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Novel Compounds from *Homalium letestui* (Flacourtiaceae)

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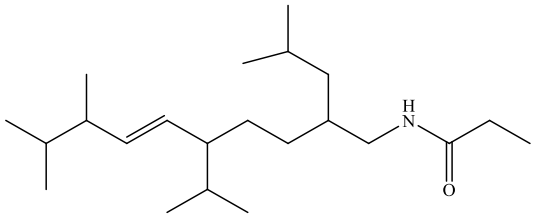
ABSTRACT
Homalium letestui is used traditionally in Nigeria as a medicinal plant. N-(2-Isobutyl-5-isopropyl-8,9-dimethyldec-6-enyl)propionamide, **I** (ringonamide), and 4-(cyclohex-3-enyl)-3-hydroxy-6-isopropyl-8-methylnonanoic acid, **II** (banitoic acid) have been isolated from the methanol extracts of Homalium letestui. Their structures were elucidated by spectrometric techniques including Electrospray Ionisation Time-of–Flight Mass Spectroscopy (ESI-ToF-MS), UV, IR, ^{1}H, ^{13}C, and 2D NMR experiments. The isolated compounds exhibited moderate antimicrobial and antioxidant activities.

***Keywords*:** *Homalium letestui*, fractionation, structure determination, bioassay.

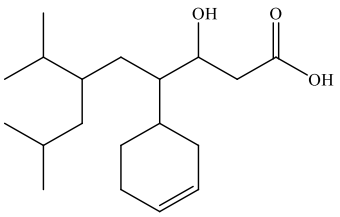
1. Introduction
Homalium letestui Pellegr (Flacourtiaceae) is a forest tree growing up to 25–30 m and found in the rain forest of West Africa [1]. In the Niger Delta region of Nigeria, the stems and roots are used in traditional medicine to treat various ailments. [2-5]. Extracts of this plant have shown promising activities in various studies [2-13]. Biologically active compounds have been isolated from the genera *Homalium* [14-20]. Previously isolated compounds from the genera include oleanane lactones, benzyl alcohol glycosides [14], salireposide, salirepin, catapol, vaccinin, homalosides A,B, C,D and 1-hydroxyl-6-oxocyclohex-2enoic acid methyl ester [15], coumarin, 6-deoxycochinolide and urolithin B [16], cochinolide and its β-glycopyranosides [17], quercetin, luteolin, cochinolide pyranosides, salirepin 6-deoxycochinolide and 1-deoxy-4,5,6-dehydrocochinolide [18].

To the best of our knowledge, previous studies on *Homalium letestui* have been restricted to the activities of the crude extracts. In this study, we report the isolation of *N*-(2-isobutyl-5-isopropyl-8,9-dimethyldec-6-enyl)propionamide, **I** (ringonamide), and 4-(cyclohex-3-enyl)-3-hydroxy-6-isopropyl-8-methylnonanoic acid, **II** (banitoic acid), from the methanol extracts of the root and stem, respectively, of *Homalium letestui*, together with their biological activities.

2. Results and Discussion
The methanol root extract of *Homalium letestui* was subjected to silica gel column chromatography repeatedly to yield *N*-(2-isobutyl-5-isopropyl-8,9-dimethyldec-6-enyl)propionamide, **I**. Also, 4-(cyclohex-3-enyl)-3-hydroxy-6-isopropyl-8-methylnonanoic acid, **II**, was obtained after successive column chromatographic separations of *Homalium letestui* methanol stem extract and purified by preparative TLC (PTLC).



I (ringonamide)



II (banitoic acid)

Compound **I** was obtained as a yellow oil. Its molecular formula was established as $C_{22}H_{43}NO$ from the ESI-ToF-MS at *m/z* = 338.34185 $[M + H]^+$ indicating two degrees of unsaturation. IR absorptions showed evidence of C-H (2854 and 2925cm^{-1}), C=O (1737 cm^{-1}), N-H (3473 cm^{-1}), C-N (1437 cm^{-1}) and C=C (3054 cm^{-1}) groups. UV revealed an n-π* (λ_{max} = 215 nm) typical of an amide. Analysis of the ^{13}C-NMR (Table 1) spectrum and HSQC experiments showed evidence of 22 carbon atoms involving a quartenary (δ_C 173.41), eight methyl (δ_C 22.68, 11.97, 29.15, 14.11, 24.96, 24.84, 24.69 and 24.58), five sp^3 methylene (δ_C 29.35, 34.04, 29.51, 29.45 and 31.92), six sp^3 methine (δ_C 29.25, 34.04, 34.13, 29.59, 33.80 and 29.69), and two sp^2 methine (δ_C 129.75, 130.01) carbon atoms. The ^{1}H-NMR showed resonances for eight methyl (δ_H 0.93, 0.85, 0.81, 0.87, 0.88, 1.02, 0.83, 0.90), five methylene (δ_H 2.35, 1.14, 1.26, 1.04, 3.67), six methine (1.62, 2.31, 2.32, 2.29, 1.30 and 2.37) groups and protons attached to the double bond (δ_H 5.36, 5.36). Three partial structures **a** (C1-C13), **b** (C2-C22) and **c** (C4-C19) were deduced from ^{1}H-^{1}H-COSY and HSQC experiments (Fig.1). The HMBC correlations for H-2 and H$_2$-1 of C-11 (δ_C 174.41), for H-3 of C-4(δ_C 29.45), for H-20 of C-2 (δ_C 34.04) and C-3 (δ_C 29.51), for H-2 of C-4 (δ_C 29.45), for H$_3$-19 of C-15 (δ_C 29.59), for H$_3$-18 of C-19 (δ_C 24.84) and C-5 (δ_C 34.13) indicated the connectivities of **a**, **b** and **c**, hence, **I** was accepted as the proposed structure.

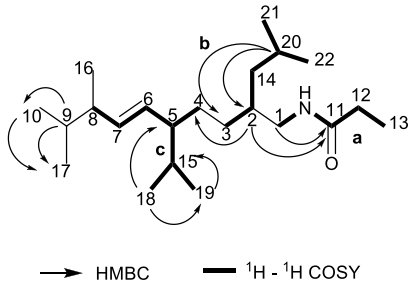
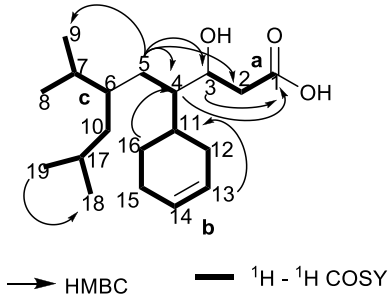


Fig.1: Selected ^{1}H-^{1}H-COSY and HMBC correlations for **I**

4-(Cyclohex-3-enyl)-3-hydroxy-6-isopropyl-8-methylnonanoic acid, **II** was isolated as a light yellow oil and had a molecular formula of $C_{19}H_{34}O_3$ as determined by ESI-ToF-MS at *m/z* = 311.25804 $[M + H]^+$, indicating three degrees of unsaturation. UV spectra showed n-σ* (205nm) and n-π* (300nm) typical of carbonyl groups. IR spectra exhibited bands at 3455, 3055, 2926 and 1737 cm^{-1} due to hydroxyl and carboxylic groups. ^{13}C-NMR and HSQC experiments revealed the presence of nineteen carbon atoms corresponding to one carboxylic group, four methyls, six sp^3 methines, six sp^3 methylenes and two sp^2 methylene. The ^{1}H-NMR, signals for the four methyl groups occurred at δ_H 0.80, 0.83, 0.86 and 0.89. The six sp^3 methylene protons resonated at δ_H 0.88, 0.89, 1.25, 1.61, 1.63 and 2.28, while signals for the sp^3 methine protons were observed at δ_H 3.67, 2.36, 2.30, 2.35 and 1.30.

^{1}H–^{1}H-COSY allowed the deduction of three partial structures **a**, **b** and **c**. The HMBC correlations for H-3 and H-4 of C-1(δ_C 174.38), for H$_2$-5 of C-9 (δ_C 14.13), C-3 (δ_C 51.45). C-4(δ_C 31.94), C-2 (δ_C 34.13), for H$_2$-16 of C-4 (δ_C 31.94), for H$_3$-18 of C-19 (24.75) and for H-13 of C-11(δ_C 29.37) indicated the connectivities of **a**, **b** and **c**. Thus, **II** was proposed as structure for the compound.



I and **II** were subjected to antimicrobial and antioxidant assay using standard methods. **I** revealed variable antimicrobial activity (Table 1) against the tested pathogens with inhibition zone ranges of 9-27mm and activity index (AI) ranges of 0.20-0.69. MIC values indicated that **I** was bacteriocidal (MIC < 0.8mg/ml) against *S. aureus* and *S. pyogenes* and bacteriostatic (MIC =0.8-2 mg/ml) against *S. typhii*, *E. coli* and *C. albicans*. **II** was most sensitive against *S. aureus* (MIC = 0.09mg/ml), *S. pyogenes* (MIC = 0.26mg/ml and *C. albicans* (MIC = 0.33mg/ml). Moderate activity was observed against *S. typhii* (MIC = 0.90 mg/ml) and P. *vulgaris* (MIC = 1.3 mg/ml).

Table 1. Antimicrobial Activities of **I** and **II**

	I			**II**			**RA**		
Microorganism	**IZ**	**AI**	**MIC[a]**	**IZ**	**AI**	**MIC[a]**	**IZ**	**AI**	**MIC[b]**
Staphylococcus aureus	23	0.50	0.70	35	0.76	0.09	46	1	11.00
Streptococcus pyogenes	27	0.69	0.20	21	0.53	0.26	39	1	10.00
Salmonella typhi	15	0.63	1.80	16	0.37	0.90	43	1	8.50
Escherichia coli	16	0.55	1.90	13	0.33	NT	40	1	11.20
Proteus vulgaris	9	0.20	NT	15	0.38	1.30	45	1	8.60
Candida albicans	19	0.50	1.20	23	0.61	1.40	38	1	9.10

IZ= inhibition zone in mm; AI= activity index, MIC[a] = minimum inhibitory concentration in mg/ml; MIC [b] = minimum inhibitory concentration in µg/ml. RA = reference antibiotics; *Staphylococcus aureus*- Penicillin; *Streptococcus pyogenes* – Gentamicin; *Salmonella typhi*- Ciprofloxacin; *Escherichia coli*- Penicillin; *Proteus vulgaris* – Ciprofloxacin; *Candida albican*s – Fluconazole

Antioxidant assay (Table 2) revealed moderate DPPH radical scavenging activity, metal chelating ability and ferric reducing potential for **I** and **II** respectively which were lower than values obtained from the controls.

Table 2. Antioxidant Activities of **I**, **II** and Reference Compounds

	EC_{50} Values (mg/ml)*		
Com-pound	DPPH Radical Scavenging Activity	Metal Chelating Ability	Ferric Reducing Antioxidant Power
I	24.00	18.00	21.02
II	23.82	20.58	12.14
BHA	0.38	-	0.60
Vit. E	0.50	-	-
Vit.C	-	-	0.05
EDTA		0.01	-

*EC_{50} value, the effective concentration at which 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were scavenged by 50%, ferrous ions were chelated by 50%, the absorbance was 0.5 for ferric reducing antioxidant power. EC_{50} value was obtained by interpolation from linear regression analysis.

3. Experimental

The NMR spectra of **I** and **II** were acquired on a Bruker AVANCE 400 instrument operating at 400 MHz for proton and 400 MHz for carbon using Me_4Si (TMS) as internal lock standard. Structural assignments were based on the interpretation of ^1H, ^{13}C, ^1H-^1H-COSY, ^1H-^{13}C HSQC, and ^1H-^{13}C HMBC experiments. Accurate molecular weight was determined using an electrospray ionization time-of-flight mass spectrometer (ESI-ToF mass spectrometer; Druker Daltronics MICR OTOF II ESI-ToF) in the positive ionization mode. UV-VIS spectra were recorded on a Specord S600 spectrophotometer. Infrared spectra were recorded on a 5500 series compact FT-IR (Agilent Technologies) instrument. Column chromatography was performed using silica gel (70-230 mesh, Sigma Aldrich) in glass columns of varying sizes fitted with Teflon taps. Analytical thin layer chromatography (TLC) was performed on pre-coated aluminium sheets with florescence (Silica gel $60F_{254}$ 0.2 mm thickness, Sigma Aldrich); PTLC was done with plates coated with florescence (Silica gel $60F_{254}$; 1 mm thickness). Detection was with iodine crystals or by visualization under ultraviolet light at wavelengths of 254 and 366 nm.

3.1 Plant Materials

H. letesui was collected in the wild in Uyo, Akwa Ibom State, Nigeria in December 2010 and identified by Dr. (Mrs) M. Bassey of the Department of Botany and Ecological Studies, University of Uyo, Nigeria (Voucher Specimen No. FPUU382).

3.2 Extraction and Isolation

The air dried roots of *H. letestui* (2000 g) were pulverized and then extracted first with petroleum ether and later with methanol (4 l X 3) at room temperature. Filtration and concentration to dryness (*in vacuo*) gave the crude petroleum ether and methanol extracts respectively. The methanol extract (22.61g) chromatgraphed in a column loaded silica gel and eluted with a gradient of hexane/EtOAc/MeOH, yielded 195 fractions (250 ml)(A_1-A_{195}), As a result of thin layer chromatography (TLC) analysis, fractions A_1-A_4 (1.76 g) were pooled and re-chromatographed on a silica gel column and eluted with hexane/chloroform/EtOAc, yielding 11 fractions (B_1-B_{11}). Fractions B4-B7 were pooled, re-dissolved in chloroform and filtered. Evaporation of the solvent gave **I** (0.243 g, 1.07%) as a yellow oil. λ_{max} (MeOH)/nm 203.5 nm (A 0.961); ν_{max}/cm^{-1}: 1737, 1437, 3473, 3054, 2854, 2925 cm^{-1}; δ_H (400Mz, $CDCl_3$): 3.67 (2H, s); 2.31 (1H, t); 1.14 (2H, m); 1.26 (2H, m); 2.32 (1H, m); 5.36 (1H, d); 5.36 (1H, d); 2.37 (1H, m); 1.30 (1H, m); 0.90 (3H, m); 2.35 (2H, m); 0.93 (3H, m); 1.04 (2H, m); 2.29 (1H, m); 1.02 (3H, m); 0.83 (3H, m); 0.88 (3H, m); 0.87 (3H, m); 1.62 (1H, m); 0.85 (3H, m); 0.81 (3H, m); δ_C (400Mz, $CDCl_3$, Me_4Si): 51.44, 34.04, 29.51, 29.45, 34.13, 129.75, 130.01, 33.80, 29.69, 22.68, 174.41, 29.35, 11.97, 31.92, 29.15, 14.11, 24.96, 24.84, 29.25, 24.69, 24.58; ESI-MS: $[M + H]^+$ Found: 338.34185, $C_{22}H_{43}NO + H^+$ requires 338.34227.

Air-dried stems of *H. letestui* (2000 g) were powdered, defatted with petroleum ether and macerated with methanol (4 l X 3) for 72 h at room temperature. Filtration and evaporation of solvent *in-vacuo* gave the crude methanol extract (13.72 g). The extract was chromatographed in a column loaded with silica gel and eluted with a gradient of hexane/chloroform/ EtOAc/MeOH. Based on TLC analysis, 63 main fractions were obtained (AA_{01}-AA_{63}). Fractions AA_6-AA_{20} (1.71 g) were re-chromatographed on a silica gel column eluted with a gradient of hexane/EtOAc/MeOH to obtain 11 fractions (AC_1-AC_{11}). Fractions AC_4 – AC_8 were pooled and subjected to PTLC using hexane:EtoAc (1:2) to afford **II** (0.13g, 0.94%). as a light yellow oil (0.13g, 0.94%). λ_{max} (MeOH)/nm 205.2 nm (A 1.448); 224.5nm (A 0.956); ν_{max} /cm^{-1}: 1265, 1737, 2854, 2926, 3055 and 3455cm^{-1}; δ_H (400Mz, $CDCl_3$): 0.88 (1H, exchangeable); 2.28 (2H, s); 3.67 (1H, m); 2.36 (1H, m); 0.88 (2H, m); 2.35 (1H, m); 2.32 (1H, m); 0.89 (3H, m); 0.83 (3H, m); 0.89 (2H, m); 2.30 (1H, m); 1.63 (2H, m); 5.35 (1H, s); 5.35 (1H, s); 1.61 (2H,m); 1.25 (2H, m); 1.30 (1H, m); 0.86 (3H, m); 0.80 (3H, m). δ_C (400Mz, $CDCl_3$, Me_4Si): 174.38, 34.13, 51.45, 31.94, 29.53, 29.46, 29.60, 27.17, 14.13, 29.71, 29.37, 29.32, 130.01, 129.76, 29.16, 27.22, 22.70, 24.75; ESI-MS: $[M + H]^+$. Found: 311.25804. $C_{19}H_{34}O_3 + H^+$ requires 311.25787.

3.3 Antimicrobial Activity.

I and **II** were evaluated for their antimicrobial activity using the disc diffusion method and the minimum inhibitory concentration (MIC) determined by the microdilution technique. Two Gram positive (*Staphylococcus aureus*, *Streptococcus pyogenes*), three Gram-negative bacteria (*Salmonella typhi*, *Proteus vulgaris* and *Escherichia coli*) and one fungus (*Candida albicans*) were assayed. The bacterial innocula were prepared with fresh isolates of microbial strains and cultured on tryptic soy agar for 24 hours at 37^{o}C. Yeast culture was prepared on Sabouraud/Dextrose agar and incubated at 28^{o}C for 48 hours. The density of the innocula was set to 0.5McFarlands unit. Empty sterilized disks of 6mm diameter were impregnated with 1ml of **I** and **II** each. Thereafter, 1 ml of the innocula was mixed with 17 ml of Muller-Hinton agar and Sabouraud/Dextrose agar, respectively, and poured into sterile petri dishes. The disc impregnated with the sample was then placed on top of the inoculated agar and pressed slightly. Plates were incubated at 37 ^{o}C for 24 hours (for bacterial strains) and at 28^{o}C for 48hours (for yeast strain). Inhibition zones formed on the medium were evaluated in

millimetres. Gentamicin, ciprofloxacin, penicillin and fluconazole were used as positive controls [19]-[20]. The lowest concentration of **I** and **II** that inhibited the growth of microorganism was taken as its MIC, in accordance with the Clinical and Laboratory Standards Institute [21].

3.4 Evaluation of Antioxidant activity:

Antioxidant activities of **I** and **II** were measuring using three models: the DPPH radical scavenging ability, ferric reducing activity and metal chelating potential.

DPPH Assay: Aliquots (1 ml) of varying concentrations of **I** and **II** were mixed with 1 ml of 0.004% methanol solution of DPPH. The mixture was vigorously shaken and allowed to stand for 30 min at room temperature in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm. The procedure was repeated for the blank and control. The radical scavenging activity was calculated using the equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A_{blank} - A_{sample}}{A_{blank}} X\ 100$$

Concentration providing 50% activity (EC_{50}) was calculated [22]. Butylated hydroxyl anisole (BHA) and Vitamin E were used as positive controls.

Evaluation of Metal Chelating Ability: Metal chelating ability was determined according to the method of Decker and Welch [23], with some modifications. Briefly, 0.5 ml of **I** and **II** were mixed with 0.05 ml of 2 mM $FeCl_2$ and 0.1 ml of 5 mM ferrozine. The total volume was diluted with 2 ml methanol. The mixture was then shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine – Fe^{2+} complex formation was calculated using the formula:

$$\text{Scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} X100$$

where $A_{control}$ = absorbance of ferrrozine – Fe^{2+} complex,

and A_{sample} = absorbance of sample.

EDTA was used as a positive control.

Evaluation of Ferric Reducing Antioxidant Power (FRAP): The reducing power was determined according to the method of Oyiazu [24]. Varying concentrations of **I** and **II** (0.1-20 mg/ml) in ethanol (2.5 ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.60) and 2.5 ml of 1% potassium ferricyanide. The resulting mixture was incubated at 50 ^{o}C for 20 min. Thereafter, 2.5 ml of 10% trichloroacetic acid (w/v) was added, and the mixture centrifuged at 200 g for 19 min. The upper layer (5 ml) was mixed with 5 ml of deionised water and 1 ml of 0.1% ferric chloride and the absorbance measured at 700 nm against a blank. A higher absorbance indicated a higher reducing power. EC_{50} value (mg/ml) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation. Ascorbic acid and BHA were used as positive controls.

4. Conclusion

The present investigation was undertaken to characterize bioactive compounds from *Homalium letestui*, which has been used locally in traditional medicine for the treatment of various ailments. Repeated chromatographic separation resulted in the isolation of **I** and **II** which exhibited moderate antimicrobial and antioxidant activities in separate models, lending support to their use in traditional medicine.

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