ISOLATION AND ANTIMICROBIAL ANALYSIS OF A STEROIDAL TERPENE FROM THE BUTANOL FRACTION OF *BYROPHYLLUM PINNATUM* (LAM.) OKEN

Olawale H. Oladimeji[a]* and Kufre E. Eberefiak[a]

Keywords: Butanol, chromatography, terpene, bacteriostatic, *Bryophyllum pinnatum*.

Bryophyllum pinnatum (Lam.) Oken is a plant used in treatment/management of ear-ache, cough, gastro-intestinal disorders and inflammation. Prior to this study, reports of the isolation of cardiac glycosides from the ethyl-acetate fraction of the plant abound. However, very scanty or no literature exists on other organic fractions from where chemical constituents could also be obtained. Hence, the chemical and biological properties of the butanol fraction of the plant were studied. The silica gel column chromatography of the fraction led to a steroidal terpene whose identity has been revealed to be 3-hydroxy-(3β, 17β)-spiro(androst-5-ene-17,1′-cyclobutan)-2′-one using the MS and IR spectral techniques. This compound was strongly bacteriostatic against *Staphylococcus aureus* and *Candida albican* sbut recorded no activity against *Escherichia coli*.

* Corresponding Authors
Tel: +2347038916740, +2348180035112, +2348173486285
E-Mail: wale430@yahoo.co.uk, hakeemoladmeji@uniuyo.edu.ng
[a] Department of Pharmaceutical & Medicinal Chemistry, Faculty of Pharmacy, University of Uyo, Uyo, Nigeria.

Introduction

Bryophyllum pinnatum (Lam.) Oken syn, (*Cotyledon pinnatum*, *Crassula pinnatum* and *Kalanchoe pinnatum)* is known as miracle leaf or life plant grows as succulent perennial herb in the tropical climatic zones of Africa, Latin America and Asia. However, the plant is now cultivated on a large scale and sold to the pharmaceutical industry for economic benefits.[1] The plant is used in the treatment of ear-ache, cough, gastro-intestinal disorders and leucorrhoea. Furthermore, extracts of the plant are employed in the treatment /management of inflammations such as cardiac problems, wounds, sores, diabetes, liver problems, certain cancers and kidney troubles.[2] Previous chemical investigations of the plant have led to the isolation of three cardiac glycosides namely, bryophillia A, bersaldegenin -3-acetate and bryophillin C while the fractionation of ethyl-acetate marc yielded seven kaempferol rhamnosides.[3] The present study was carried out to isolate chemical constituent(s) in the butanol fraction which showed a higher antimicrobial activity than that by the ethyl-acetate fraction[4] and also screen the compound(s) for possible antibacterial and antifungal activities.

Experimental

Collection of plant material

The fresh leaves of *B. pinnatum* were collected in the month of July, 2016 from inside the University of Uyo Main Campus, Akwa Ibom State, Nigeria. The plant had previously been identified[4] and a voucher specimen of the plant (No. H 045) was deposited in the Herbarium Unit of the Faculty of Pharmacy. Immediately after collection, the leaves were dried in a laboratory oven at 40 ^{0}C for 48 h and the resultant material powdered on an electric mill (Uniscope, England).

Extraction and isolation

The dried powder (0.5 kg) was exhaustively extracted with 50 % EtOH (3 x 5 L) at room temperature (27±2 ^{0}C) for 72 h. The resultant crude extract mixture was filtered, concentrated *in vacuo* on a rotary evaporator, when 47 g of dried crude extract was obtained and stored in a desiccator prior to further use.

Subsequently, 7.8 g of the extract was partitioned using H_2O: BuOH (3 x 200 mL). The combined butanol fractions were evaporated to dryness to give a brown solid residue. Then 0.8 g of the fraction was chromatographed on a silica gel 254 column (Pyrex, USA; 8 g pre-swollen in 100 % toluene; 2 g concentration zone + 6 g separation zone; 10.2 x 4 cm) and eluted with a gradient of 20 % $(CH_3)_2CO$: toluene (36 mL), 40 % $(CH_3)_2CO$: toluene (36 mL), 60 % $(CH_3)_2CO$: toluene (36 mL) and 80 % $(CH_3)_2CO$: toluene (36 mL). Fractions of 6 mL each were collected and monitored on silica plates in $(CH_3)_2CO$:toluene:H_2O (10:20:1) using $FeCl_3/CH_3OH$ and vanillin-H_2SO_4 as spray reagents. Hence, fractions with similar TLC characteristics (R_f values, reaction with vanillin-H_2SO_4 spray) were bulked and dried. Three sub-fractions coded KF-1, KF-2 and KF-3 were obtained. Further TLC examinations of these sub-fractions in $(CH_3)_2CO$:toluene:H_2O (10:20:1) and $(CH_3)_2CO$:EtOAc (35:65) indicated a single spot in **KF-2**(yellow compound; R_f(0.57); 43 mg) while the others showed multi-component TLC profiles and were not processed any further in the course of this study.

Structural elucidation

The mass spectrum of the compound was run on Kratos MS 80 (Germany) while the IR analyses were done on Shimadzu FTIR 8400S (Japan).

Table 1. Antimicrobial screening of crude extract, butanol fraction, KF-2 at different concentrations on test microbes.

Test microbe	CE	BT	KF-2	DW	SP	NY
S. aureus (ATCC 21824)	11	16	18	5	27	5
E. coli (ATCC 23523)	5	5	5	5	31	5
C. albicans (NCYC 106)	10	12	15	5	5	28

CE= Crude ethanolic extract (20 mg mL^{-1}), BT = Butanol fraction (10 mg mL^{-1}), KF-2 (2 mg mL^{-1}), DW = Deionised water, SP = Streptomycin (10 μg mL^{-1}), NY = Nystatin (1 mg mL^{-1}).

Antimicrobial tests

The micro-organisms used in this study were limited to three *viz*: one Gram(+), one Gram(-) and a fungus. *Staphylococcus aureus* (ATCC 21824), *Escherichia coli* (ATCC 23523) and *Candida albicans* (NCYC 106) were clinically isolated from specimens of diarrheal stool, abscesses, necrotizing fascitis, urine and wounds obtained from the Medical Laboratory, University of Uyo Health Centre, Uyo. The clinical isolates were collected in sterile bottles, identified and typed by convectional biochemical tests.[5,6] These clinical microbes were then refrigerated at -5 ^{0}C prior to use. The agar plates used were prepared by adhering to the manufacturer's instructions. The media and plates were sterilized in an autoclave at 121 °C for 15 min. The hole-in-plate agar diffusion method was used observing standard procedure with Nutrient Agar-CM003, Mueller-Hinton-CM037 (Biotech Limited, Ipswich, England) and Sabouraud Dextrose Agar (Biomark, India) for the bacteria and fungus respectively. The inoculum of each microorganism was introduced into each petri-dish (Pyrex, England). Cylindrical plugs were removed from the agar plates by means of a sterile cork borer (Simax, India) to produce wells with diameter of approximately 5 millimeters. The wells were equidistant from each other and the edge of the plate.[7, 8] Concentrations of 20 mg mL^{-1} of crude extract, 10 mg mL^{-1} of butanol fraction, 2 mg mL^{-1} of KF-2 were introduced into the wells. Different concentrations of 10 μg mL^{-1} Streptomycin (Orange Drugs, Nigeria), 1 mg mL^{-1} of nystatin (Gemini Drugs, Nigeria) and deionized water were also introduced into separate wells as positive and negative controls respectively.[9-13] The experiments were carried out in triplicates. The plates were labelled on the underside and left at room temperature for 2 h to allow for diffusion. The plates were then incubated at 37±2 ^{0}C for 24 to 48 h. Zones of inhibition were measured in mm with the aid of a ruler.

Results and Discussions

Spectroscopic data

KF-2**:** $C_{22}H_{32}O_2$; yellow compound; R_f (0.57). MS [ES+-MS] *m/z* 328 $[M]^+$ (3.14 %), 285 $[M\text{-}2CH_3\text{-}OH+4]^+$ (5.25%), 273$[M\text{-}2CH_3\text{-}CO+3]^+$ (6.04%), 271 $[M\text{-}2CH_3\text{-}CO+1]^+$ (29.46%), 242 $[M\text{-}2CH_3\text{-}2CH_2\text{-}CO]^+$ (11.24 %) and 183 $[M\text{-}2CH_3\text{-}5CH_2\text{-}CO\text{-}OH]^+$ (100.00%). FTIR cm^{-1}: 913, 856 (alkyl substitution), 1612 (-C=C) and 3219 (-OH). The chemical structure of KF-2 was established by a combination of spectroscopic techniques as highlighted above. These data were matched with those in the library data of organic compounds. Furthermore, the obtained data were found to be consistent with those reported in literature.[14, 15] Therefore KF-2 has been identified to be 3-hydroxy-(3β,17β)-spiro(androst-5-ene-17,1′-cyclobutan)-2′-one. (Due to the nature of the matrix, many fragmented peaks appeared in the MS of the compound but those that are easily identifiable include $[M]^+$ at *m/z* 328 (3.14 %), while fragments at 285 (5.25 %), 273 (6.04 %) and 271(29.46 %) represent the losses of methyl groups and a hydroxy unit and methyl groups and carbonyl units from the molecular ion respectively. Furthermore, the ion a 242 (11.24 %), in addition to the excisions of methyl and carbonyl units also indicates the removal of methylene groups from the molecular matrix. However, the base peak at 183 (100.00 %) reveals the removal of methyl, carbonyl, methylene and hydroxy units from the $[M]^+$. The IR spectrum of KF-2 shows absorptions at 913, 856, 1612 and 3219 cm^{-1} indicating characteristic alkyl substitutions, endocyclic -C=C and -OH functional groups respectively. It is interesting to note that this steroidal terpene has been isolated from *Saccharium spontaneum* (L.) and *Rauwolfia vomitoria* (Afzel) using gas-chromatography/mass spectrophotometry.[14,15]

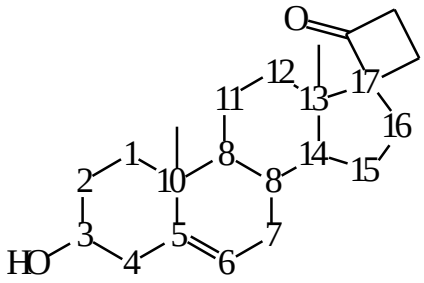

Figure 1. 3-Hydroxy-(3β,17β)-spiro(androst-5-ene-17,1′-cyclobutan)-2′-one

Antimicrobial Screening

The spectrum of microbes employed in the sensitivity tests was narrowed down to one Gram positive (*S. aureus*), Gram negative (*E. coli*) bacterial strains and fungus (*C. albicans*). The results displayed in the Table 1 show that the crude extract, butanol fraction and KF-2 are remarkably bacteriostatic against *S. aureus* and *C. albicans* while no activity was recorded against *E. coli*. Furthermore, the butanol fraction also inhibited the growth of *S. aureus* and *C. albicans* but was inactive against *E. coli*. Gram negative bacteria are well known for their unique resistance to antimicrobial agents. This resistance is believed to be due to the nature of the cell envelope of these organisms which unlike Gram positive organisms possess asophisticated

three-layered envelope which does not allow permeation of external agents.[16] Derivatization studies are currently on-going in our laboratories with the aim of improving on the observed activities.

Conclusion

The isolation of 3-hydroxy-(3β, 17β)-spiro(androst-5-ene-17,1′-cyclobutan)-2′-one is being reported for the first time from the butanol fraction of the plant. Hence, the compound is expected to serve as chemotaxonomic marker for this species and the genus, *Bryophyllum*in general. Furthermore, the results of the antimicrobial sensitivity tests lend some justification to the use of this plant especially in the treatment/management of bacterial disease. However, the compound and derivatives expected to be obtained in another study will be further screened against other bacterial and fungal strains with the aim of obtaining improved activity and widening the spectrum of antimicrobial activity.

Acknowledgements

We thank the Shimadzu Training Centre for Analytical Instruments (STC) Lagos, Nigeria for the assistance rendered in obtaining the spectra of the compounds. The expertise of E. Akpan, Principal Technologist, Pharmaceutical Microbiology Unit, University of Uyo, Nigeria in the conduct of the antimicrobial screening is warmly appreciated. The gifts of reagents by the Department of Pharmaceutical/Medicinal Chemistry, Faculty of Pharmacy, University of Uyo, Nigeria is heartily acknowledged.

References

[1]Hutchinson, J., Daniel, J. M., *Flora of West Tropical Africa*. 2nd edn., Crown Agents for Overseas Governments and Administrations, **1972**.

[2]Okezie, A., Agyrakwa, C. W., *A Handboook of West African Weeds*. International Institute of Tropical Agriculture Press, **1998**.

[3]Taylor, L., *The Healing Power of the Rain-forest Herbs*. http://raintree.com/coirama.htm, **2007.**

[4]Oladimeji, H. O., Nia, R., Edoho, E. J., *Afr. J. Pharm. Res. Dev*., **2006**, *2(2)*, 102-108.

[5]Gibson, L., Khoury, J., *Lett. Appl. Microbiol.*, **1986**, *3*, 127-129. https://doi.org/10.1111/j.1472-765X.1986.tb01565.x

[6]Murray, P., Baron, E., Pfaller, M., Tenover, F., Yolken, R., *Manual of Clinical Microbiology*. American Society of Microbiology Press, **1995**.

[7]Washington, J., *The Agar Diffusion Method*. In: *Manual of Clinical Microbiology*. 4th ed., American Society of Microbiology Press, **1995**.

[8]NCCLS, *Performance Standard for Antimicrobial Susceptibility Test*. 8th edition, Approved Standard, The Committee, **2003**.

[9]Oladimeji, H. O., *Chemical and Biological Studies on Cyathula prostrata (L.) Blume*. Ph.D. Thesis, University of Uyo, **2012**, 189.

[10]Oladimeji, H. O., Tom, E. U., Attih, E. E., *Eur. Chem. Bull.*, **2014**, *3(8)*, 788- 791. DOI: 10.17628/ecb.2014.3.788-791

[11]Oladimeji, H. O., Udo, F. I., *Eur. Chem. Bull*., **2014**, *3(11)*, 1060-1063. DOI: 10.17628/ecb.2014.3.1060-1063

[12]Oladimeji, H. O., Johnson, E. C., *J. Pharm. Biores*., **2015**, *12(1)*, 48-53. https://doi.org/10.4314/jpb.v12i1.7

[13]Oladimeji H. O., Attih, E. E., Udo, U. C., *Eur. Chem. Bull.*, 2016, 5(4), 147-150. DOI: 10.17628/ecb.2016.5.147-150

[14]Devi, J. A. I., Muthu, A. K., *Int. J. Pharmacy Pharm. Sci*., **2014**, *6(2)*, 755-759.

[15]Okereke, S. C., Ijeh, I., Arunsi, U. O., *Afr. J. Pharmacy Pharmacol.*, **2017**, *11(2)*, 25-31.

[16]Brown, M. R., *Pharm. J.*, **1975**, *215*, 239-242.

Received: 21.05.2017.
Accepted: 12.07.2017.