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Research Article

Isolation, characterization and antibacterial evaluation of Zymosterol from the Root of *Pachystela Brevipes* (Sapotaceae)

Ezuruike, I.T [*], Aba, O.Y [2], Habila J.D [3], Ndukwe G.I.[4]
[1,3,4]Department of Chemistry Ahmadu Bello University, Zaria, Nigeria
[2]Department of Chemistry Kaduna State University, Kaduna, Nigeria

***Corresponding author**
Ezuruike I.T.
Email: eucaichem@gmail.com

Abstract: The root of *Pachystelabrevipes,* which has some medicinal applications was investigated. The results of the phytochemical analysis showed the presence of carbohydrates, cardiac glycosides, saponins, steroids/ triterpenes, flavonoids, tannins and alkaloids. The crude extracts showed zone of inhibition in the range, 16-19 mm (pet.ether), 20-24 mm (chloroform), 20-27 mm (ethylacetate) and 20-22 mm (methanol), against the test organism*; Nettricillin resistant staphylococcus aureus, Staphylococcus aureus, Escherichia coli; Salmonella typhi, Shigelladysenterea, Pseudomonas aeruginosa, klebsiella pneumonia, Candida stellatoidea, Candida tropicalis, Candida krusei, Proteus mirabilis, Proteus vulgaris* and *Streptococcus feacalis.* The minimum inhibitory concentration (MIC) of 2.5 mg/mL was recorded for the chloroform, ethylacetate and methanol fraction against the entire test organism except *S. pyogenes, C. krusei, P. mirabilis and P. vulgaris.* The petroleum ether fraction showed MIC of 5.0 mg/mL. The minimum bactericidal/fungicidal concentration (MBC/MFC) determination showed that a concentration of 5-10 mg/mL of the pet.ether, chlororform, ethylacetate and methanol fraction could completely kill all the test organism except *S. pyogenes, C. krusei, P. mirabilis and P. vulgaris.*Zymosterol (3β-hydroxy-4β-methyl-5α-cholesta-6,22-diene-4α-carboxylic acid) was isolated from the ethyl acetate fraction (EAF) and confirmed purely by spectral techniques.
Keywords: *Pachystela brevipes*, Zymosterol, NMR spectral anaysis, phytochemical, antimicrobial.

INTRODUCTION

Pachystela brevipes (Sapotaceae), commonly known as star apple of the forest is a much – branched evergreen tree with a dense, wide – spreading crown of dropping branches. It can occasionally be as much as 25 meters tall, often in lowland forest and riverine forest, damp sites, swamp forest and beside streams or other sites with permanently high water -table, at elevations up to 1,500meters, distributed from Senegal to .W Cameroons, and widespread in Sudan, E Africa, S. central Africa and Mozambique. The species of the tree is not very well known in Nigeria. The plant is used in traditional medicine in treatment of Malaria, Pneumonia, Coughs, Cold, and Hookworm infection of the small intestine, Jaundice, Nausea, Hernia, Oedema, Stomach complaints and Swellings [1-4]. Because of this observation and the regular uses of the plant by the natives prompted this research possibly to find out if there could be more metabolites that can be of pharmacological relevance. This paper deals with the phytochemical and antimicrobial activities as well as the isolation, purification and characterization of Zymosterol from the extracts of the root part of *Pachystela brevipes*. Before the initiation of this study, it was found that there were no chemical studies of *Pachystela brevipes* in the literature. However, some other members of the family that are related to *Pachystela brevpes* have been explored to some extent.

MATERIALS AND METHODS

Collection and identification of the plant material of plant material

The root of *Pachystela brevipes* was collected from Okpokwu, in Okpokwu Local Government Area of Benue State, Nigeria in March 2013. This was identified at the Herbarium of the Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Zaria Nigeria. The voucher specimen with number 7106 was deposited at the Herbarium. The sample was air-dried, pulverized using wooden pestle and mortar. Finally, this was stored in an air-tight polyethene bag and kept away from moisture until when needed for extraction.

Preparation of the extract

The root was dried at room temperature under the shade for three weeks and size reduced manually using mortar and pestle. The size reduced root (2kg) was subjected to extraction with Methanol in a soxhlet apparatus and after evaporation of the solvent, 80g of

the extract was obtained. The extract was then suspended in distilled water and filtered. The water insoluble portion was washed with n-Hexane, chloroform and ethyl acetate to yield n-Hexane fraction (nHxF), chloroform fraction (CF) and ethyl acetate fraction (EAF), respectively.

Phytochemical analysis of the plant material

The root of the plant was screened for plant metabolites using the pulverized materials respectively. Standard techniques of Chemical tests were carried out on the extracts using standard procedures to identify the constituents as described by Sofowora[5] and Trease and Evans[6]. These metabolites include carbohydrates, cardiac glycosides, tannins, saponins, flavonoids, Alkaloids, steroids/triterpenes and anthraquinones.

Antimicrobial Screening

Pure clinical isolates of *Nettricillin resistant staphylococcus aureus, Staphylococcus aureus; Streptococcus pyrogenes; Escherichia coli; Salmonella typhi; Shigella dysenterea; Pseudomonas aeruginosa; klebsiella pneumoniae Candida krusei Candida stellatoidea, Candida tropicalis, Streptococus feacalis Proteus vulgaris, proteus mirabilis* were gotten from Ahmadu Bello University Zaria Teaching Hospital. All the micro-organisms were checked for purity and maintained in slants of agar.

Determination of Minimum Inhibitory Concentration

The minimum Inhibition Concentration of the extract was carried using broth dilution method. Mueller Hinton broth was prepared; 10mls was dispersed into test tube and was sterilized at 37^{o}C for 15minutes, the broth was allowed to cool. Mc – farland's standard turbidity scale number 0.5 was prepared to give turbid solution. Normal saline was prepared, 10mls was dispensed into sterile test tube and the test microbe was inoculated and incubation was made at 37^{o}C for 6 hours. Dilution of the test microbe in the normal saline was made until the turbidity marched that of the Mc-farland's scale by visual comparison at this point the test microbe has a concentration of about 1.5 x 10^{8}cfu/ml. Two fold serial dilution of the extract in the sterilized broth was made to obtain the concentration of 10, 5, 2.5, 1.25 and 0.625mg/ml. The initial concentration was obtained by dissolving 0.1g of the extract in 10mls of the sterile broth. Having obtained the different concentrations of the extract in the sterile broth, 0.1ml of the test microbe in the normal saline was then inoculated into the different concentrations, incubation was made at 37^{o}C for 24 hours, after which each test tube was observed for turbidity (growth) the lowest the lowest concentration of the extract in the broth which shows no turbidity was recorded as the minimum inhibition concentration.

Determination of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

Minimum bactericidal concentration /fungicidal concentration were carried out to determine whether the test microbes were killed or only their growth was inhibited. Mueller Hinton agar was prepared, sterilized at 121^{o}C for 15minutes, poured into sterile Petri dishes and was allowed to cool and solidify. The content of the MIC in the serial dilution were then sub cultured onto the prepared medium, incubation was made at 37^{o}C for 24 hours, after which the plates of the medium was observed for colony growth, the MBC/MFC were the plates with lowest concentration of the extract without colony growth.

Isolation and purification of compound

A small quantity of EAF was dissolved in ethylacetate and the solution was spotted on TLC plates. The plates were developed using several solvent systems; the solvent systems of Hexane / Ethyl acetate (8:2 and 7:3) gave better separation of the components, and were used in the TLC monitoring of the Column Chromatography. 12g of the ethyl acetate fraction (EF) was subjected to column chromatography on a silica gel (60 – 120 mesh) with gradient elution using Hexane and Ethyl acetate [7]. Eluents were collected in 25ml aliquots and TLC was used to monitor the fractions.

A total of 97 collections were made and pooled into 7 major fractions, based on their TLC profiles. Fraction 3 indicated significant proportion of the compound of interest and was further subjected to purification by preparative TLC using the solvent system Hexane / Ethylacetate (8:2 and 7:3). A single homogenous spot was obtained on TLC with two different solvent systems Hexane / Ethylacetate (8:2). This compound, coded (IKE1), appeared as white crystalline and was subjected to spectral analysis.

Spectroscopic characterisation

Different spectroscopic methods were used to elucidate the structure of IKE1, including1H NMR, 13C NMR and 2D NMR techniques. The NMR spectra were recorded on a Brucker AVANCE-300 Japan (100MHz and 400MHz) in deuterated chloroform with TMS as internal standard at the University of Kwazulu Natal, Westville Campus, Durban.

RESULTS AND DISCUSSIONS

Biological Results

Phytochemical analysis conducted on the plant extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities [7]. Analysis of the plant extracts revealed the presence of phytochemicals such as carbohydrates, tannins, saponins, cardiac glycosides, steroid and triterpenes, and alkaloids. The presence of these could account for high antimicrobial activity demonstrated by the plant. Tannins, saponins and alkaloids have been

reported to have pronounced physiological effect particularly on the nervous system [8].

Tannins encompass a heterogeneous group of compounds and polymers (polyphenols). In general their non-specific activity has been ascribe to their ability to complex metal ions, scavange radicals and reduce active oxygen species and form tight complexes with a wide array of proteins and polysaccharides [9]. Hence, they have antioxidative properties.

The plant extracts were also revealed to contain saponins which are known to produce inhibitory effect on inflammation [10]. Saponins have the property of precipitating and coagulating red blood cells. Some of the characteristics of saponins include formation of foams in aqueous solutions, hemolytic activity, cholesterol binding properties and bitterness [11, 12].

Steroid and Triterpenes have been reported to have antibacterial properties [13], and they are very important compounds especially due to their relationship with compounds such as sex hormones [14]. Tritepenes have important biological roles, for instance the triterpene lanosterol is the precursor from which steroid hormones are made in nature and the tetraterpene β-carotene is a major dietary source of vitamin-A and reported that triterpenes have some chemotaxonomic properties.

Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity [15]. Several workers have reported the analgesic, antispasmodic and antibacterial [11] properties of alkaloids.

Glycosides are known to lower the blood pressure according to many reports [16]. The presence of these phytochemicals in *Pachystela brevipes* extracts suggests that the plant is pharmacologically active ,supporting the claim by the traditional healers proving the plant to be an increasingly valuable reservoir of bioactive compounds of substantial medicinal merit.

The antimicrobial and sensitivity test of the four crude extracts of the root parts of *Pachystela brevipes* were carried out using fifteen pathogens namely:- *Staphylococcus aureus, Streptococcus feacalis, Streptococcus pyogenes, Nettricillin resist. staph. aureus, Corynebacterium ulcerans, Escherichia coli, Salmonella typhi, Shigella dysenteriea, Pseudomonas aeroginosa, Klebsiella pneumonia, Candida tropicalis, Candida stellatoidea, Candida krusei, Protus mirabilis, Proteus vulgaris* . The results obtained are shown in 2 – 5.

Streptococcus pyogenes Candida krusei, Protus mirabilis, and Proteus vulgaris were resistant to all the four extracts. However, *Staphylococcus aureus, Streptococcus feacalis, Nettricillin resist. staph. aureus, Corynebacterium ulcerans, Escherichia coli, Salmonella typhi, Shigella dysenteriea, Pseudomonas aeroginosa, Klebsiella pneumonia, Candida tropicalis, and Candida stellatoidea,* were sensitive to all the four extracts. This indicates that the root extracts of *Pachystela brevipes* has broad spectrum of activity. This result paved way for subsequent antimicrobial tests such as zones of inhibitions (ZI), MIC and MBC tests.

The four crude extracts had significant zones of inhibition against all the tested microorganisms except *Streptococcus pyogenes Candida krusei, Protus mirabilis, and Proteus vulgaris* which did not show any zone of inhibition in the four extracts. The ethylacetate extract had the highest zone of inhibition ranging from 20 – 27mm, followed by chloroform 20 – 24mm, then methanol 20 – 22mm. Petroleum ether had the lowest zone of inhibition range of 16 – 19mm.

Minimum inhibitory concentration is important in diagnostic laboratory to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. The results of the minimum inhibitory concentration (MIC) for Chloroform, Ethyl acetate and Methanol extracts revealed that these extracts inhibited the growth of *Staphylococcus aureus, Streptococcus feacalis, Nettricillin resist. staph. aureus, Corynebacterium ulcerans, Escherichia coli, Salmonella typhi, Shigella dysenteriea, Pseudomonas aeroginosa, Klebsiella pneumonia, Candida tropicalis,* and *Candida stellatoidea,* at 2.5mg/ml. Petroleum ether extract inhibited the growth of *Staphylococcus aureus, Streptococcus feacalis, Nettricillin resist. staph. aureus, Corynebacterium ulcerans, Escherichia coli, Salmonella typhi, Shigella dysenteriea, Pseudomonas aeroginosa, Klebsiella pneumonia, Candida tropicalis,* and *Candida stellatoidea,* at 5mg/ml. This shows that the extract is the least active in terms of Minimum Inhibition Concentration (MIC). However, *Candida krusei, Protus mirabilis, Proteus vulgaris* and *Streptococcus pyogenes* did not show any Minimum Inhibitory Concentration in any of the four extract that is Petroleum ether, Chloroform, Ethyl acetate and Methanol respectively.

The result of minimum bactericidal concentration (MBC) and Minimum fungicidal concentration (MFC) for petroleum spirit showed concentration of 10mg/ml for *Staphylococcus aureus, Streptococcus feacalis, Nettricillin resist. staph. aureus, Corynebacterium ulcerans, Escherichia coli, Salmonella typhi, Shigella dysenteriea, Pseudomonas aeroginosa, Klebsiella pneumonia, Candida tropicalis,* and *Candida stellatoidea.* This shows that this extract has antifungal effect at the highest concentration against the test fungi used in this research.

The result of the minimum bactericidal concentration (MBC) and minimum fungicidal

concentration (MFC) for Chloroform extract as shown in showed concentrations of 5mg/ml for *Staphylococcus aureus, Escherichia coli, Shigella dysenteriea, Klebsiella pneumonia,* and *Candida stellatoidea* and 10mg/ml for *Nettricillin resist. staph. aureus, Corynebacterium ulcerans, Salmonella typhi, Pseudomonas aeroginosa, Streptococcus feacalis* and *Candida tropicalis* respectively.

The result of the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) for Ethyl acetate extract showed concentrations of 5mg/ml for *Staphylococcus aureus, Escherichia coli, Shigella dysenteriea, Klebsiella pneumonia,* and *Candida stellatoidea, Nettricillin resist. staph. aureus, Corynebacterium ulcerans, Salmonella typhi, Pseudomonas aeroginosa,* and 10mg/ml for *Streptococcus feacalis* and *Candida tropicalis* respectively.

The result of the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) for Methanol extract showed concentrations of 5mg/ml for *Nettricillin resist. staph. aureus, Corynebacterium ulcerans, Shigella dysenteriea* and *Klebsiella pneumonia,* and 7mg/ml for *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Pseudomonas aeroginosa, Candida stellatoidea, Streptococcus feacalis* and *Candida tropicalis* respectively.

From the results of the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the four extracts, it is evident that the four extracts has antibacterial and antifungal activity, Methanol and Petroleum ether extracts being the highest, then Ethyl acetate and Chloroform. The result shows that the four extracts of the root of *Pachystela brevipes* can be used to treat infections caused by these test bacteria and fungi. For example, infections caused by *salmonella typhi* and *Escherichia coli,* such as typhoid fever (enteric fever), food poisoning, gasto-enteritis, urinary tract infections and other infections in which ciprofloxacin is used for treatment.

The broad spectrum exhibited by the root of the plant *Pachystela brevipes,* as shown in the antimicrobial results lend credence to the traditional uses of the plant in folk medicine. The plant contains anti-pathogenic substances which can be isolated and their toxicities studied to ensure their safe usage, and to ascertain that they are not toxic to humans.

Table 1: Phytochemical Screening

Phytochemicals	Pet Ether	Chloroform	Ethyl acetate	Methanol
Carbohydrate	+	+	+	+
Cardiac glycosides	+	++	+	+
Saponins	-	+	-	-
Steroids/Triterpenes	-	-	+	+
Flavonoids	-	-	-	-
Tannins	-	-	-	+
Alkaloids	-	+	-	-
Anthraquinones	-	-	-	-

Table 2: Zones of inhibition (mm)

Test organisms	Pet ether	Chloroform	Ethyl acetete	Methanol	Ciprofloxacin
N.R.S. Aureus	17	20	23	21	35
S. Aureus	19	21	21	20	34
S. Feacalis	18	21	20	20	38
S. pyogenes	0	0	0	0	37
C. Ulcerans	16	20	22	21	0
E. Coli	19	22	24	20	35
S. Typhi	18	20	22	20	40
S. Dysenteriea	19	23	25	22	39
P.Aeroginosa	17	20	22	21	37
K.Pneumonia	19	24	27	22	42
C. Tropicalis	16	20	20	20	0
C. Stellatoidea	18	22	24	20	0
C. Krusei	0	0	0	0	0
P. Mirabilis	0	0	0	0	32
P. Vulgaris	0	0	0	0	0

Table 3: MIC

Test organisms	Pet ether	Chloroform	Ethyl acetete	Methanol
N.R.S. Aureus	5.00	2.50	2.50	2.50
S. Aureus	5.00	2.50	2.50	2.50
S. Feacalis	5.00	2.50	2.50	2.50
S. pyogenes	0	0	0	0
C. Ulcerans	5.00	2.50	2.50	2.50
E. Coli	5.00	2.50	2.50	2.50
S. Typhi	5.00	2.50	2.50	2.50
S.Dysenteriea	5.00	2.50	2.50	2.50
P.Aeroginosa	5.00	2.50	2.50	2.50
K.Pneumonia	5.00	2.50	2.50	2.50
C. Tropicalis	5.00	2.50	2.50	2.50
C.Stellatoidea	5.00	2.50	2.50	2.50
C. Krusei	0	0	0	0
P. Mirabilis	0	0	0	0
P. Vulgaris	0	0	0	0

Table 4: MBC

Test organisms	Pet ether	Chloroform	Ethyl acetete	Methanol
N.R.S. Aureus	10.00	10.00	5.00	5.00
S. Aureus	10.00	5.00	5.00	10.00
S. Feacalis	10.00	10.00	10.00	10.00
S. pyogenes	0	0	0	0
C. Ulcerans	10.00	10.00	5.00	5.00
E. Coli	10.00	5.00	5.00	10.00
S. Typhi	10.00	10.00	5.00	10.00
S.Dysenteriea	10.00	5.00	5.00	5.00
P.Aeroginosa	10.00	10.00	5.00	10.00
K.Pneumonia	10.00	5.00	5.00	5.00
C. Tropicalis	10.00	10.00	10.00	10.00
C.Stellatoidea	10.00	5.00	5.00	10.00
C. Krusei	0	0	0	0
P. Mirabilis	0	0	0	0
P. Vulgaris	0	0	0	0

Spectral Result

The compound (IKE1, 3mg) appeared as a white crystalline substance; ^{1}H NMR (CDCl3, 400MHz): δ 3.78 (1H, m), 5.01, 5.19, 5.21 (3H, m), 0.35- 2.30 (1H, m, s); ^{13}C NMR ($CDCl_3$, 100MHz): δ 177.4(C-29), 139.6 (C-5), 138.2 (C-22), 129.5 (C-23), 117.5 (C-6), 71.1 (C-3), 55.9 (C-14), 55.2 (C-17), 51.3 (C-9), 43.3 (C-3&13), 40.3 (C-20), 39.5 (C-12&24), 37.2 (C-1), 34.3 (C-10), 31.9 (C-8&25), 31.8 (C-7), 31.5 (C-2), 28.5 (C-16), 24.7 (C-15), 23.0 (C-27), 22.7 (C-26), 21.4 (C-21), 21.1 (C-11), 19.0 (C-19), 14.1 (C-28), 12.1 (C-18).

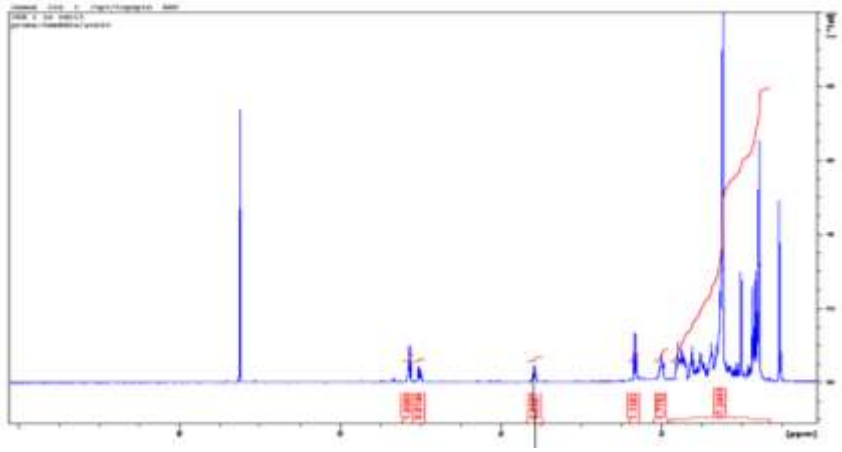

Fig-1: Proton NMR of IKE1

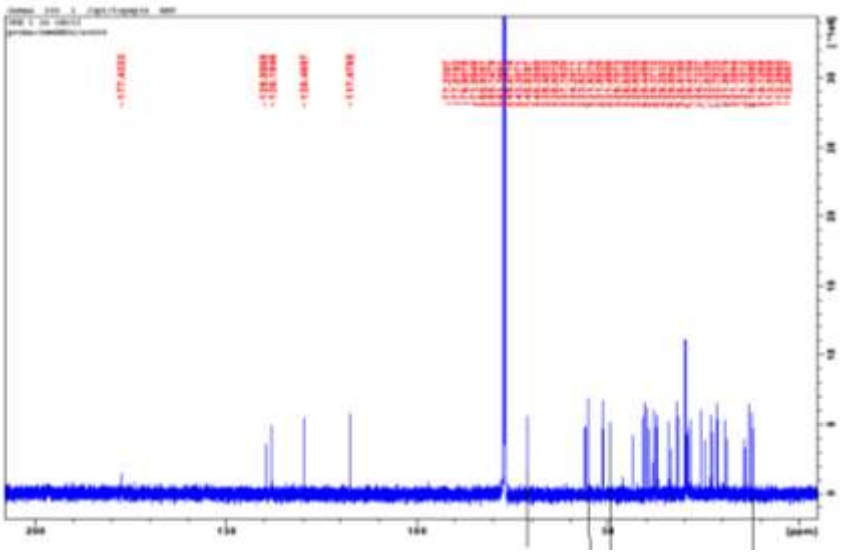

Fig-2: Carbon-13 NMR of IKE1

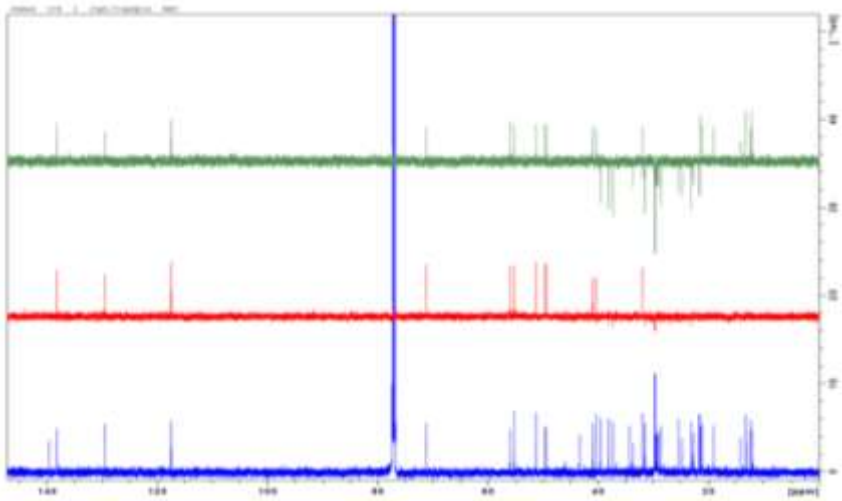

Fig-3: COSY of IKE1

From the spectral analysis of IKE1, the ^{13}C NMR shows the presence of twenty nine carbon atoms. The chemical shift at δ 71.09 indicated the presence of an oxymethine carbon. The signal at δ 139.59, 117.48 138.18 and 129.47 respectively indicates the presence of olefinic carbons. The signals from δ 21.10, 31.48, 37.16, 31.89, 39.48, 24.73, 28.52, 39.48 and 31.93 are probably due to CH_2 groups, while the CH_3 methyl groups are indicated by the following signals δ 12.06, 19.00, 21.39, 22.70, 23.03 and 14.12. The chemical shift signal at δ 177.43 indicated the presence of a carboxylic acid respectively.

From the ^{1}H NMR spectra, it showed the presence of one oxymethine proton at δ 3.78 (multiplet). It also showed the presence of olefinic protons at δ 5.01, 5.19 and 5.21 (multiplet). The signal from δ 0.35-2.30 (multiplet) are due to the presence of overlapping methyl, methylene and methine protons respectively.

Norlanosta dien oic acid; this was a white crystalline substance. The NMR analyses (1D and 2D) confirmed that the compound was 3β-hydroxy-30-norlanosta-6, 22-dien-29oic acid or 3β-hydroxy-4β-methyl-5α-cholesta-6, 22-diene-4α-carboxylic acid. The proton decoupled ^{13}C-NMR spectrum showed 29 carbon atoms. The Distortionless Enhancement Polarization Transfer (DEPT) subspectrum indicated 10 methine (CH) carbon, eight methylene (CH_2) and six methyl (CH_3) groups. Quartenary 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 five (5) quaternary carbons were identified. The findings in this work have justified the use of this plant in ethnomedicinal treatment of diseases caused by some of these organism used in this study.

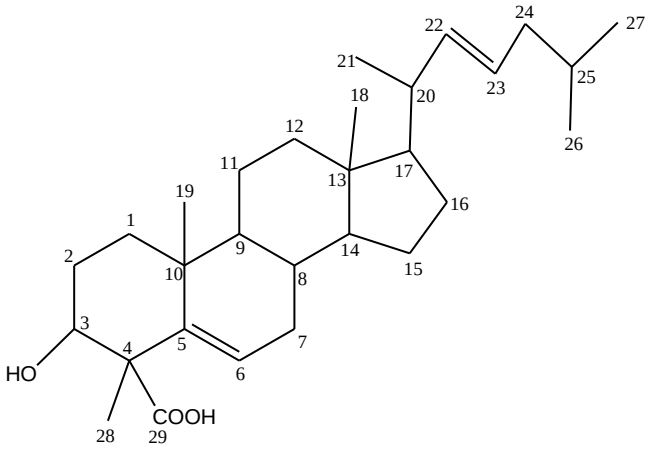

Figure 4: IKE 1 Cholesta-diene-acid or Zymosterol: ($C_{29}H_{46}O_3$, 442.7 g/mol) Name: 3β-hydroxy-4β-methyl-5α-cholesta-6, 22-diene-4α-carboxylic acid Or 3β-hydroxy-30-norlanosta-6, 22-dien-29 oic acid

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