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A novel antimicrobial phenanthrene alkaloid from *Bryopyllum pinnatum*

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ABSTRACT

From the ethanolic extract of the leaves of Bryophyllum pinnatum (Syn. B. Calcinum Kalanchoe pinnata) a versatile Nigeria medicinal plant was isolated a phenenthrene alkaloid identified as 1-ethanamino 7 Hex-1-yne-5^{I}-one phenanthrene. The structure was elucidated using NMR spectroscopy in combination with IR, UV, and MS spectral data. Antimicrobial studies showed that the isolated compound successfully inhibited Psuedomonas aeruginosa, Klebsiella Pneumonia, Staphylococcus aureus, Escherichia coli, Candida albicans and Aspergillus niger. This result authenticates the use of Bryophyllum pinantum in phytomedicine for disease prevention and treatment of infections.

Keywords: *Bryophyllum pinnatum*, Phenanthrene alkaloid, Antibacterial activity, Antifungal activity, Phytomedicine.

INTRODUCTION

As part of our project on the study for the use of Nigeria medicinal plants for drug discovery, [1, 2]. We have previously described two novel flavonoids isolated from *B. pinnatum* (Syn. B. Calcinum Kalanchoe). These flavonoids have remarkable biological activities, including inhibitory effects on enzymes, modulatory effect on some cell types, protection against allergies, antibacterial, antifungal, antiviral, anti-malarial, antioxidant, anti-inflammatory and anti-carcinogenic properties. [3,4]. *B. pinnatum* has been noted for its versatile medicinal value in traditional medicine in Nigeria. It has been employed for the treatment of earache, burns, abscesses, ulcer, insect bites, whitlow, diarrhea and lithiasus. [1,5,7]. In southern Nigeria, the

herb is used to facilitate the dropping of the placenta of newly born baby [1]. The lightly roasted leaves are used externally for skin fungus and inflammations and the leaf infusion is an internal remedy for fevers [8]. The herb is considered a sedative, wound-healer, diuretic and cough suppressant [8]. It is used for the treatment of all sorts of respiratory conditions; asthma, cough and bronchitis. *B. pinnatum* is an active ingredient as the decoction is used presently by herbalist in Eastern Nigeria for the treatment of gonorrhea, genital, vaginal and muscosal candidiasis as well as asthma and cough [1,8]. Several studies [11,14] have documented the scientific basis for the efficacy of plants in phytomedicine. The study seeks to ascertain the usefulness of *B. pinnatum* in the treatment of infection conditions caused by common pathogens. The study involves the isolation, structural elucidation and characterization of the bioactive constituents in the plant and consequently evaluates the antibacterial and antifungal activity against some pathogenic organism for possible development of new drugs for the prevention and treatment of infections.

EXPERIMENTAL SECTION

General Experimental Procedure

The IR spectrum was determined on Thermo Nicolet Nexus 470 FTIR spectrometer. The ^{1}H and ^{13}CNMR spectra were recorded on a Bruker Avnce 400FT NMR spectrometer using TMS as internal standard. Chemical shift are expressed in part per million (ppm).

LC-ESIMS spectra were determined in the positive ion mode on PE-Biosynthesis API 165 single quadruple instruments. HRESIMS (Positive ion mode) spectrum was recorded on a thermo Finniga Mat 95XL mass spectrometer Column chromatography was carried out with silica gel (200-300 mesh) and to monitor the preparative separations analytical thin layer chromatography (TLC) was performed at room temperature on pre-coated 0.25mm thick silica gel $60F_{254}$. aluminum plates 20 x 20 cm Merck Darmstadt, Germany. General UV spectrum were recorded on Shimadzu 160A spectrophotometer. Reagents and solvents like ethanol, chloroform, diethyl ether, hexane were all of analytical grades and procured from Merck Darmstadt, Germany. TLC aluminum sheets, silica gel $60F_{254}$ was also purchased from Merck. The nutrient agar was purchased form Scharlan Chemic APHA, Spain.

Plant materials

Fresh leaves of *B. pinantum* were harvested from the Botanical garden of Michael Okpara University of Agriculture Umudike, Nigeria on 6th April 2007. The plant samples (leaves and stems) were identified by Dr. A Nmeregini of Taxonomy Section, Forestry Department of the University. A voucher specimen No BP/122 has been deposited at the forestry Department Herbarium of the University.

Extraction and Isolation of Plant Material

Plant materials were treated and analyzed at the Chemistry laboratory, Michael Okpara University of Agriculture Umudike, Nigeria. Mature leaves (1kg) of *B. pinnatum* were dried on the laboratory bench for 10 days. The dry samples were milled and ground into powered (860g) using Thomas Willey machine (Model 5 USA). The powdered plant samples (500g) were packed into a Soxhlet apparatus (2L) and extracted exhaustively with 1000 ml ethanol for 24h. The ethanol extract was concentrated using rotary evaporator at 45^{O}C and left on the laboratory

bench for 2 days to obtain a dry dark green pigment (68g). The column was packed with silica gel and the dark green pigment (40g) of the isolated plant material was placed on top of silica gel and eluted with methanol: chloroform: petroleum ether (20:30:50) to afford three fractions comprising compound 1 (dark green pigment 0.52g Rf 0.2965); Compound 2 (dark green pigment 0.48g Rf 0.3906) and compound 3 (yellow pigment 0.45g Rf 0.3012). Compound 1 and 2 have earlier been reported. Compound 3 was crystallized from hexane (0.42 mg Rf 0.3012 IR Vmax 1744 cm^{-1} (C=O), 1483 cm^{-1} (C=C aromatic) UV λmax MeOH: 325nm HREIMS m/z 312.3021 [M+] calculated for $C_{22}H_{19}ON$ (m/z 313) and m/z 57.0701 base peak calculated for C_3H_5O (m/z 57). The ^1H and ^{13}CNMR of compound 3 were determined.

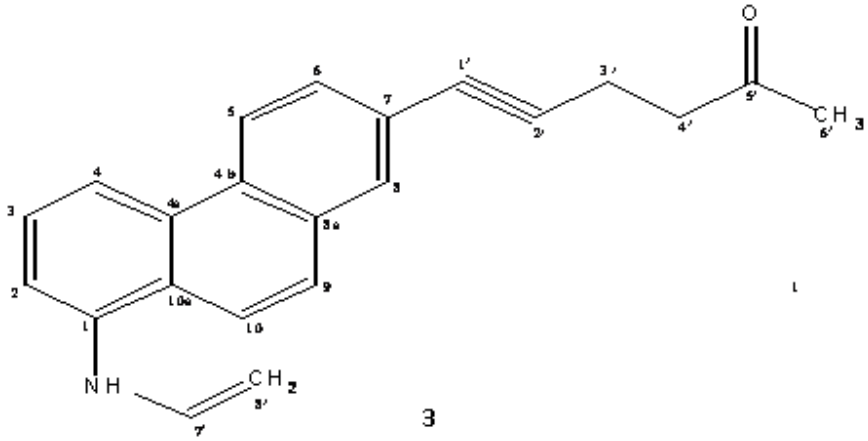
Bioassay

The *in vitro* antimicrobial activity of compound 3 were carried out for 24 hrs culture of four selected bacteria and two fungi. The bacteria used were three gram-negative organism comprising *Escherichia coli, Pseudomonas aerugonosa* and *Klebisella pneumonia and a gram positive Staphylococcus aureus*. The two fungi used were *Candidia albicans* and *Aspergillus niger*. All the test organisms are clinical isolates of the pathogens obtained from Federal Medical Centre (FMC) Umuahia, Nigeria. Cultures were brought to laboratory conditions by resuscitating the organism in buffered peptone broth (Scharlan Chemie) and thereafter nutrient agar (Peptone 5g/l and meat 3g/l) and inoculated at 37^OC for 24 hrs. The antimicrobial activity was performed by filter paper disc diffusion technique. The medium (7g nutrient agar in 250ml distilled water, autoclaved at 115^OC. 20ml of the medium was poured into a sterile Petri dish and allowed to solidify. It was observed for contamination. The sterility of the medium was tested using autoclave at 121^OC 15Psi for 15 mins. Nutrient agar (Scharlan Chemie) was used for bacteria while subourands agar (Scharlan Chemie) was used for fungi. The isolated sample (Compound 3) was dissolved in 1ml of absolute ethanol and made up to 10ml with distilled water to give a concentration of 100 mg/ml (10% dilution). A colony of each organism was sub-cultured on nutrient broth which contains peptone (5g/l) and meat extract of (3g/l) and incubate aerobically at 37^OC for 8 hrs. 30 mls of the nutrient broth was used to flood the agar plates. A sterilized Whatman No 1 filter paper disc soaked in compound 3 (0.02ml) was used to test for the sensitivity or antimicrobial effect of the compound. The plates were incubated at 37^OC for 24 hrs. After incubation, plates were observed for zones of inhibition (in mm diameter). The minimum inhibitory concentration was determined. Plates containing agar medium without the addition of compound 3 were used as control. Each test tube was replicated three times.

RESULTS AND DISCUSSION

Compound 3 was obtained as yellow pigment. The compound showed IR peak at 2954, 2923 and 2852 cm^{-1} for aliphatic CH stretching. The IR spectrum also displayed peaks at 1744 cm^{-1} (C=O), 1463 cm^{-1} (C=C aromatic) and 1167cm^{-1} (N-H) stretching absorptions. The UV absorption occurred at 325nm representing a phenanthrene nucleus[10]. The Compound 3 was assigned the molecular formular m/z 312.3021 calculated for $C_{22}H_{19}ON$ (m/z 312) with base peaks at m/z 57.0701 calculated for C_3H_5O (m/z 57). Apart from the molecular ion peak and base peak, the high resolution mass spectrum gave fragment peaks at m/z 41.0393 and 43.0547 corresponding to amine detachment at C_2H_4N (m/z 42) and carbonyl alpha cleavage at C_2H_3O (m/z 43) respectively. Also alpha cleavage from the phenathrene nucleus resulted to the peak at

m/z 47.1014 calculated for C_6H_{10} (M/z 95). The fragmentation pattern of compound 3 is shown n figure 1.



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The ^{1}HNMR spectrum showed the presence of olefinic proton as a doublet at ∂H 4.2837 (2Hd) and triplet at ∂H 5.3496 (1Ht). The nine aromatic protons produce peaks at ∂H 7.2566-7.7161. The methylene protons at C_3 and C_4 produce peaks at ∂H 1.2548 (2Ht) and 6.921 (2Ht) respectively. The methylene protons at C_6 produce the peaks at ∂H 2.3860. Analysis of the ^{13}CNMR spectrum showed the carbonyl carbon at ∂C 173.272 with fourteen aromatic carbon which showed their peaks from ∂C 129.672-132.505. There are two olefinic carbons at ∂C 124.294 and ∂C 127.914 while the acetylene carbons appeared at ∂C 77.347 and 77.029. These data were consistent with phenathrene frame work [9]. All the protons and carbon resonances were assigned as reported in Table 1 by careful analysis of ^{1}HNMR and ^{13}CNMR spectra. This analysis confirmed compound 3 to be a phenantherene alkaloid (1-ethenamino-7-Hex-1-yne-5-one phenanthere) as the measured spectral properties are in accordance with the available literature [9,10]. This compound may be one of the physiologically active compounds of *B. pianntum*.

Table 2 shows the antimicrobial activity of compound 3 isolated from *B. pinnatum* leaves. The compound has activity against *Staphylococcus auresu, Psuedomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Candida albicans*. *C. albicans* and *A. niger* are fungi. P. aeruginosa, *E. coli* and *K. pneumonia* are gram negative bacteria while *Staphylococcus aureus* is a gram positive bacterium. In general, the order of activity against the bacteria was *Staphylococcus aureus > Psuedomonas aeruginosa> Klebsiella pneumonia> Escherichia coli*. This results agreed with the findings of Egeronu and Mokwe[8] who reported that the leaf of *B. pinnatum* demonstrated significant antibacterial activity towards the above organisms, including several strains of multi-drug resistant bacteria. It can be concluded that the compound has activity against both gram positive and gram negative bacteria as well as fungi. The above results led credence to the common use of *B. pinnatum* in phytomedicine as an antibacterial and antifungal crude drug in Nigeria.

Table 1: 1H (400 MHz) and 13CNMR (75MHz) of Compound 3

Position	δC Chemical shift	Carbon	δH Chemical shift		Multiciplicity	proton
1	132.505	C				
2	130.862	CH	7.7161	CH	1Hd	
3	130.228	CH	7.7072	CH	1Ht	
4	130.862	CH	7.7161	CH	1Hd	
4a	130.031	C				
4b	129.692	C				
5	130.228	CH	7.6836	CH	1Hd	
6	130.862	CH	7.6836	CH	1Hd	
7	130.031	C				
8	129.698	CH	7.2566	CH	1Hs	
8a	129.692	C				
9	130.862	CH	7.5369	CH	1Hd	
10	130.228	CH	7.5286	CH	1Hd	
1^{1}	77.347	C				
2^{1}	77.029	C				
3^{1}	14.073	CH_2	1.2548	CH_2	2Ht	
4^{1}	14.117	CH_2	0.9215	CH_2	2Ht	
5^{1}	173.272	C=O				
6^{1}	24.888	CH_3	2.3860	CH_3	3Hs	
7^{1}	124.294	CH	5.3496	CH	1Ht	
8^{1}	127.914	CH	4.2837	CH_2	2Hd	
			4.1660	NH	1Hs	

Table 2: Diameter of zones of inhibition (mm) of compound 3 isolated from *Bryophyllum pinnatum*

Pathogens	Concentration of compound 3 mg/ml					
	100.0	**50.0**	**25.0**	**12.5**	**6.25**	*MIC* (mg/ml)
	Zone diameter of inhibition (mm)					
Staphyllococus aureaus	7.1 ± 0.01^{a}	2.5 ± 0.01^{b}	-	-	-	50
Escherichia coli	1.0 ± 0.01^{c}	-	-	-	-	100
Pseudomonas aeruginosa	3.2 ± 0.01^{b}	1 ± 0.01^{c}	-	-	-	50
Klebsiella pnuemonia	2.1 ± 0.01^{c}	-	-	-	-	100
Aspergillus niger	2.0 ± 0.01^{c}	-	-	-	-	100
Candida albicans	3.0 ± 0.01^{b}	-	-	-	-	100

Values are mean ± standard deviation of triplicate determinations, values with superscript that are the same in each row are not significantly different ($p<0.05$)
- = No inhibition

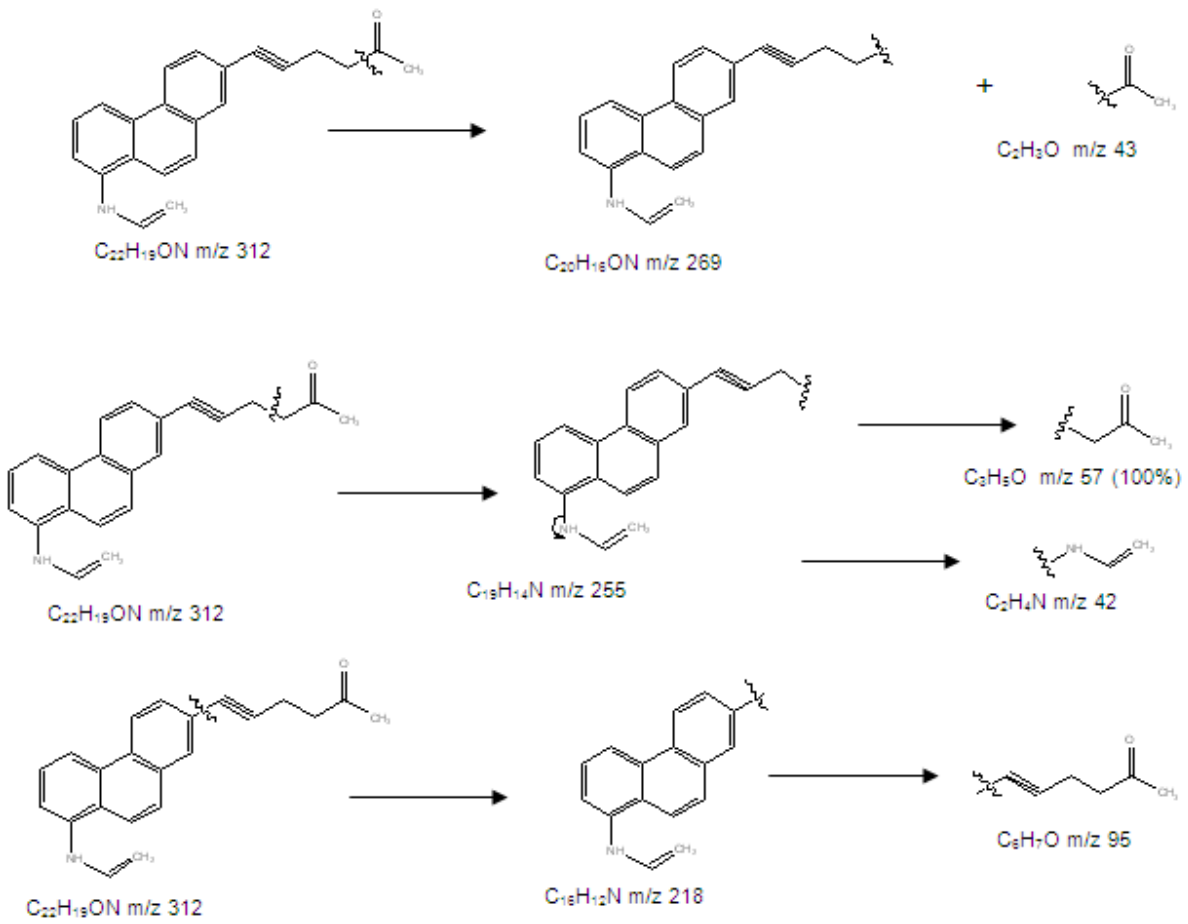

Figure 1: Fragmentation pattern of compound 3

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