A Bioactive Triterpene from *Laggera pterodonta* (Asteraceae) (DC.) Sch. Bip.

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ABSTRACT

Laggera pterodonta (DC.) Sch. Bip. aerial part was extracted successively with hexane, ethyl acetate and methanol. The ethyl acetate extract was subjected to chromatography to give a pentacyclic triterpenoid ester which was identified as taraxasteryl acetate based on spectroscopic evidence. The triterpenoid exhibited antimicrobial activity against Staphylococcus aureus, Klebsiella pneumoniae, Klebsiella ozaenae, Bacillus cereus, Shigella dysenteriae, Escherica.coli and Streptococcus faecalis with MICs ranging from 25-50µg/ml. This is the first time taraxasteryl acetate has been isolated from the genus, Laggera.

INTRODUCTION

Laggera pterodonta(DC.) Sch. Bip. (Asteraceae/Compositae), belongs to the genus *Laggera* which consists of over 20 species. The plant is spread throughout the sub-Saharan Africa and the tropical countries of Asia, especially South-East Asia[1]. In Nigeria, it is used traditionally for the treatment of athletes-foot, skin infections, pediatric malaria and wounds. It has also been reported for ethnomedicinal use in China as anti-inflammatory agent for treatment of hepatitis, arthritis, bronchitis and nephritis.[2,3,4,]

Several works have been carried out by the Chinese researchers on the Asian species with the reported isolation of a number of flavonoids and sesquterpenes[3,4,5,6,] However, the folkloric use as an anti-infective has not been fully investigatd, especially on Nigerian species of the plant. Reports of chemical constituents of the oil from the species in Benin and Cameroon showed variation in composition[7,8,] The activity of crude methanol extract against BCG strains of *Mycobacterium tuberculosis*, and the broad spectrum activity of the hexane, ethyl acetate and methanol extracts of the Nigerian species of the plant have earlier been reported[9,10] Previous phytochemical screening of the methanolic extract showed the plant to contain terpenes, sterols and glycosides[9] . This study aimed at isolating the bioactive compounds of the aerial part of the plant which is used locally as a phytomedicine.

EXPERIMENTAL

Materials

The aerial part of the plant was collected in November, 2009 from Chaza village, Suleja, Niger State, Nigeria, and authenticated at the National Institute for Pharmaceutical Research and Development (NIPRD) Abuja, Nigeria. The plant was assigned a voucher specimen number NIPRD/H/6403. It was air-dried for two weeks, and then crushed with a mechanical grinder. The powdered plant part was kept in an air-tight cellophane bag until used.

All the solvents and reagents were of Analar grade, and unless otherwise stated, were sourced from Zayo-Sigma, Abuja. Melting points (mps) were measured on Barnstead Electrothermal BI 9100 and were uncorrected. The ^{1}H NMR and ^{13}C NMR (500 MHz) spectra were run on a Bruker DRX 500

spectrometers using $CDCl_3$ as solvent and TMS as internal standard.The IR spectrum was on a Shimadzu Fourier Transformed Infra-Red spectrometer (FTIR) 8400S while the UV was obtained on Shimadzu UV-160A.. Column chromatography was performed on ChemGlass and Kontes glass columns. The organisms screened *Staphylococcus aureus, Escherichia coli, Bacillus cereus, streptococcus feacalis, shigellia dysentae* and *Klebsiella pneumonia* were obtained from the department of Medical Microbiology, Ahmadu Bello University, Zaria Nigeria. The positive controls were sparfloxacin (0.2 mg/ml), erythromycin (0.5 mg/ml) and flouconazole (0.5 mg/ml), all of Sigma Chemicals, United Kingdom. Mueller Hinton agar was used for bacteria while saboraud dextrose agar (SDA) was used for fungi and were both obtained from Oxoids Limited Basingstoke, Hampshire, England.

Methods

Extraction of plant material:

The powdered material was macerated successively for 24hrs in hexane, ethyl acetate and methanol. The extracts were concentrated to dryness using a Rotavapor.

Chromatographic separation of ethyl acetate extract:

50g of ethyl acetate extract was adsorbed on 25 g of silica gel, 230-400 mesh. The adsorbed extract was chromatographed on a column of length 30 cm and diameter 4 cm, loaded with silica gel of the same mesh size. The eluents were mixtures of n-hexane and ethyl acetate, followed by methanol in increasing polarity. Eluates were collected in volumes of 150-200 ml and a total of 36 fractions were collected. The fractions were labelled LPE_1 to $LPE_{36.}$ The fraction LPE_7 was re-fractionated using mixtures of hexane and ethyl acetate on a column of silica gel. A compound, HOE 25A crystallised out of sub-fractions $LPE_{7.\ 2\text{-}5}$ and was further purified by re-crystallisation in methanol to give white crystals(81mg), mp 233 – 233.5 °C; TLC: R_f 0.78 (hexane-ethyl acetate (10:1));

*Spectral analyses of HOE 25A (compound **1**)*

λ_{max} ($CHCl_3$) nm: 244; $\bar{V}_{max}$ (Neat-$CHCl_3$) cm^{-1}: 1586 (C=C), 1710 (C=O), 2924 (C-H); ($CDCl_3$) δ: 1.29 (1H, m, H-1a), 1.19 (1H, m, H-1b), 1.66-1.75 (2H, m, H-2), 4.48-4.53 (1H, dd, J = 7.4.0,7.8 Hz, H-3), 0.84 (1H, m, H-5), 1.52 (1H, m, H-6a), 1.41 (1H, m, H-6b), 1.44 (2H, m, H-7), 1.39 (1H, m, H-9), 1.58 (1H, m, H-11a), 1.32 (1H, m, H-11b), 1.73 (1H, m, H-12a), 1.15 (1H, m, H-12b), 1.60 (1H, m, H-13), 1.73 (1H,m, H-15a), 0.88 (1H, m, H-15b), 1.18 (1H, m, H-16a), 1.06 (1H, m, H-16b), 0.99 (1H, m, H-18), 2.12 (1H, m, H-19), 2.43 (1H, m, H-21a), 2.21 (1H, m, H-21b), 1.78 (1H, m, H-22a), 1.48 (1H, m, H-22b), 0.87 (3H, s, H-23), 0.88 (3H, s, H-24), 0.88 (3H, s, H-25), 0.96 (3H, s, H-26), 0.95 (3H, s, H-27), 1.04 (3H, s, H -28), 1.05 (3H, d, J = 3.85 Hz, H-29), 4.61-4.65 (2H, m, H -30), 2.06 (3H, s, H-2'); ^{13}C-NMR ($CDCl_3$) δ:38.5 (C-1), 23.7 (C-2), 81.0 (C-3), 37.8 (C-4), 55.5 (C-5), 18.2 (C-6), 34.0 (C-7), 40.9 (C-8), 50.4 (C-9), 37.1 (C-10), 21.5 (C-11), 26.2 (C-12) , 39.2 (C-13), 42.1 (C-14), 26.7 (C-15), 38.3 (C-16), 34.5 (C-17), 48.8 (C-18), 39.4 (C-19), 154.7 (C-20), 25.6 (C-21), 38.9 (C-22), 28.0 (C-23), 16.3 (C-24), 16.5 (C-25), 15.9 (C-26), 14.7 (C-27), 19.5 (C-28), 25.5 (C-29), 107.1 (C-30), 21.3 (C-2'), 171.0 (C-1').

Bioassay of extractives:

The method employed was basically that of Gatsing *et.al*[11].

Preparation of test stock concentration: 0.1 g of extract and 2 mg of the pure compound were each weighed and dissolved in 10 ml of distilled water or dimethylsulphoxide to obtain a concentration of 10 mg/ml or 0.2 mg/ml, respectively.

Preparation of the inoculums: For screening of crude extracts, a loopful of the test organism was taken from their respective agar slants and subcultured into test-tubes containing nutrient broth for bacteria while sabouraud dextrose liquid for fungi. The test-tubes were incubated for 24hrs at 37°C for bacteria (48hrs at 30°C for the fungi). The concentrations of these organisms in the broth were standardized using normal saline until the turbidity matched that of the Mc-Farland's scale by visual comparison, to obtain a population density of 1.5 x 10^8cfu/ml for the bacteria. For the fungi, fungal spores were harvested after 7 days old SDA slant culture was washed with 10 ml normal saline in tween 80 with the aid of glass beads to help in

dispersing the spores. The spores' suspensions were standardized to 10^5cfu/ml. The nutrient broth was substituted with Mueller Hinton broth for screening of isolated pure compounds.

Preparation of media:

A 20 ml of the sterilized medium were poured into sterile petri-dishes and allowed to cool and solidify. The plates were labeled with the test microorganism (each plate with a test microbe). The microbes were spread evenly over the surface of the medium with the aid of a glass spreader. The plates were incubated at 37^{o}C for 30 minutes in a laminar flow station.

Zones of inhibtion using well diffusion method:

A standard cork borer of 5mm in diameter was used to cut well at the center of each inoculated plate and the agar removed from the well. 0.1 ml of the test solution was then introduced into the well created at the center of each plate. The bacteria plates were incubated at 37^{o}C for 24hrs while the fungal plates were incubated at 30^{o}C for 1-7days, and observed for the zone of inhibition of growth. The zones were measured with a transparent ruler and the results recorded in millimeters. Sterilized distilled water and DMSO were used as negative control.

Minimum inhibitory concentration (MIC) using broth dilution method:

MIC of the extracts was carried out using broth dilution method as described by Ibekwe *et. al*[12]. The nutrient broth and sabouraud dextrose liquid are prepared according to the manufacturer's instruction. 10 ml of each broth were dispensed into separate test-tubes and sterilized at 121^{o}C for 15 minutes and then allowed to cool. Two-fold serial dilutions of samples in broth were made from the stock concentration in a test-tube. 0.1 ml of the standardized inoculums of the microbes was inoculated into the different concentrations of the samples in the broth and incubated at 37^{o}C for 24 hours and 30^{o}C for 7days, for bacteria and fungi, respectively, and observed for turbidity or growth. The lowest concentration which showed no turbidity in the test-tube was recorded as the minimum inhibitory concentration (MIC).

Minimum bactericidal/fungicidal concentration using broth dilution method

Mueller Hinton and sabouraud media were prepared according to manufacturer's instruction. The media were sterilized at 121^{o}C for 15 mins and poured into sterile petri-dishes to cool and solidify. The contents of the MIC in the serial dilution were then subcultured onto the media and incubated at 37^{o}C for 24 hours and 30^{o}C for 7 days, for bacteria and fungi, respectively, and observed for colony growth. The MBC/MFC was the plate with the lowest concentration of test sample and without colony growth.

RESULTS AND DISCUSSION

The ^{1}H and ^{13}C NMR spectra of compound HOE 25A (Figures 2 - 6) shows that it has an ursane-type pentacyclic structure[13]with a vinyl and an exo-methylene carbons signals at 154.7 and 107.1, and the exo-methylene protons at 4.61-4.65, respectively. The methyl singlet at 2.06 in the proton NMR spectrum and the carbon signal at 171.0 in the ^{13}C NMR spectrum suggested the presence of an acetate group at carbon-3. The full assignment of the protons and carbons signals was accomplished by the COSY, HSQC and HMBC NMR spectral analyses. The compound tested positive to Liebermann-Burchard test which suggested it to be a terpene. The melting point of 233-233.5°C recorded for compound HOE 25A is within the range 232-234°C reported for taraxasteryl acetate by Khalilova and co-workers[14]. In addition the spectral characteristics are in agreement with those reported for 3β,18α,19μ-urs-20(30)-en-3-ol,3-acetate (taraxasteryl acetate), (**1**)[15,16] .

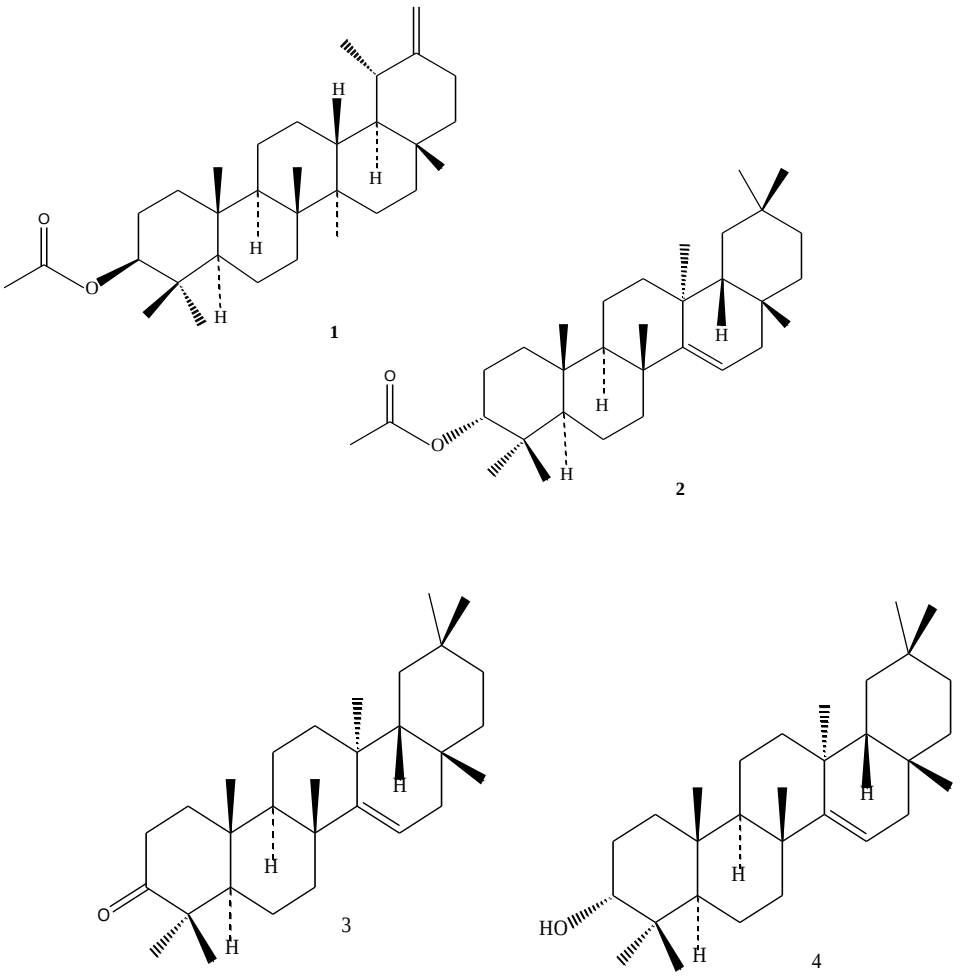

Figure 1: Structures of taraxasterol acetate (**1**), taraxeryl acetate (**2**), taraxerone (**3**) and taraxerol (**4**)

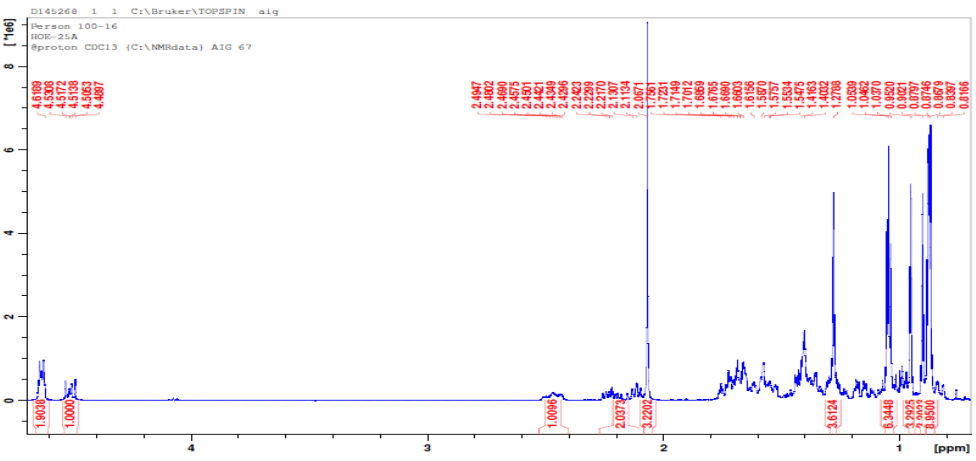

Figure 2: ^{1}H NMR spectrum of compound HOE 25A

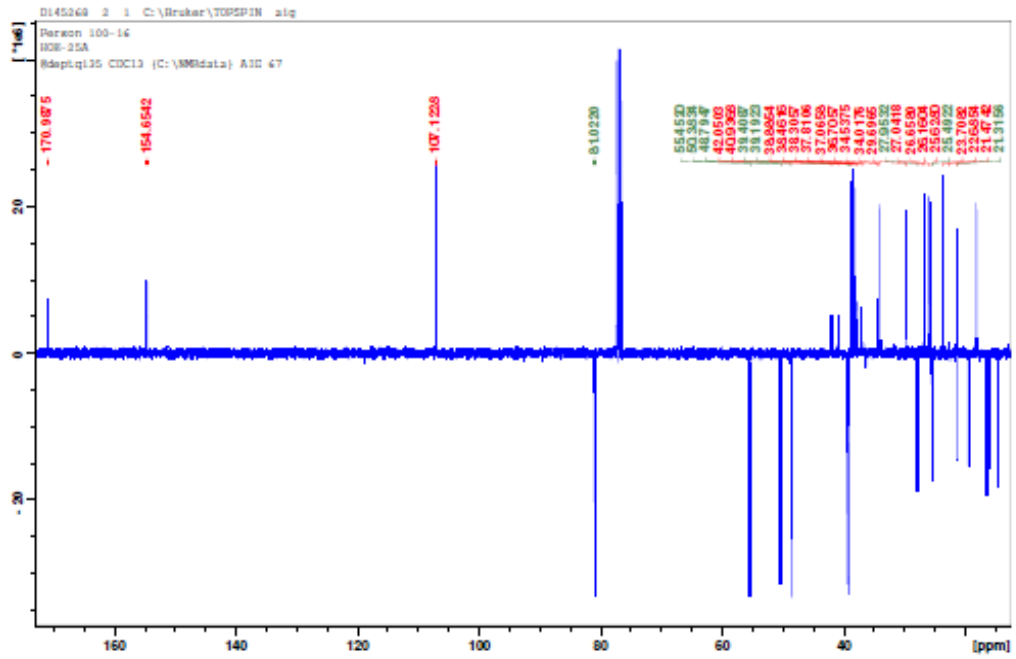

Figure 3: ^{13}C NMR (DEPT) spectrum of compound HOE 25A

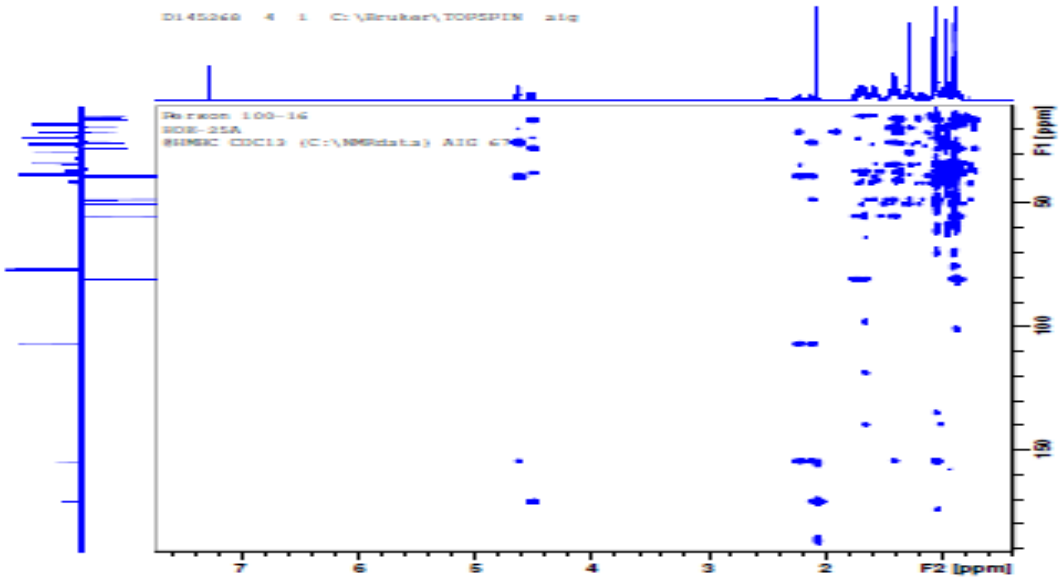

Figure 4: HMBC NMR spectrum of compound HOE 25A

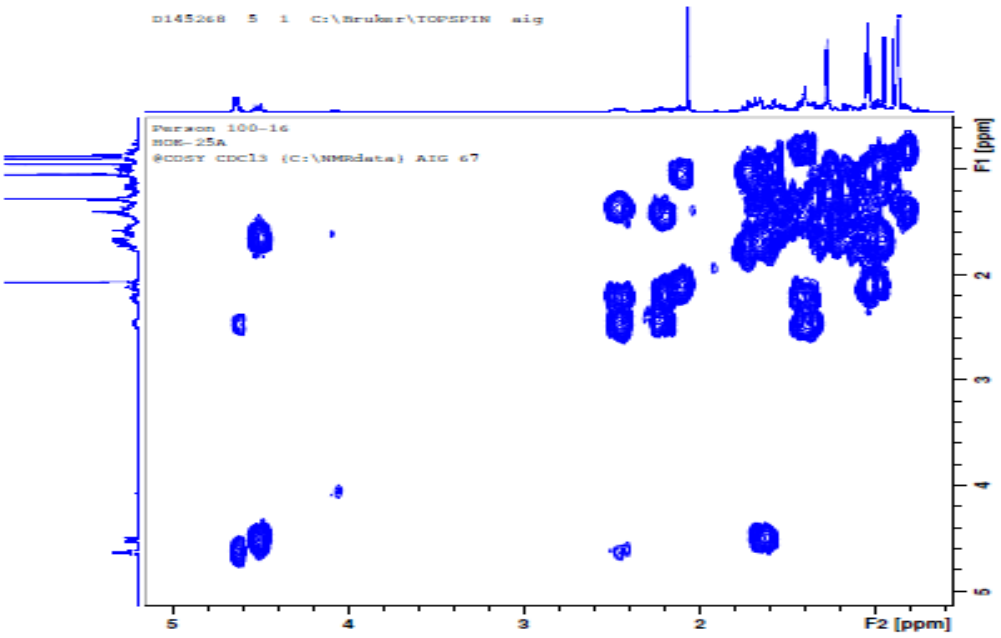

Figure 5: ^{1}H COSY NMR spectrum of compound HOE 25A

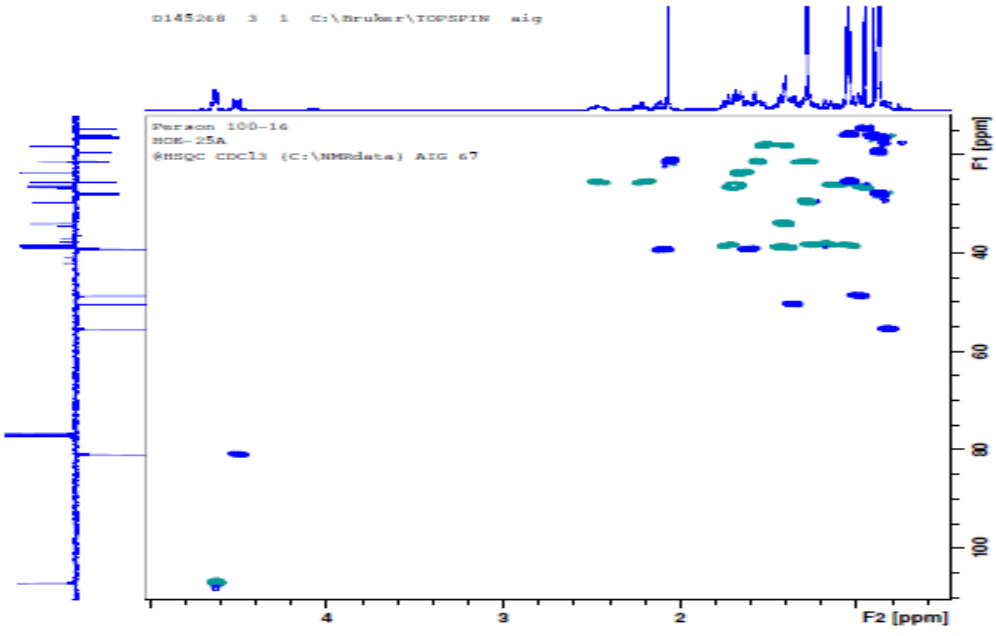

Figure 6: HSQC NMR spectrum of compound HOE 25A

Taraxasterol and derivatives have previously been isolated from a number of plant genera[15,16,17,18] but it has not been reported from the genus *Laggera*.

The antimicrobial screening results (Table1) of HOE 25A showed strong activities against *Staphylococcus aureus, Esherichia coli, Bacillus cereus, Streptococcus feacalis, Shigellia dysentae* and *Klebsiella pneumonia* which are known to be highly implicated in upper respiratory tract infections and other pulmonary diseases such as pneumonia and gastroenteritis. The biological activities of taraxasteryl esters including its acetate have been variedly reported by several workers[19,16,18,15]. The compound taraxasterol has been reported as a cancer chemopreventive agent and exhibit inhibition against EBV-EA induction. The compound may be responsible for the anti-tuberculosis, anti-viral and anti-bacteria properties of the plant. However the anti-tubercular and anti viral activity of the compound is yet to be verified. Two similar compounds reported from the plant species in Thailand, taraxeryl acetate (**2**) and taraxerone (**3**), were reported to be active against herpes simplex virus, HSV I and II. However the alcohol, taraxerol (**4**) was inactive[20].

Table 1: Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of HOE 25 A

S/N	Organisms	MIC µg/ml	MBC µg/ml
1	*S. aureus NCTC 6571*	25	50
2	*K. pneumonia ATCC 10031*	25	100
3	*S. aureus ATCC 13704*	50	100
4	*S. aureus* (isolates)	50	200
5	*S. feacalis* (isolates)	50	200
6	*B. Cereus* (isolates)	50	200
7	*E. Coli* (isolates)	50	200
8	*S. dysenterae* (isolates)	50	200
9	*K. pneumonia* (isolates)	50	200
10	*K. ozaenae* (isolates)	50	200

CONCLUSION

The isolation and characterization of tarasteryl acetate and its antimicrobial activities shows that the plant possesses a very potent bioactive triterpene which may be responsible for the activity of the crude extract. This justifies the local application of the plant as an anti-infective for gastroenteritis and respiratory diseases. This is the first report of taraxasteryl acetate from the genus *Laggera*.

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