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TRITERPENOIDS FROM *GUTENBERGIA NIGRITANA(BENTH).OLIV AND HIERN*

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Abstract

Gutenbergia nigritana (Benth).Oliv and Hein (Asteraceae) is a Nigerian ethnomedicinal plant which has long being used medicinally in traditional systems of medicine.The plant has diverse ethnomedicinal uses which include: The Plant is used in the Ijaw area of Niger Delta region of Nigeria to treat Malaria,convulsion,diarrhea and as a remedy for skin infection. The antibacterial study on dichloromethane extract and chromatographic fractions:dichloromethane soluble part(DC1),ethyl acetate soluble part(E1) and N-butanol soluble portions of methanolic extract were investigated using agar diffusion assay method by measuring the zone of inhibition against clinical isolates: *Bacillus subtillis,Escherichia coli,Staphylococcus aureus* and *Pseudomonas aeruginosa*.Extensive Chromatographic separation and preparative TLC were employed in the isolation steps. Column chromatograpy of the dichloromethane extract, gel filteration on sephadex and preparative TLC led to the isolation of two triterpenoids: Lupenol(1) and Lupenol acetate(2). The structures were elucidated by NMR spectroscopy and compared with literature. The dichloromethane extract exhibited antibacterial activity against all the test microorganisms with zones of inhibition ranging between 12-26mm. The chromatographic fraction (Fr-6) exhibited activity against *E.coli*,*Ps.aeruginosa* and *S.aureus* but in-active against *B.subtilis*, while the methanol soluble portions: dichloromethane and ethyl acetate showed weak activity while the n-butanol portion was in active. Dichloromethane extract of *Gutenbergia nigritana* leaves exhibited anti-bacterial activity, though the compounds isolated did not show activity, it is likely that the activity might reside in other components present in the dichloromethane extract.

Key words: Lupenol, Lupenol acetate,antibacterial activity.

Introduction

Widespread antimicrobial use continues to cause significance increases in resistant bacteria, particularly resistant Gram-positive organisms. The emergence of these resistant bacteria has caused a major concern and thus the urgent need for new antibacterial agents (Davis,1994).Plants are known to have defense systems against phytopathogenic bacteria(Smith et al,2005).Medicinal plants that are commonly used may be a good source for safe antibacterial agents(Choi et al.,2009).

Gutenbergia nigritana (Benth).Oliv and Hein, locally referred to as bush bitter leave in Amassoma kingdom in the southern Ijaw area of Bayelsa-Nigeria is an erect branching herb of 1.3m high with wrinkled leaves and mauve to reddish purple reddish florets in head. The plant is widely distributed in Guinea,Sierra Leone,Ghana and in Southern Nigeria particularly in the Ijaw speaking area. In the Ijaw area of Bayelsa state the leaves of the plant is used to treat Malaria and Convulsion (Personal communication). In the Igala area of Kogi state, the decoction of leaves of the plant is used as a medicine to treat fever and bacterial infection in children (Burkhill, 1985). The genus Gutenbergia consists of about 25 species which include *Gutenbergia cordifolia, Gutenbergia macrolephala* and *Gutenbergia aderocarpa*. Sesquiterpene lactones and Germacranolides have been isolated from *Gutenbergia cordifolia* (Yoshinori et al.,1987; Zidero and Bohlmann, 1989), there is however no documented evidence on the Phytoconstituents of *Gutenbergia nigritana*. As part of our continuing studies on medicinal plants used ethno medicinally in the Ijaw area of Bayelsa state-Nigeria,we report here the isolation of triterpenoids from the leaves of Gutenbergia nigritana and the antibacterial studies of the extract, fractions and isolated compounds.

Materials and Method

Instrument

The NMR experiments were recorded in $CDCl_3$ and TMS as internal standard on a Bruker Avance 600MHz and 150MHz spectrometer. UV was carried out on a Pye-Unicam genesis spectrophotometer, while IR was performed on Genesis series FT infra-red spectrophotometer.Thin layer chromatography(TLC) was performed on pre coated silica gel TLC plates(0.25mm) and preparative TLC silica gel glass plates(0.5mm) Merck , column chromatography was carried out on silica gel G 230-400 mesh size, Fluka.

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Plant material

Guternbergia nigritana leaves were collected in the month of November, 2010 in Amassoma and authenticated by Mr B. D. Musa of Herbarium section Department of Biological Sciences, Ahmadu Bello University, Zaria-Nigeria. A voucher specimen (973) was deposited.

Preparation of extracts

The air dried powdered leaves (210 g) was extracted to exhaustion with dichloromethane (2.5L) at room temperature for seven days, the combined dichloromethane extract was concentrated at room temperature to give a dark green sticky mass (12.63g, 6.01%w/w). The dried marc was then extracted with 70%methanol (2.5L) using the same process for seven days and the combined extract concentrated at room temperature to give a dark brown mass (21.2g, 10.09%). A portion of the methanol extract (15 g) was suspended in 100 ml of water and partitioned successively with 1L each of dichloromethane , Ethyl acetate and n-butanol (five times each with 200 ml) to give dichloromethane soluble part DC1(0.54 g), Ethyl acetate soluble portion EL(1.22 g) and n-butanol soluble part(NB) 2.04 g. The dichloromethane extract (DC), DC1,EL and NB were screened for antibacterial activity at stock concentration of 50mg/ml using DMSO(dimethyl sulphoxide) as the solvent,while the isolated compounds were screened at 1mg/ml using microbiological procedure.

Isolation

A portion of the dichloromethane extract (10.5g) was packed in a column (50cmx2.5cm) with silica gel (50g), and eluted gradiently with n-hexane and n-hexane: ethyl acetate mixtures(95:5;90:10;80:20;70:30;60:40;50:50;30:70;10:90), ethylacetate(100%) and finally 10% methanol in ethyl acetate using flash chromatography. 100mls aliquot were collected.The separation were monitored on TLC using the solvent systems I: N-hexane: ethyl acetate(9:1) and II: N-hexane :ethyl acetate(3:1) . Fractions(1-5) and fraction (6) which showed prominent spots on the TLC were screened for antibacterial activity at concentration of 20mg/ml, fraction 6 (0.25g) which showed better activity against the test pathogens,was subjected to gel filteration over sephadex LH-20 eluting with dichloromethane(100%), 20 fractions (10ml) each were collected and separation were monitored using solvent system 1.Fractions 8-15 pooled together based on their TLC profile was further purified using preparative TLC with the solvent system N-hexane:ethylacetate (15:1) to afford a white crystalline solid (12mg) compound I and a white amorphous solid (9mg) compound II.Liebermann-buchard test on the two compounds gave positive test for triterpenes.

Antibacterial test

Clinical isolates of *Bacillus subtillis*,*Staphylococcus aureus*,*Escherichia coli* and *Pseudomonas aeruginosa* obtained from the Department of Pharmaceutical Microbiology and Biotechnology,Niger Delta University,Wilberforce island,Yenagoa-Nigeria were used in the experiment.

Agar well diffusion assay described by (Mendoza et al, 1997) was adopted in the antibacterial studies with dichloromethane extract, dichloromethane soluble portion (DCI),ethylacetate soluble portion(EI) and n-butanol soluble portion of methanol extract at concentrations of 50mg/ml using DMSO a polar aprotic colourless solvent with no antibacterial activity as the solvent for antibacterial activity, while the chromatographic fractions and the isolated triterpenoids were tested at concentration of 20mg/ml and 1000µg/ml respectively using the same dissolving solvent. Gentamycin 10µg and Ciprofloxacin 5µg discs were used as standard antibacterial agents against the four test bacteria (*Bacillus subtilis*,*Staphylococcus aureus*,*Eschericia coli* and *Pseudomonas aeruginosa*).The bacteria isolates were standardized using colony suspension method by matching the isolates suspension with 0.5McFarland standard (Duraipandiyan and Ignacimuthu,2009). 50ml each of sterilized Mueller Hinton agar was transferred to sterilized plates and were differently inoculated with the standardized suspension of each of the test organisms after solidification: using a cork borer of 8mm diameter,wells were made in the inoculated plates and the bottoms sealed with few drops of molten agar. Then 0.1,0.2,0.3,0.4ml each of the extract,fractions and isolated compounds were poured aseptically into the four well labelled wells on each of the inoculated agar plate while the standard antibiotic discs were placed on the surface of the same plate. The plates were then incubated at 37^{o}C for 24hours and after which zones of inhibition were measured to the nearest millimetres.

Results

Compound I, a white crystalline solid (12mg).
UV ($CHCl_3$) λmax(nm): 235
IR(Nujol) cm-^{1}: 3307.1(OH),1637.5(C=C)
^{1}H-NMR ($CDCl_3$) δ: Singlet methyl protons: 0.77(H-24), 0.79(H-25), 0.83(H-28), 0.94(H-23), 0.95(H-26), 1.1 (H-27), 1.71(H-30). 3.17 m (H-3), 4.61(s), H-29b), 4.70(s), H-29a).
^{13}C-NMR($CDCl_3$),δ: 38.7(C-1),27.4(C-2),79.1(C-3),38.9(C-4),53.3(C-5),18.3(C-6),29.3(C-7),40.8(C-8),50.4(C-9),37.2(C-10),20.9(C-11),25.2(C-12),38.2(C-13),42.3(C-14),27.5(C-15),35.6(C-16),43.1(C-17),48.4(C-18),48.1(C-19),150.9(C-20),29.8(C-21),40.0(C-22),28.1(C-23),15.5(C-24),16.1(C-25),15.9(C-26),14.6(C-27),18.0(C-28),109.4(C-29),19.3(C-30).

Compound II a white amorphous solid (9mg).

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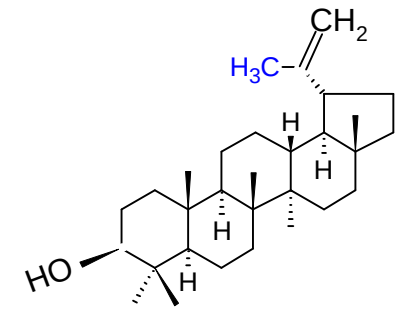
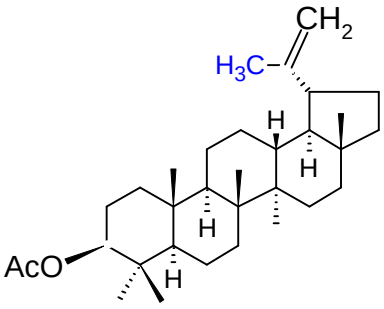
UV ($CHCl_3$) λmax(nm): 238, 288
IR (Nujol) cm-^{1}: 1739(C=O), 1641.5(C=C).
^{1}H-NMR($CDCl_3$) δ: Singlet methyl protons: 0.80(H-25),0.82(H-26),0.84(H-28),0.88(H-23),0.95(H-26),1.08(H-27),1.69 (H-30),2.05(H-2^{1}),4.57(s),H-29b),4.61(s),H-29a).
^{13}C-NMR ($CDCl_3$),δ:171.3(C-1^{1}),38.6(C-1),21.7(C-2),28.2(C-21),81.2(C-3),38.1(C-4),55.6(C-5),18.4(C-6),34.4(C-7),41.1(C-8),50.5(C-9),37.3(C-10),21.1(C-11),24.0(C-12),36.2(C-13),43.0(C-14),25.3(C-15),35.8(C-16),43.7(C-17),48.5(C-18),48.2(C-19),151.2(C-20),30.0(C-21),40.2(C-22),27.6(C-23),16.7(C-24),16.4(C-25),16.2(C-26),14.7(C-27),18.2(C-28),109.6(C-29),19.5(C-30).



Compound I : Lupenol



Compound 2: Lupenol acetate

Figure 1: Isolated compounds

Table 1: Antibacterial activity of *G. nigritana* extracts,column fractions and isolated compounds #*

Extract/Compound	**Conc.(µg/disc**	***S. aureus***	***B. subtilis***	***E. coli***	***Ps. aeruginosa***
Dichloromethane	20000	25.0±0.83	26.0±0.71	12.0±0.71	16.5±0.71
Dichloromethane(DCI)	20000	-	17.3±0.6	-	11.0±0.0
Ethylacetate (EI)	20000	13.3±0.6	13.7±0.9	-	13.0±0.0
N-butanol (NB)	20000	-	-	-	-
Fr1-5	4000	-	16.0±0.0	11.3±0.6	16.0±0.0
Fr 6	4000	12.0±0.0	-	18.0±0.7	21.6±0.9
Compound I	250	-	-	17.0±0.0	-
Compound II	250	-	-	16.3±0.6	12.0±0.0
Ciprofloxacin	5	32.0±1.2	25.8±0.9	25.0±0.0	36.0±1.0
Gentamycin	10	20.2±0.5	21.3±0.6	21.2±0.6	16.0±.0

-: No inhibition
#* Values are zone of inhibition diameter in millimetre and mean of triplicates
Diammeter of cork borer =8.0mm

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Discussion.

The dichloromethane extract of the leaves of *Gutenbergia nigritana* was repeatedly fractionated over column chromatography,gel filteration over sephadex LH-20 and preparative TLC to afford compounds 1 and 2.The ^{1}H and ^{13}C-NMR spectral data for these compounds revealed that both belong to the Lupane series of triterpenoid. Compound 1, a white crystalline solid. The ^{1}H-NMR spectrum revealed the presence of seven tertiary methyl protons singlets at δ 0. 77, 0.79, 0.83, 0.94, 0.95, 1.1 and 1.71ppm. Two protons appeared at δ 4.70 and 4.61 as singlets representing exocyclic double bond protons assigned to H-29a and H-29b respectively. The signal at δ 3.17 is typical for a triterpenoid with a 3-hydroxy substitution (Cavie et al, 1968), the presence of a hydroxyl group was confirmed by the IR spectrum which showed a broad peak at 3307.1cm-^{1} and a weak peak at 1637.5cm-^{1} assigned to the double bond group. ^{13}C-NMR showed 30 signals for the triterpenoid Lupane skeleton which was represented by seven methyl carbon signals in the range 16-22ppm (Cavie et al,1968).The carbon bonded to the hydroxyl group C-3 appeared at δ 79.1, while the vinylic carbon signals appeared at δ 155.1 and 109.5ppm. Based on these and other spectral data from literature (Reynolds et al, 1986), compound 1 was found to be Lupenol.

Compound 2, a white solid, the IR spectrum revealed the absence of a hydroxy peak, but a sharp peak at 1739cm-^{1} which was assigned to ester carbonyl group, while the vinylic peak was also observed at 1641.5cm-^{1}.The ^{1}H-NMR spectrum showed the presence of eight methyl singlets at δ 0.80,0.82,0.84,0.88 ,0.95,1.08,1.69 and 2.05 ppm. The extra methyl singlet at δ 2.05 is assigned to the acetyl methyl protons indicating that compound 2 must be an ester derivative of compound 1. Two protons appeared at δ 4.57 and 4.61 as singlets representing the exocylic double bond protons H-29a and H-29b as in compound I. The absence of a signal at δ 3.19 in the ^{1}H-NMR spectrum and the appearance of a carbonyl signal at δ 171.3 and a signal at 81.2 in the ^{13}C-NMR of compound 2 confirmed it to be an acetylated derivative and the point of acetylation is the 3-hydroxy position of triterpenoid.The signal at δ 151.2 and 109.6 is due to the vinylic carbon or the exocyclic double bond. Thus compound 2 was found to be Lupenol acetate which was consistent with the NMR data reported in the literature (Pakrashi et al, 1968, Jamal et al, 2008).

The antibacterial studies of the Dichloromethane extract,the column eluate(Frs 1-5 ,and 6) and the soluble portions from the methanolic extract(DC1,EL1 and NB) revealed that the dichloromethane extract is the most active of the four extracts screened, its activity was more on the gram positive organisms at concentration of 20 mg/disc which was comparable to the standard antibiotic ciprofloxacin (5µg/hole), the extract showed better activity than gentamycin(10µg/hole), activity was particularly noted against *Ps.aeruginosa* with mean zone of inhibition at 16.50mm. The fractions from the column(1-5 and 6) showed that fraction 6 from which compounds 1 and 2 were isolated showed significant activity against *E.coli* and *Ps.aeruginosa* with zones of inhibition at 18 and 21.6mm respectively comparable to the standard antibiotics used,while activity against *S.aureus* was weak and no activity was observed against *B.subtilis*.

The methanol soluble fractions: DC1, E1 and NB, antibacterial activity was more on the ethyl acetate soluble fraction E1, though the activity was weak in comparison to the dichloromethane extract and the standard antibiotic ciprofloxacin and gentamycin. DC1 showed more activity on *B.subtilis* in comparison to E1 while the n-butanol soluble fraction(NB) did not show any activity, thus it is likely that the antibacterial properties of this plant resides in the dichloromethane extract however, the two compounds isolated from the extract showed weak antibacterial activity especially compound 2 against *E.coli* and *Ps. aeruginosa* with mean inhibition zones at 16.3mm and 12mm suggesting that activity might be on other compounds present in the extract, though Lupenol and its acetate have not been reported to possess antibacterial activity, more compounds need to be isolated from the dichloromethane extract and their antibacterial activity investigated.

Conclusion

Chromatographic separation of the active dichloromethane extract led to the isolation of two compounds,Lupenol and Lupenol acetate for the first time from this plant,the two compounds showed weak activity,the dichloromethane extract might contain other components responsible for the observed antibacterial properties,this might justify the use of this plant ethnomedicinally.

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