35]. They also interact with collagen proteins from the cuticle of nematodes and this interaction may be responsible for the nematotoxic effects [36]. At high concentrations, the isolate significantly inhibited the development of L1 and L2 larvae. These substances are able to penetrate the cuticle of the nematode and to prevent the absorption of the glucose, or block post synaptic receptors thus paralyzing the larvae. The activity of CB 47 can be attributed to the carboxylic acid group which has the ability of hydrolysing the P-gylcoprotein gateway. The activity of CB 47 can be linked to the property of terpenoids, terpenoids are most likely to undergo lipid peroxidation which results in damage to the cell membrane, mostly the end product of lipid peroxidation may be mutagenic and carcinogenic [37].

4.3 Spectra Results

The UV absorption band is an indication of weak auxochromes that are pH dependent like OH and an isolated olefinic group showed UV absorption of about 290 nm.

The IR spectra of the isolated compound CB 47 indicated prominent absorption frequencies characteristic of certain functional groups. The band of weak intensity at 3036 cm^{-1} (in the IR) suggests the presence of hydroxyl group. The broad band of very medium intensities at 901 and 718 cm^{-1} are characteristic of O-H bending of alcohols, carboxylic acids, esters, ethers groups and C–H rock of alkanes respectively. It is obvious that there is the absence of carbonyl stretching vibration in the spectrum of CB 47.

^{1}H (Fig. 2) and ^{13}C NMR (Fig. 3) spectra are characteristic feature of an oleanane triterpenoids [39]. CB 47 was obtained as a white amorphous solid. The ^{13}C-NMR spectrum showed 30 carbon atoms while the Distortionless Enhancement Polarization Transfer (DEPT) subspectrum indicated five methine (CH) carbons, nine methylene (CH_2) and eight methyl (CH_3) groups. Quaternary carbon atoms do not contain attached protons hence do not appear in DEPT subspectrum. They may be identified as the signals which are additionally in the proton broadband decoupled ^{13}C-NMR spectra, therefore eight quaternary carbons were identified (Fig. 4).

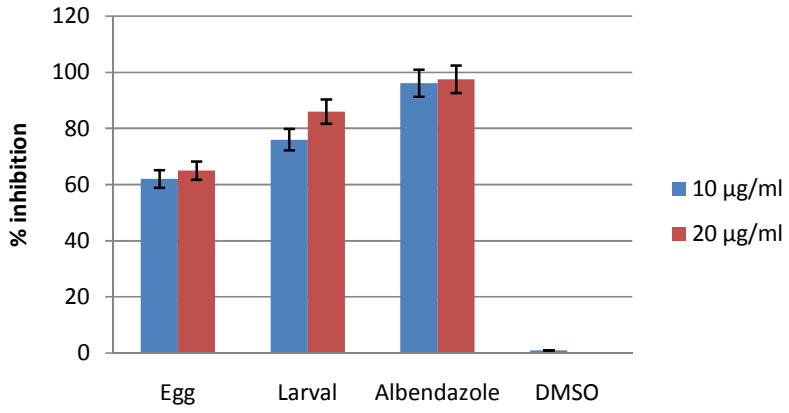


Fig. 1. % inhibition of eggs and larval of *A. suum* by CB47

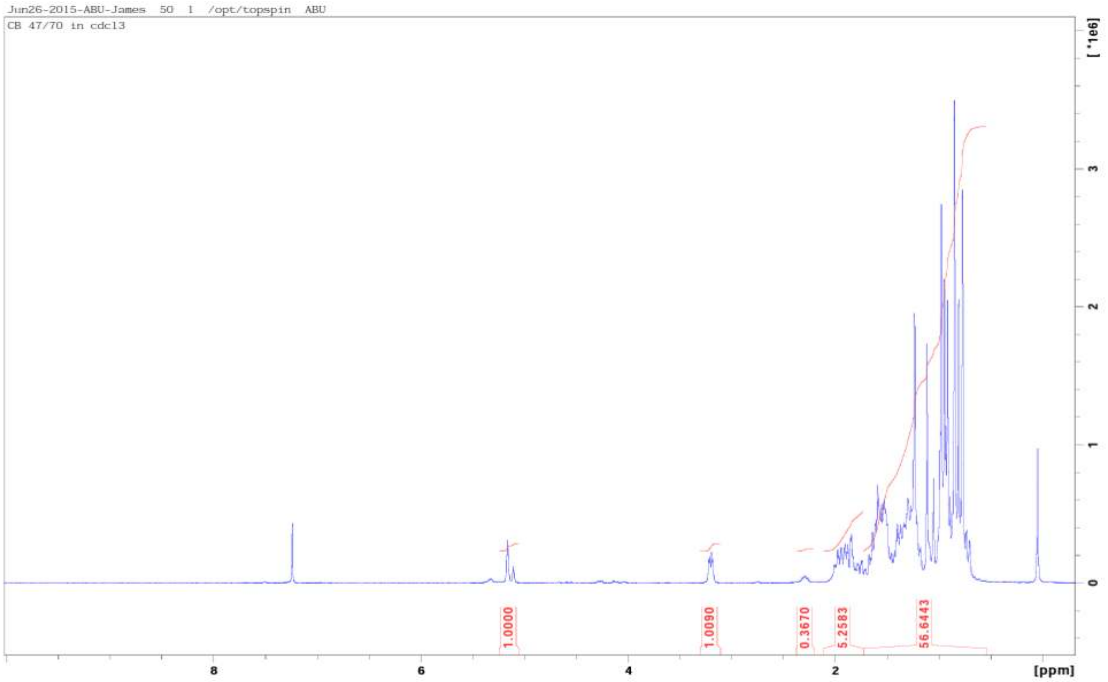


Fig. 2. ^{1}HNMR

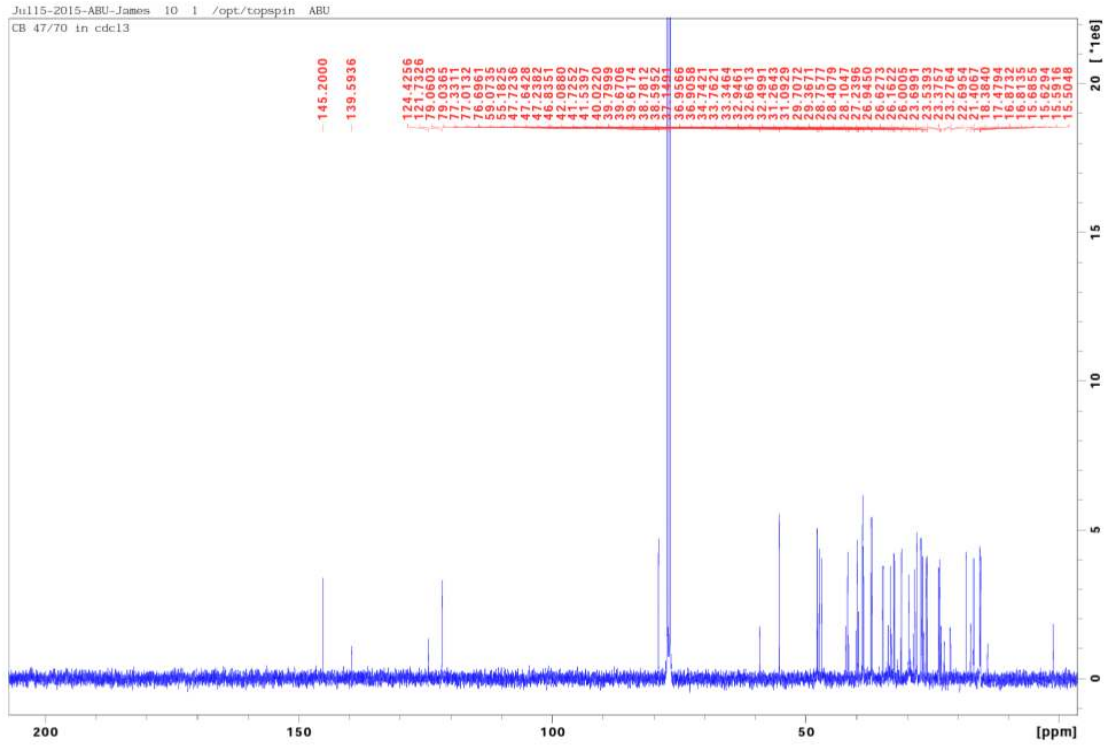


Fig. 3. ^{13}C NMR

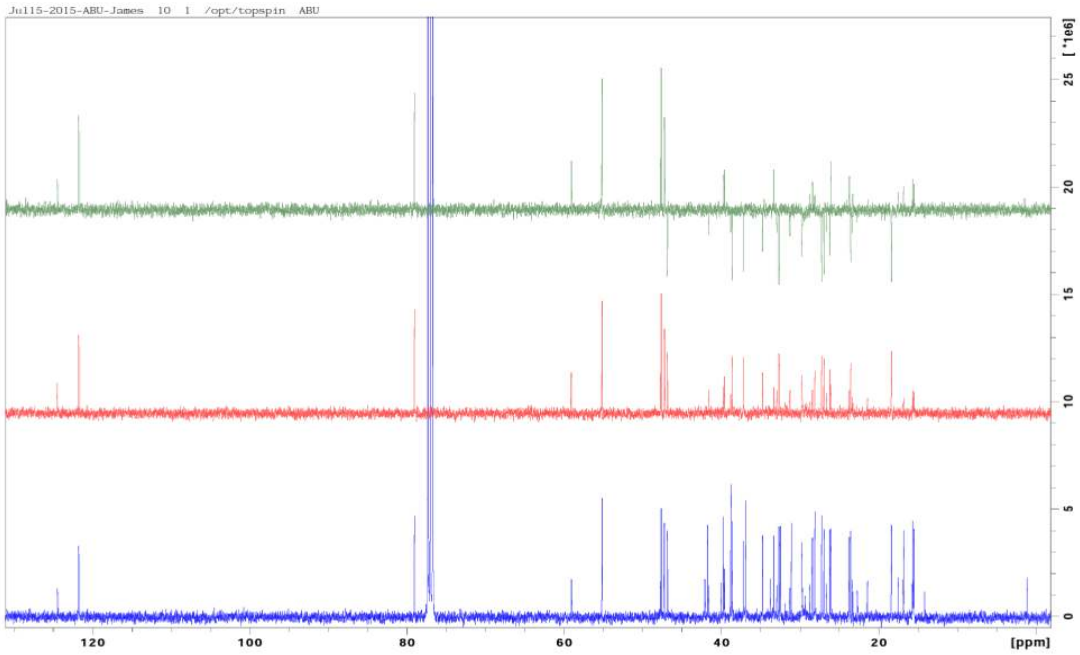


Fig. 4. DEPT

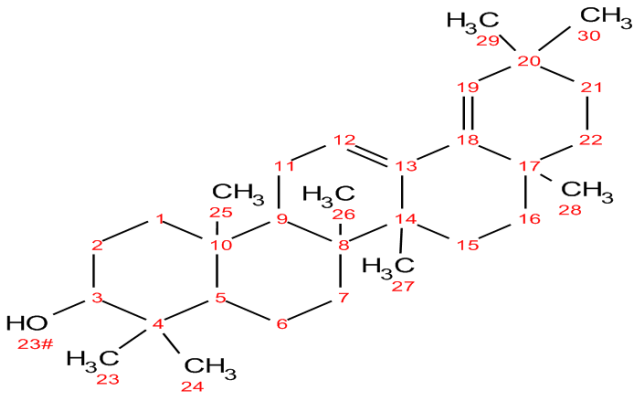


Fig. 5. Structure of 3β-hydroxyl olean-12,18-diene

The ^{1}H NMR (400 MHz, CD_3Cl) spectrum showed the presence of an oxymethine proton resonating at δ 3.40 (1H (t) H-3), two olefinic protons at δ 5.20 (1H (t) H-12), δ 5.35 (1H (s) H-19) and hydroxyl proton at δ 2.25 (1H (d)). The signal from δ 0.70-1.98 (multiplet) are due to the presence of overlapping methyl, methylene and methine protons (Fig. 1).

The ^{13}C NMR data (400 MHz, CD_3Cl) spectrum showed the presence of four olefinic carbons resonating at δ 124.43 (C-19), δ 139.59 (C-13), δ 121.73 (C-12) and δ 145.20 (C-18). The oxymethine carbon signals at δ 79.1 (C-3) while the signals at δ 47.72, δ 55.18, are methine group. The signals from δ 37.20, 27.66, 23.70, 28.10, 34.74, 31.26 and 38.60 are probably due to $-CH_2-$ groups, while the CH_3- groups are indicated by the following signals δ 21.41, 17.48, 18.38, 22.70, 21.41, 23.53, 23.70 and 16.87 (Fig. 2).

Based on this information and on the HSQC, COSY, HMBC, and NOESY experiments, **CB 47** was deduced to be 3β-hydroxyl olean-12,18-diene (Fig. 5 above). The complete ^{1}H and ^{13}C NMR spectroscopic assignments are consistent with the reported literature values (Table 4) [40].

Table 4. ^{13}C NMR data of the isolated compound

Carbon atoms	Experimental δc(ppm)	*Literature of Comp δc(ppm)	DEPT
C1	38.79	38.51	CH_2
C2	27.24	27.43	CH_2
C3	79.06	79.20	CH
C4	39.62	39.02	C
C5	55.18	55.75	CH
C6	18.38	18.31	CH_2
C7	34.74	34.75	CH_2
C8	40.02	40.81	C
C9	47.72	51.31	C
C10	38.60	37.31	C
C11	22.60	22.20	CH_2
C12	121.73	123.88	CH
C13	139.30	141.50	CH
C14	42.09	42.84	C
C15	29.71	30.85	CH_2
C16	39.62	39.01	CH_2
C17	34.74	35.65	C
C18	145.20	142.25	C
C19	124.42	129.76	CH
C20	32.95	32.92	C
C21	33.35	33.22	CH_2
C22	37.15	37.52	CH_2
C23	29.37	28.81	CH_3
C24	21.41	20.81	CH_3
C25	15.63	15.62	CH_3
C26	16.81	16.40	CH_3
C27	23.70	23.73	CH_3
C28	26.00	24.12	CH_3
C29	30.09	30.71	CH_3
C30	28.77	28.71	CH_3

**[39] and [40]*

5. CONCLUSION

The compound 3β-hydroxyl olean-12,18-diene was isolated from chloroform fraction of *C. schweinfurthii* leave. It was the most potent against MRSA and VRE (MIC 6.25 µg/mL and MBC 12.5 - 25 µg/mL), and helmintic (54% -65% egg inhibition and 64%- 81% larva inhibition). The data obtained from the study proved that the uses of the *C. schweinfurthii* in the treatment helmintic by ethnomedical practitioners have been justified.

We wish to recommend that the test compounds be further evaluated to confirm their in vivo activity and toxicity on the host and the mechanism(s) through which the compounds exert therapeutic effects should be clearly elucidated.

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COMPETING INTERESTS

Authors have declared that no competing interests exist

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