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Antimicrobial Marine Natural Products from the Sponge, *Axinella infundibuliformis*

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Abstract: The antimicrobial activity of the extracts and pure compounds from the sponge *Axinella infundibuliformis* collected Mombasa, the Kenyan Coast have been reported. The pure compounds were purified and characterized through various chromatographic and spectroscopic techniques. Three triterpenoid compounds were isolated and identified from *Axinella infundibuliformis*. These were 3β-Hydroxylup-20(29)-ene (**1**), 3β-Hydroxylup-20(29)-en-28-oic acid (**2**) and 3-Oxo-lup-20(29)-en-28-oic acid (**3**). The hexane, dichloromethane and methanol crude extracts (10 mg/ml) of *Axinella infundibuliformis* showed strong antibacterial activity against *methicilin* resistant *Staphylococcus aureus* with inhibition zone diameters of 24.7 ± 0.05, 22.0 ± 0.35 and 12.7 ± 0.09 mm respectively. The MIC values for dichloromethane and hexane extracts were then evaluated as at 6.25 mg/mLand 3.12 mg/mLrespectively. The antifungal tests for *Axinella infundibuliformis* against *Candida albicans* by all the three extracts showed mild activity with inhibition zone diameters of 6.7 ± 0.02, 6.0 ± 0.04, and 5.7± 0.03 mm respectively. In addition, the dichloromethane and hexane extracts exhibited low activities against *Microsporum gypseum* (6.3 ± 001 mm) and *Cryptococcus neoformans* (6.3 ± 0.07mm) respectively. Of the three compounds isolated, 3β-Hydroxylup-20(29)-ene (24.0 ± 0.09 mm diameter) exhibited strong activity against *Pseudomonas aeruginosa,* while 3β-Hydroxylup-20(29)-en-28-oic acid (7.0 ± 0.06 mm diameter) and 3-Oxo-lup-20(29)-en-28-oic acid (10.7 ± 0.08 mm diameter) showed moderate activity against *P. aeruginosa*. Gentamycin (Standard drug 10 µg/disc) had an inhibition zone diameter of 16.0 ± 0.01 mm.

Keywords: sponge; *Axinella infundibuliformis*; *antimicrobial activity; 3β-Hydroxylup-20(29)-ene, 3β-Hydroxylup-20(29)-en-28-oic acid; 3-Oxo-lup-20(29)-en-28-oic acid.*

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1. Introduction

Sponges are simple animals that live permanently attached to a location in water, they are sessile as adults. They come in an incredible variety of shapes, sizes and colours. Most sponges are predominantly marine, only about 150 species live in fresh water and this constitute the *Spongillidae family*. Sponges evolved over 500 million years ago and are the oldest metazoan group [1].

The body of a sponge is a collection of a few different types of cells loosely arranged in a gelatinous protein or carbohydrate matrix called mesohyl, mesoglea or mesenchyme [2]. The sponges range in size from a few millimetres to 2 meters tall. The mesohyl is the connective tissue of a sponge body and is supported by the skeletal elements. Throughout this body, run canals through which water flows. The canals have openings to the outside called pores; those through which water enters the sponges are called 'ostia', while those through which water leaves are called 'oscula'. Sponges have skeletons whose main components are a protein; sponging and spicules; non-living aggregates of a chemical nature secreted and made from either silica or calcium carbonate. Spicules are important in classification of sponges [3].

All sponges are filter feeders of planktons and most are sedentary or immobile as adults thus they have defensive chemical weapons (secondary metabolites) for their protection. Intensive evolutionary pressure from competitors that threaten overgrowth, poisoning, infection of predation have armed sponges with an arsenal of potent chemical defence agents which not only play various roles in the metabolism of the producer but also in their strategies to the given environment. Studies have shown sponges to be rich in terpenoids, steroids, alkaloids, peptides and poly-ketides [1]. Ecological reproduction is either by sexual means or asexual means [3].

Axinella infundibuliformis (Linnaeus) is commonly called "funnel or cup sponge." It is variable in shape but is usually cup-like or lamellate with a short thick stalk. The funnel can grow to 10 cm in diameter and walls have a regular thickness of 3-4 mm and terminate with rounded firm edges [4]. The inner surface of the cup has many exhalant openings which are visible with naked eyes. They are to be found usually on upward facing bedrock or stable boulders in the circumlittoral zone from 20 to 630m. However, they are found mainly on horizontal surfaces in semi sheltered conditions where they have to be tolerant to the present sediments. The main identification features are presence of a well formed cup with rounded edge reaching 25 cm in diameter (Figure 1) and a large exhalant pores inside the cup [5].

Figure 1. *Axinella infundibuliformis* in ocean base.

On the basis of solid taxonomy and ecological data, the high value of this phylum for human application becomes obvious, especially with regard to the field of chemical ecology and the desire to find novel potential drugs for clinical use. Marine organism especially those associated with sponges despite their high potential as sources for new bioactive compounds have remained largely untapped and thus the need to highlight the biomedical potential of these organisms.

2. Materials and Methods

2.1.1 Sponge Sample

The fresh samples of *Axinella infundibuliformis* were collected in ocean waters off the coast of Jomo Kenyatta public beach and the English Channel next to Kenya Marine and Fisheries Research Institute (KEMFRI) in Mombasa (Latitude: 04°02'S, Longitude: 039°37'E), Kenya. The voucher specimens S2PL was deposited at the Jomo Kenyatta University of Agriculture and Technology, Zoology department museum, where taxonomic identification was also carried out with help from Kenya Marine and Fisheries Research Institute (KEMFRI) museum department.

2.1.2 Extraction and Isolation

The sample was washed with distilled water, air dried and chopped into small sizes before being ground into fine powder. Sequential extractions with hexane (C_6H_{14}), dichloromethane (CH_2Cl_2) and methanol (CH_3OH) were carried out with each solvent extraction being carried out in triplicates for 48 hours per soaking. The air dried ground sponge it gave 0.12%, 0.50% and 1.47% of hexane, dichloromethane and methanol extracts respectively.

A combination of chromatographic techniques, VLC, CC and TLC were used for isolation. The extracts were separately fractionated by VLC performed on Kieselgel silica gel with various proportions of a solvent gradient of changing polarity, starting with 100% n-hexane through ethyl acetate and down to 100% methanol (15 ml portions of solvent with 25% change in polarity).

Further purification was achieved by use of CC done on silica gel 60 and eluted with a slow gradient of solvent system.

Analytical pre-coated plastic TLC plates were used throughout the purification process. These were mainly for establishment of optimum solvent systems for separations, complexity of extracts and purity of isolated compounds. Spots on the chromatograms were detected under UV light at 254 nm for UV active compounds and visualized upon development by separately spraying vanillin-sulphuric acid (25% sulphuric acid) and heating for 10 min. at 110 ^{o}C in an oven. Fractions that showed homogeneity were combined and concentrated together to give pure compounds or semi pure compounds for further purification.

The isolated compounds were identified by spectroscopic methods and comparing the values with reported literature.

2.2. Antimicrobial activity

2.2.1. Microbial test cultures and growth conditions

Escherichia coli (ATCC 25922), Salmonella typhi (clinical isolate), *Pseudomonas aeruginosa* (ATCC 27853) gram negative bacteria and *Staphylococcus aureus* (ATCC 22923), Methicilin resistant *Staphylococcus aureus* (clinical isolate) gram positive bacterial strains were used. *Candida albicans* (ATCC 90028), *Candida tropicalis* (ATCC 750), *Cryptococcus neoformans* (ATCC 66031), and

Microsporum gypseum (Clinical isolate) were used as fungal test microorganisms. Bacterial strains were maintained on Nutrient Agar (NA) petri dishes at 4 ^{o}C, while fungi were maintained on Sabourand Dextrose Agar (SDA) petri dishes. Antifungal and antibacterial in-vitro assays were done using disc diffusion [6, 7]. All the procedures were done according to Clinical laboratory standard Institute Standards procedures and quality control [8]. The fresh cultures were obtained by growing the test strains overnight at 37 ^{o}C for bacteria while fungi were grown at 28 ^{o}C for 48 hours.

2.2.2 Media preparation

Mueller Hinton Agar (MHA) (OXOID, UK) was used for bacteria bioassay while SDA (OXOID, UK) was used for fungi. MHA was prepared by dissolving 19 g in 500 ml of distilled water and brought to boil to completely dissolve. Sterilization was achieved by autoclaving at 121 ^{o}C for 15 minutes. SDA was prepared by dissolving 65 g in 1 litre of distilled sterilized water, brought to boil to ensure complete dissolution. The media was autoclaved at 121 ^{o}C for 15 minutes to ensured sterilization. The media was dispensed (20 ml) onto the pre-sterilized petri dishes yielding uniform depths. They were then covered and allowed to cool and solidify at room temperature. Filter paper discs (5 mm diameter) were prepared and sterilized by autoclaving.

2.2.3 Antimicrobial activity assay

Disc diffusion method was employed in the preliminary antimicrobial screening of both the crude organic extracts and selected isolated purified compounds. Test strains suspension of 0.5 McFarland was prepared from fresh cultures using normal saline. The plates were aseptically streaked with the test microorganism using a sterile swab and allowed to dry for a few minutes. Test samples (100 mg) were dissolved in 1ml of DMSO. Sterile 3 mm diameter filter paper discs were impregnated with 100 mg/mL(sample sizes ranged from 2 mg to 100 mg) of the test samples from *A. infundibuliformis* and using sterile forceps the discs placed aseptically on the inoculated agar plates samples. The plates were then incubated for 24 hours at 37 ^{o}C for bacteria and at 28 ^{o}C for fungi. The experiments were carried out in triplicates. Presence of a clear circular zone around the sample impregnated disc was used as an indicator of activity. The results (mean values, n = 3) were recorded by measuring zones diameter in millimetres. Disc impregnated with the solvent used (DMSO) was included as negative controls. For comparative purposes standard drugs Gentamycin (10 µg/disc) and fluconazole (40 µg/disc) were included as positive controls in the assay for antibacterial and antifungal assays respectively.

2.2.4. Minimal inhibitory concentration (MIC)

Minimal inhibitory concentration of the active extracts was determined by serial micro-dilution to obtain concentration ranging from 100-2 mg/ml. The procedure was carried out as described by Ratanavalachai and Wongchai [9]. This was carried out on extracts whose activity was deemed to have significant activity in comparison with the standard drug.

2.2.5. Instrumentation

The 1D [^{1}H (300 MHz), ^{13}C (75.4MHz), DEPT] and 2D [COSY, TOCSY, ROESY, HMBC, and HMQC] spectra were acquired on Bruker Avance DPX 300 spectrophotometer and referenced to a residual solvent signal. The spectra were recorded in $CDCl_3$, Mass spectra were run as autospec time of flight (TOF) on a GCT Premier Mass spectrometer, while Infra-red (IR) absorptions were measured on a Shimadzu FTIR 8000 SCSI spectrophotometer as KBr pellets after background correction in the range 4000 - 400 cm^{-1}. Melting points were recorded using Gallen Kamp Griffins melting point apparatus and are uncorrected.

Thin layer chromatography (Tlc) experiments were developed on ready made 0.25 mm thick layer of silica gel 60 F_{254} (Merck) coated aluminium sheets and visualized by observation under UV-light (254 nm and 365 nm) and spraying with vanillin-sulphuric acid spray. Normal column chromatography (CC) was conducted using different sizes of columns packed with (Merck) silica gel 60 (size 0.040-0.063mm). Preparative Tlc were run on 0.5 mm thick layer (Merck) silica gel 60 HF_{254} containing gypsum (CaSO4 binder) coated on 20 x 20 cm glass plates.

*2.2.6. 3β-Hydroxylup-20(29)-ene (**1**) - Lupeol ($C_{30}H_{50}O$):* White powder, mp: (212-216 ^{o}C) uncorrected; R_f: 0.67, $CHCl_3$/EtOAc 9:1 v/v; IR (KBr pellets) ν_{max} cm^{-1};3346, 2935, 1705, 1639, 1460, 1379, 1190, 1035, 881;TOF-MS (m/z); 426.72 $[M]^{+}$.

*2.2.7. 3β-Hydroxylup-20(29)-en-28-oic acid (**2**) - Betulinic acid ($C_{30}H_{48}O_3$)*:White powder, mp: (320-322 ^{o}C) uncorrected; R_f: 0.63, $CHCl_3$/EtOAc 9:1 v/v; IR (KBr pellets); ν_{max} cm^{-1} 3070, 2943, 1693, 1456, 1375, 1244, 732. TOF-MS (m/z); 456.70 $[M+1]^{+}$.

*2.2.8. 3-Oxo-lup-20(29)-en-28-oic acid (**3**) - Betulonic acid ($C_{30}H_{46}O_3$)*:White powder, mp: (247-249 ^{o}C) uncorrected; R_f: 0.64, $CHCl_3$/EtOAc 9:1 v/v; IR (KBr pellets); ν_{max} cm^{-1} 3446, 3070, 2947, 1705, 1454, 1377, 1244, 883. TOF-MS (m/z); 454.70 [M] +; 455.70 $[M+1]^{+}$

3. Results and Discussion

3.1 Identification of the Pure Compounds

The results of the 1D NMR (^{1}H and ^{13}C) (Table 1. and 2) and 2D experiments together with the MS data confirmed that this compounds to have chemical structures for 3β-hydroxylup-20(29)-ene (**1**), 3β-Hydroxylup-20(29)-en-28-oic acid (**2**) and 3-oxo-lup-20(29) - en-28-oic acid (**3**).

3β-hydroxylup-20(29)-ene (**1**), NMR data is consistent with the already identified data for lupeol [10]. Most of the data reported previously for lupeol, has been the ^{13}C NMR data. In this paper we have assigned all ^{1}H NMR peaks for lupeol with the help of the 2-D NMR spectra (Table 1). The ^{13} C NMR spectral data (Table 2) are consistent with the already reported data.

3β-Hydroxylup-20(29)-en-28-oic acid (**2**), NMR data is consistent with the already identified data for betulinic acid [11, 12]. The data reported previously for betulinic acid was the ^{13}C NMR data. In this paper we report with precision the ^{1}H NMR peaks for betulinic acid with the help of the 2-D NMR spectra (Table 1). The ^{13} C NMR spectral data (Table 2) are consistent with the already reported data.

Table 1. ^{1}H (300 MHz) NMR chemical shifts (δ_H, ppm) for 3β-Hydroxylup-20(29)-ene (**1**), (3β) -Hydroxylup-20(29)-en-28-oic acid (**2**) and 3-Oxo-lup-20(29)-en-28-oic acid (**3**) in $CDCl_3$.

Position	1	2	3
1 *a*	0.99 *m*	0.98 *m*	1.00 *m*
e	*1.05 m*	*1.05 m*	*1.03 m*
2.*a*	1.85 *m*	1.83 *m*	2.35 *m*
e	1.95 *m*	1.96 *m*	2.43 *m*
3	3.18 *dd*	3.40 *br,s*	
5	0.69 *t*	0.68 *t*	0.67 *t*
6 *a*	1.39 *m*	1.40 *m*	1.40 *m*
e	1.51 *m*	1.50 *m*	1.59 *m*
7 *a*	1.35 *m*	1.35 *m*	1.36 *m*
e	1.42 *m*	1.41 *m*	1.42 *m*
9	1.71 *m*	1.68 *m*	1.28 *m*
11 *a*	1.29 *m*	1.26 *m*	1.25 *m*
e	1.60 *m*	1.42 *m*	1.42 *m*
12 *a*	1.38 *m*	1.41 *m*	1.41 *m*
e	1.52 *m*	1.52 *m*	1.60 *m*
13	0.68 *m*	0.69 *m*	0.69 *m*
15 *a*	1.40 *m*	1.41 *m*	1.41 *m*
e	1.50 *m*	1.52 *m*	1.58 *m*
16 *a*	1.34 *m*	1.33 *m*	1.35 *m*
e	1.41 *m*	1.42 *m*	1.42 *m*
18	1.79 *dd*	1.77 *dd*	1.77 *dd*
19	2.38 *dt*	3.01 *dt*	3.02,*dt*
21 *a*	1.41 *m*	1.41 *m*	1.40 *m*
e	1.51 *m*	1.51 *m*	1.52 *m*
22 *a*	1.35 *m*	1.35 *m*	1.36 *m*
e	1.42 *m*	1.41 *m*	1.42 *m*
23	0.76 *s*	0.81 *s*	0.84,*s*
24	0.78 *s*	0.83 *s*	0.94,*s*
25	0.82 *s*	0.93 *s*	0.93,*s*
26	0.94 *s*	0.99 *s*	0.99 *s*
27	0.96 *s*	1.02 *s*	1.02 *s*
28	1.03 *s*		
29 [a]	4.69 *d*	4.74 *br,s*	4.74,*br,s*
29 [b]	4.57 *d*	4.65 *br,s*	4.61,*br,s*
30 (CH_3)	1.68 *s*	1.69 *s*	1.69,*s*

Assignments were done by COSY, TOCSY, ROESY, HMQC and HMBC. 29[a] and 29 [b] are interchangeable.

Table 2. ^{13} C (75.4MHz) NMR chemical shifts (δ_H, ppm) for 3β-Hydroxylup-20(29)-ene (**1**), (3β)-3-Hydroxylup-20(29)-en-28-oic acid (**2**) and 3-Oxo-lup-20(29)-en-28-oic acid (**3**) in $CDCl_3$.

Position	1	2	3
1	38.7 (*t*)	37.1 (*t*)	37.0 (*t*)
2	25.2 (*t*)	25.4 (*t*)	32.1 (*t*)
3	79.0 (*d*)	76.3 (*d*)	218.1 (*s*)
4	38.9 (*s*)	37.3 (*s*)	47.3 (*s*)
5	55.3 (*d*)	50.3 (*d*)	54.9 (*d*)
6	18.3 (*t*)	18.3 (*t*)	19.6 (*t*)
7	34.3 (*t*)	34.2 (*t*)	34.1 (*t*)
8	40.9 (*s*)	40.9 (*s*)	40.6 (*s*)
9	50.5 (*d*)	49.3 (*d*)	49.8 (*d*)
10	37.2 (*s*)	37.5 (*s*)	36.9 (*s*)
11	20.9 (*t*)	20.7 (*t*)	21.3 (*t*)
12	27.4 (*t*)	25.5 (*t*)	29.7 (*t*)
13	38.1 (*d*)	38.4 (*d*)	38.5 (*d*)
14	43.0 (*s*)	42.5 (*s*)	42.5 (*s*)
15	27.5 (*t*)	29.7 (*t*)	25.5 (*t*)
16	35.6 (*t*)	30.6 (*t*)	33.6 (*t*)
17	42.9 (*s*)	56.4 (*s*)	54.9 (*s*)
18	48.3 (*d*)	49.1 (*d*)	49.2 (*d*)
19	48.0 (*d*)	46.9 (*d*)	46.9 (*d*)
20	151.0 (*s*)	150.4 (*s*)	150.3 (*s*)
21	29.9 (*t*)	30.6 (*t*)	30.5 (*t*)
22	40.0 (*t*)	33.2 (*t*)	39.6 (*t*)
23	15.4 (*q*)	16.0 (*q*)	20.9 (*q*)
24	28.0 (*q*)	28.2 (*q*)	26.6 (*q*)
25	16.1 (*q*)	15.9 (*q*)	15.1 (*q*)
26	16.0 (*q*)	16.0 (*q*)	16.1 (*q*)
27	14.6 (*q*)	14.8 (*q*)	14.6 (*q*)
28	18.0 (*q*)	181.5 (*s*)	181.9 (*s*)
29	109.3 (*t*)	109.7 (*t*)	109.7 (*t*)
30	19.3 (*q*)	19.4 (*q*)	19.3 (*q*)

Assignments were confirmed by HMQC, HMBC and DEPT experiments

*3-oxo-lup-20(29) - en-28-oic acid (**3**),* was isolated as white solid powder of melting point 247-249 °C. TOF-MS spectrum displayed molecule ion peaks at m/z 454.70 $[M]^+$, 455.70 $[M+1]^+$, consistent with a molecular formula $C_{30}H_{46}O_3$. The IR spectrum showed absorption bands ν_{cm}^{-1} due to O-H (3446 cm^{-1}), keto carbonyl C=O (1705 cm^{-1}), carboxylic carbonyl C=O (1643 cm^{-1}), and vinyl C=C- (1456 cm^{-1}) stretch.

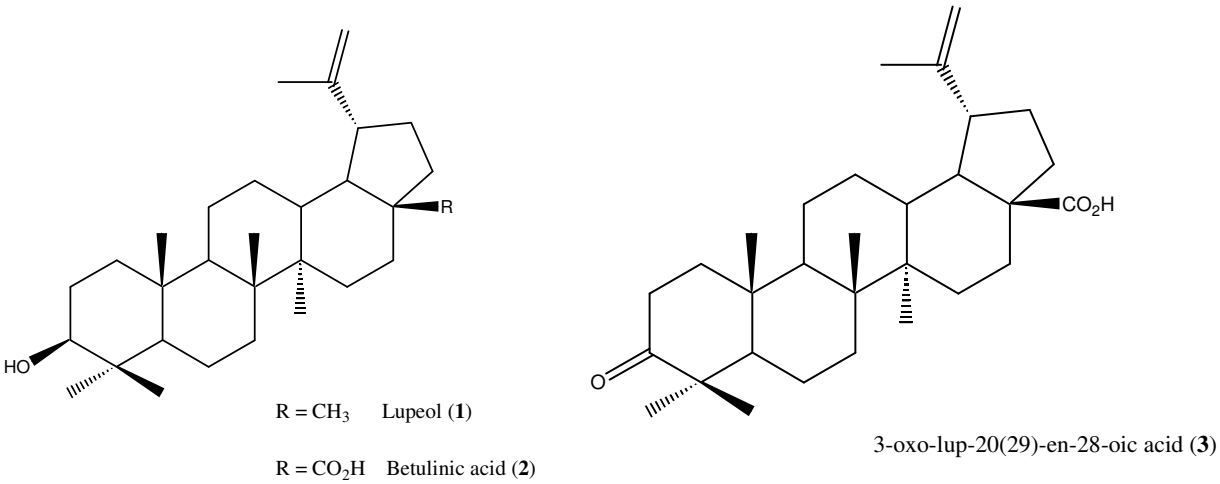

Figure 2. Isolated compounds from *Axinella infundibuliformis*

3.2 Antimicrobial activity

The antimicrobial activity results were tabulated as diameter in mm of the developed Discs from the extracts, pure compounds isolated and the standards as elaborated in Figure 3 below. The antimicrobial activity results of *A. infundibuliformis* extracts are recorded in Table 3 and those for pure isolated compounds in Table 4

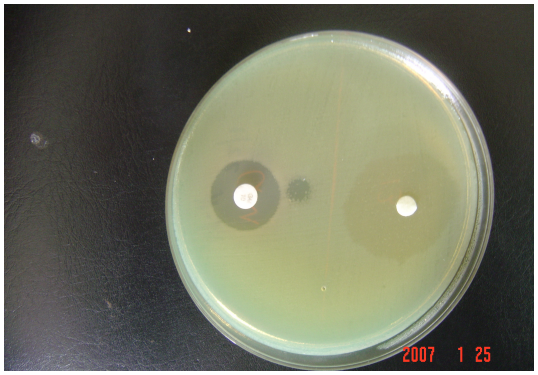

Figure 3. Disc showing zones of inhibition by Gentamycin (left) and 3β-hydroxylup-20(29)-ene-28-oic acid (**2**) (right) against *Pseudomonas aeruginosa.*

Results of the extracts tested (Table 3) indicated that all the extracts of *Axinella infundibuliformis* have potential activity. This was observed strongly against MRSA, where an inhibition zone of diameter 6.7 ± 0.01 mm, 11.0 ± 0.03 mm and 12.7 ± 0.03 mm for methanol, dichloromethane and hexane respectively was observed. Comparison of the observed activities with that of standard drug Gentamycin, shows inhibition zone 7.0 ± 0.01 mm. The extracts were run at concentrations of 5.0 mg/ml. The MIC of dichloromethane extract and hexane extract of *Axinella infundibuliformis* on MRSA were determined to be 4.25 mg/mLand 3.12 mg/mLrespectively.

Table 3. *Antimicrobial activity of A. infundibuliformis extracts. S4M (Methanol), S4D (Dichloromethane), S4H (Hexane), ND - Not Detectable*

Activity expressed in mm of inhibition zones diameter

Microorganism	(mean values n = 3)				
	S4M (5mg/ml)	**S4D (5mg/ml)**	**S4H (5mg/ml)**	*Gentamycin (10µg/disc)*	*fluconazole (40 µg/disc*
Escherichia coli	ND	ND	ND	3.5 ± 0.01	-
Staphylococcus aureus	5.6 ± 0.02	4.5 ± 0.02	5.9 ± 0.01	5.8 ± 0.01	-
Pseudomonas aeruginosa	3.5 ± 0.01	5.3 ± 0.02	3.0 ± 0.01	3.4 ± 0.01	-
Salmonella typhi	ND	ND	ND	3.1 ± 0.01	-
Methicillin resistant *Staphylococcus aureus (MRSA)*	6.7 ± 0.01	11.0 ± 0.03	12.7 ± 0.03	7.0 ± 0.01	-
Candida albicans	4.5 ± 0.01	3.0 ± 0.01	3.0 ± 0.01	-	4.6 ± 0.01
Candida tropicalis	4.6 ± 0.01	3.0 ± 0.01	3.5 ± 0.01	-	6.6 ± 0.01
Cryptococcus neoformans	3.4 ± 0.01	3.4 ± 0.01	3.0 ± 0.01	-	5.4 ± 0.01
Microsporum gypseum	3.5 ± 0.01	3.0 ± 0.01	3.1 ± 0.01	-	3.5 ± 0.01

The ^{13}C NMR spectrum showed thirty carbon signals suggesting this compound to be a triterpene. The ^{1}H-NMR displayed peaks due to an isoproprenyl group of the lupane triterpenoid skeleton, δ_H 1.69 (3H, s), δ_H 4.74 (1H, s), δ_H 4.61 (1H, s) and a typical lupene Hβ-19 proton at δ_H 3.02 (1H, *J*=10.8 and 5.4 Hz) and hence compound was confirmed as a lupeol derivative (Figure 2). Its spectral data (Tables 3.1 and 3.2) were similar to those of betulinic acid. The signals at δ_H 3.40 and δ_C 76.3 corresponding to H-3 and C-3 respectively in betulinic acid were not present but instead there was an additional carbonyl signal in the ^{13}C NMR resonating at δ_C 218.13. Similarly the IR band at 1701cm^{-1} due to presence of a six membered ring ketone confirmed the validity of the carbon signal at δ_C 218.13.

The HMBC correlation showed a long range correlation between the methyl protons at δ_H 0.84 [H-23], δ_H 0.94 [H-24], with the carbonyl carbon δ_C 218.1 confirming the assignment of this carbonyl carbon to C-3. This compound was thus identified as 3-Oxo-20(29)-lupen-28-oic acid. It is a known

compound called betulonic acid. Its structure shows it to be a ketone of betulinic acid together with which it was isolated. It has been reported from *Avicennia germinana* the black mangrove [13] and its derivative [14]. So far there has been no NMR data reported for this compound in literature

Table 4. *In-vitro* antibacterial results of isolated compounds

Compound (10 mg/ml)	**Strains**	**Activity expressed in mm of inhibition zones diameter (n=3)**
*3β-Hydroxylup-20(29)-ene (**1**)*	*Salmonella typhi*	5.0 ± 0.01
	Escherichia coli	5.0 ± 0.01
	Pseudomonas aeruginosa	24.0 ± 0.03
	Methicilin resistant *staphylococcus aureus*	0.0
*3β-Hydroxylup-20(29)-en-28-oic acid (**2**)*	*Salmonella typhi*	0.0
	Escherichia coli	0.0
	Pseudomonas aeruginosa	7.0 ± 0.01
	Methicilin resistant *staphylococcus aureus*	0.0
*3-oxo-lup-20(29)-en-28-oic acid (**3**)*	*Salmonella typhi*	0.0
	Escherichia coli	0.0
	Pseudomonas aeruginosa	10.7 ± 0.02
	Methicilin resistant *staphylococcus aureus*	0.0
Standard drug Gentamycin 10µg/disc	*Salmonella typhi*	5.6 ± 0.01
	Escherichia coli	8.2 ± 0.01
	Pseudomonas aeruginosa	16.0 ± 0.01
	Methicilin resistant *staphylococcus aureus*	10.5 ± 0.01

3.3 In-vitro antimicrobial activity test results of isolated pure compounds

The three compounds isolated and characterized were 3β-Hydroxylup-20(29)-ene (**1**), 3β-Hydroxylup-20(29)-en-28-oic acid (**2**), and 3-Oxo-lup-20(29)-en-28-oic acid (**3**). Table 4 shows the results of antibacterial tests for pure compounds isolated at 10 mg/mLconcentrations. The pure compounds exhibited undetectable antifungal activity. The compounds showed strongest activity against *Pseudomonas aeruginosa* 24.0 ± 0.03 mm (1), 7.0 ± 0.01 mm (2), 10.7 ± 0.02 mm (3), and low or no activity against the other bacterial strains. Whereas the zones of inhibition due to the standard drug Gentamycin were clear, zones due to these compounds were not as clear (Figure 3). These results are consistent with the already published data on lupeol, which is known to show antiinflammatory and antiarthritic activities [8, 15], inhibits growth of highly aggressive human metastatic melanoma cells [16], Antiangiogenic activity [17], antioxaluric and anticalciuric activity [18]. Lupeol derivatives have also shown to have antimalarial activity [19, 20]. The Anti-Human Immunodeficiency Virus Activity of betulinic acid derivative has also been reported [21]. The Effect of lupeol, betulinic on protein phosphorylation has been reported [22] and the antimalarial activity of betulinic acid reported from *Triphyophyllum peltatum* [11].

The activity of betulonic acid is being reported for the first time. The activity has been reported in comparison with isolated samples of lupeol and betulinic acid from the sponge A.infundibuliformis. Previously Boc-lysinated-betulonic acid derived from betulinol, showed a highly specific anti-prostate cancer activity in vitro cell cultures [14].

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