A Novel Antimicrobial Phenanthrene Alkaloid from *Bryophyllum pinnatum*

DONATUS EBERE OKWU* and FRED UCHENNA NNAMDI

Department of Chemistry
Michael Okpara University of Agriculture Umudike
P.M.B 7267 Umuahia Abia State, Nigeria
okwudonatus@yahoo.com

Received 3 June 2009; Accepted 1 August 2009

Abstract: Phenanthrene alkaloid identified as 1-ethanamino 7 hex-1-yne-5'-one phenanthrene was isolated from the ethanolic extract of the leaves of *Bryophyllum pinnatum* (syn. *B. calinum kalanchoe pinnata*) a versatile Nigeria medicinal plant. The structure was elucidated using NMR, 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 a 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 *Bryophyllum pinnatum* (syn. *B. calinum 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 diarrhea⁵⁻⁷. In southern Nigeria, the herb is used to facilitate the dropping of the placenta of newly born baby¹. The lightly roasted leaves are used externally for skin fungus and inflammations and the leaf infusion is an internal remedy for fevers⁸. The herb is considered a sedative, wound-healer, diuretic and cough suppressant⁸. It is used for the treatment of all sorts of respiratory conditions; asthma, cough and bronchitis. *Bryophyllum 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^{9,10} have documented the scientific basis for the efficacy of plants in phytomedicine. The study seeks to ascertain the usefulness of *Bryophyllum 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

The IR spectrum was determined on Thermo Nicolet Nexus 470 FTIR spectrometer. The ¹H and ¹³C NMR 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 0.25 mm thick silica gel 60F₂₅₄, 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 60F₂₅₄ was also purchased from Merck. The nutrient agar was purchased form Scharlan Chemic APHA, Spain.

Plant materials

Fresh leaves of *Bryophyllum 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 (1 kg) of *Bryophyllum pinnatum* were dried on the laboratory bench for 10 days. The dry samples were milled and ground into powder (860 g) using Thomas Willey machine (Model 5 USA). The powdered plant samples (500 g) were packed into a Soxhlet apparatus (2 L) and extracted exhaustively with 1000 mL ethanol for 24 h. The ethanol extract was concentrated using rotary evaporator at 45 °C and left on the laboratory bench for 2 days to obtain a dry dark green pigment (68 g). The column was packed with silica gel and the dark green pigment (40 g) 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 0.52 g R_f 0.2965); Compound 2 (dark green pigment 0.48 g R_f 0.3906) and compound 3 (yellow pigment 0.45 g R_f 0.3012). Compound 1 and 2 have earlier been reported. Compound 3 was crystallized from hexane (0.42 mg R_f 0.3012 IR V_{max} 1744 cm⁻¹ (C=O), 1483 cm⁻¹ (C=C aromatic) UV λ_{max} MeOH: 325 nm HREIMS m/z 312.3021 [M+] calculated for C₂₂H₁₉ON (m/z 313) and m/z 57.0701 base peak calculated for C₃H₅O (m/z 57). The ¹H and ¹³C NMR of compound 3 were determined.

Bioassay

The *in vitro* antimicrobial activity of compound **3** were carried out for 24 h culture of four selected bacteria and two fungi. The bacteria used were three gram-negative organism comprising *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* and a gram positive *Staphylococcus aureus*. The two fungi used were *Candidia albicans* and *Aspergillus niger*. All the test organisms were clinical isolated 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 5 g/L and meat 3g/L) and inoculated at 37 °C for 24 h. The antimicrobial activity was performed by filter paper disc diffusion technique. The medium (7g nutrient agar in 250 mL distilled water, autoclaved at 115 °C. 20 mL 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 °C 15 Psi for 15 min. 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 1 mL of absolute ethanol and made up to 10 mL 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 (5 g/L) and meat extract of (3 g/L) and incubate aerobically at 37 °C for 8 h. 30 mL of the nutrient broth was used to flood the agar plates. A sterilized Whatman No 1 filter paper disc soaked in compound 3 (0.02 mL) was used to test for the sensitivity or antimicrobial effect of the compound. The plates were incubated at 37 °C for 24 h. 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 1167 cm^{-1} (N-H) stretching absorptions. The UV absorption occurred at 325 nm representing a phenanthrene nucleus¹¹. The Compound **3** was assigned the molecular formula m/z 312.3021 calculated for $\text{C}_{22}\text{H}_{19}\text{ON}$ (m/z 312) with base peaks at m/z 57.0701 calculated for $\text{C}_3\text{H}_5\text{O}$ (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 $\text{C}_2\text{H}_4\text{N}$ (m/z 42) and carbonyl alpha cleavage at $\text{C}_2\text{H}_3\text{O}$ (m/z 43) respectively. Also alpha cleavage from the phenanthrene 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.

Figure 1. Fragmentation pattern of compound 3

The 1H NMR spectrum showed the presence of olefinic proton as a doublet at δH 4.2837 (2Hd) and triplet at δH 5.3496 (1Ht). The nine aromatic protons produce peaks at δH 7.2566-7.7161. The methylene protons at C_3 and C_4 produce peaks at δH 1.2548 (2Ht) and 6.921 (2Ht) respectively. The methylene protons at C_6 produce the peaks at δH 2.3860. Analysis of the ^{13}C NMR spectrum showed the carbonyl carbon at δC 173.272 with fourteen aromatic carbon which showed their peaks from δC 129.672-132.505. There are two olefinic carbons at δC 124.294 and δC 127.914 while the acetylene carbons appeared at δC 77.347 and 77.029. These data were consistent with phenanthrene frame work¹². All the protons and carbon resonances were assigned as reported in Table 1 by careful analysis of 1H NMR and ^{13}C NMR spectra. This analysis confirmed compound 3 to be a phenanthrene alkaloid (1-ethenamino-7-Hex-1-yne-5-one phenanthrene) as the measured spectral properties are in accordance with the available literature^{11,12}. This compound may be one of the physiologically active compounds of *B. pinnatum*.

Table 2 shows the antimicrobial activity of compound 3 isolated from *B. pinnatum* leaves. The compound has activity against *Staphylococcus aureus*, *Pseudomonas 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* > *Pseudomonas aeruginosa* > *Klebsiella pneumonia* > *Escherichia coli*. This results agreed with the findings of Egeronu and Mokwe⁸ 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 *Bryophyllum pinnatum* in phytomedicine as an antibacterial and antifungal crude drug in Nigeria.

Table 1. ¹H (400 MHz) and ¹³C NMR (75 MHz) of compound **3**

Position	δC			δH	
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 ^l	77.347	C			
2 ^l	77.029	C			
3 ^l	14.073	CH ₂	1.2548	CH ₂	2Ht
4 ^l	14.117	CH ₂	0.9215	CH ₂	2Ht
5 ^l	173.272	C=O			
6 ^l	24.888	CH ₃	2.3860	CH ₃	3Hs
7 ^l	124.294	CH	5.3496	CH	1Ht
8 ^l	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						
<i>Staphylococcus aureus</i>	7.1 ± 0.01 ^a	2.5 ± 0.01 ^b	-	-	-	50
<i>Escherichia coli</i>	1.0 ± 0.01 ^c	-	-	-	-	100
<i>Pseudomonas aeruginosa</i>	3.2 ± 0.01 ^b	1 ± 0.01 ^c	-	-	-	50
<i>Klebsiella pneumonia</i>	2.1 ± 0.01 ^c	-	-	-	-	100
<i>Aspergillus niger</i>	2.0 ± 0.01 ^c	-	-	-	-	100
<i>Candida albicans</i>	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

Acknowledgment

We are indeed grateful to Miss Nkechi Ibisi for her kind assistance in running the spectra in China and to Dr. A. Nmerigini for authenticating the plant samples.

References

1. Okwu D E, *J Med Aromat Plant Sci Biotechnol.*, 2007, **1(1)**, 90-96.
2. Okwu D E, *J Med Aromat Plant Sci Biotechnol.*, 2007, (1), 97-102.
3. Amnlou M, Ariae S and Farsam H, *J Med Aromat Plant Sci.*, 2005, **27**, 469-475.
4. Veitch N C and Grayer R J, *Natural Product Reports* 2007, **21**, 539-573.
5. Agoha R C, Medicinal plants of Nigeria. Offset Arakkenji faculficider wiskunde. The Netherlands, pp 33-41.
6. Ofokansi K C, Esimone C O and Anele C K, *Plant Root Research J.*, 2005, **9**, 23-27.
7. Okwu DE and Josiah C, *African J Biotechnol.*, 2006, **5(4)**, 357-361.
8. Egereonu U U and Mokwe N R, *J Chem Soc Nigeria*, 2005, **30(2)**, 192-196.
9. Okoli C.O, Akah P A and Okoli A C, *BMC Compliment Altern Med.*, 2001, **7**, 24-30.
10. Okwu D E and Morah F N I, *J Appl Sci.*, 2007b, **7(2)**, 306-309.
11. Kemp N. Organic spectroscopy. English Language Book Society, Macmillain Hong Kong, 1987, 207.
12. Okwu D E, Morah F N I, Anam E M, *J Chem Soc Nigeria*, 2006, **31(1-2)**, 19-21