RESEARCH ARTICLE Am. J. PharmTech Res. 2013; 3(3) ISSN: 2249-3387

AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

Phytochemical and Pharmacological Studies of *Bryophyllum daigremontianum* (Raym.)

Shazid M. Sharker[1], Md. Khalid Hossain[1], Mohammad R. Haque[1], A. N. M. Hamidul Kabir[2], Choudhury M. Hasan[1], Mohammad A. Rashid*[1]

1.Phytochemical Research Laboratory, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh

2. *Department of Applied Chemistry & Chemical Engineering, Faculty of Engineering, University of Dhaka, Dhaka-1000, Bangladesh*

ABSTRACT

A total of 4 compounds have been isolated from a methanol extract of *Bryophyllum daigremontianum*. These compounds were identified as glut-5(6)-en-3β-ol (**1**), mixture of α-amyrin (**2a**) & β-amyrin (**2b**) and stigmasterol (**3**), by extensive analysis of NMR data and by comparison with published values. The crude extract was subjected to assay for antioxidant potential through determination of total phenolic content, membrane stabilizing and thrombolytic activities, which revealed potent antioxidant and moderate membrane stabilizing activities and inhibition of clot of rat's blood.

Keywords: *Bryophyllum daigremontianum*, glutenol, α- & β-amyrin, stigmasterol, antioxidant, phenolics.

*Corresponding Author Email: rashidma@du.ac.bd

Received 15 April 2013, Accepted 29 April 2013

Please cite this article in press as: Rashid MA. *et al.*, Phytochemical and Pharmacological Studies of *Bryophyllum Daigremontianum* (Raym.). American Journal of PharmTech Research 2013.

INTRODUCTION

Bryophyllum daigremontianum (Raym.) (Synonym: *Kalanchoe daigremontiana*, local name: Pathorkuchi, English name: Mother of Thousands, Family: Crassulaceae) is an annual perennial, glabrous, succulent herb. It is a native of western Indian ocean of Africa and naturalized elsewhere in the world[1,2] and is reputed for antitumor[3], antinociceptive, anti-inflammatory, antidiabetic[4], antimicrobial[5] and cytotoxic activities[6]. Previous phytochemical studies with *B. daigremontianum* revealed the occurrences of organic acids[7], alkaloids and tannins[8] and 11-oxo-epi-β-amyrin, 21-dehydrodesmo-sterol,3,4-dihydroxy-*cis*-cinnamic acid and *p*-hydroxy benzaldehyde [1].

As a part of our systematic investigations of medicinal plants of Bangladesh[9,10], we studied *B. daigremontianum* and we, herein, report the isolation of glut-5(6)-en-3β-ol (**1**), mixture of α-amyrin (**2a**) & β-amyrin (**2b**) and stigmasterol (**3**), as well as antioxidant, total phenolic contents, membrane stabilizing and clot lysis activities of this plant for the first time.

MATERIALS AND METHODS

General experimental procedure

^{1}H NMR spectra were recorded on a Bruker AMX-400 (400 MHz) instrument in deuterated chloroform and the δ values for ^{1}H spectra are reported relative to the residual non-deuterated solvent signal. DPPH and ascorbic acid were purchased from Sigma Chemical Co. Ltd., (St. Louis, MO, USA). Lyophilized Streptokinase vial (15,00,000 I.U.) were procured from Beacon Pharmaceutical Ltd. (Bangladesh). Kieselgel 60 H, silica gel (mesh 70-230), Silica gel F_{254}, Sephadex (LH-20) and all other chemicals, solvents and reagents were of analytical grade.

Plant material

The whole plants of *B. daigremontianum* were collected from Narsingdi district in November 2009 and was identified by the experts of Bangladesh National Herbarium, where a voucher specimen representing this collection has been deposited with DACB Accession number- 35469.

Extraction and isolation

The air-dried and powdered plant material (400 gm) was macerated in methanol in a large flask (2 liters) for 15 days with occasional shaking and stirring. The whole mixture was then filtered off through Whatman filter paper number 1 and the filtrate thus obtained was concentrated at 40°C with a rotary evaporator. A portion (5.0 gm) of the concentrated methanol extract was fractionated by the modified Kupchan partitioning protocol [11] to yield petroleum ether (750 mg), carbon tetrachloride (950 mg), chloroform (350 mg) and aqueous (950 mg) soluble materials.

An aliquot of the crude methanolic extract (10.0 gm) was chromatographed over vacuum liquid chromatographic (VLC), packed with silica gel 60H. The column was then eluted with petroleum ether, followed by mixtures of petroleum ether and ethyl acetate and finally with ethyl acetate and methanol mixtures of increasing polarities. Depending on the TLC behaviors, fractions 3A-4B and 6A-6B, eluted with 10-15%, and 25% ethyl acetate in petroleum ether respectively, were selected for further investigation.

Fractions 3A-4B were mixed together and further subjected to column chromatography over silica gel (Kieselgel, mesh 70-230) and the column was eluted with petroleum ether, followed by mixtures of petroleum ether and ethyl acetate of increasing polarities. A total of 40 fractions (each 20 mL) were collected. Fractions 31 and 37 provided crystalline mass which upon repeated washing with hexane followed by recrystallization yielded compound **3** (4.5 mg) and **1** (5.0 mg), respectively. VLC fractions 6A-6B were mixed and chromatographed over Sephadex (LH-20) using *n*-hexane- dichloromethane-methanol (2:5:1) and a total of 25 fractions were collected, each 25 ml. Preparative thin layer chromatography (PTLC) of column fraction 15, over silica gel using 5% ethyl acetate in toluene provided compounds **2a** and **2b** as a mixture (3.5 mg).

Free Radical Scavenging Activity

The free radical scavenging activities of the plant extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) were estimated by the method of Brand-Williams *et al* [15]. For this 2.0 mL of a methanol solution of the extract at different concentration were mixed with 3.0 mL of a DPPH methanol solution (20 µg/mL). The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract as compared to that of BHT with a UV-Vis spectrophotometer at 517 nm. Inhibition free radical DPPH in percent *(I%)* was calculated as follows:

$$(I\%) = (1 - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test material) and A_{sample} is the absorbance of the sample. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted by inhibition percentage against extract concentration.

Total phenolics content (TPC)

Total phenolics content of the methanolic extracts of *B. daigremontianum* was measured by employing the method described by Skerget *et al.*, [16] involving Folin-Ciocalteu reagent as oxidizing agent and gallic acid as standard [17]. The intensity of the color change is measured with a UV-Vis spectrophotometer at 760 nm [18] and the phenol content of the sample was expressed as mg of GAE (gallic acid equivalent)/100 gm of the dried extract.

Membrane stabilizing activity

The membrane stabilizing activity was assessed by using hypotonic solution and heat induced hemolysis of erythrocyte, by following the established protocol [19,20].

Thrombolytic activity

The thombolytic activity of all extractives was evaluated by the method of Prasad and collaborators [21,22] using streptokinase as standard.

RESULTS AND DISCUSSION

Compound **1** was obtained as white needles that melted at 210-211 °C, which was identical to that published for glut-5(6)-en-3β-ol [12,23]. The ^{1}H NMR spectrum of **1** displayed an olefinic proton signal at δ 5.63 (1H, *d*, *J* = 6.41 Hz) and an oxymethine proton resonance at δ 3.47 (1H, dd, *J* = 3.2, 2.4 Hz) which were assigned to H-6 and H-3, respectively. The ^{1}H NMR spectrum also showed eight methyl group resonances as singlets at δ 0.85, 0.95, 0.99, 1.00, 1.05, 1.08, 1.14 and 1.17. On the basis of the above spectral data, compound **1** was characterized as glut-5(6)-en-3β-ol. The identity of this compound as glut-5(6)-en-3β-ol (**1**) was further confirmed by comparison of its spectral data with reported values as well as by co-TLC with authentic sample [12,23].

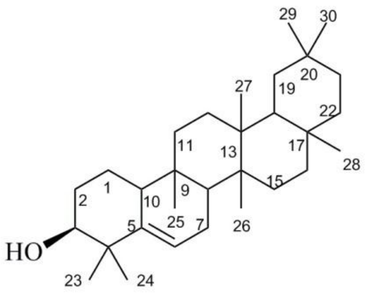
Glut-5(6)-en-3β-ol (1); White needles, m.p. 210-211 °C [lit.[12] 208-209 °C]; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.47 (dd, *J* = 3.2, 2.4 Hz, H-3), 5.63 (d, *J* = 6.41 Hz, H-6), 0.85 (3H, s), 0.95 (3H, s), 0.99 (3H, s), 1.00 (3H, s), 1.05 (3H, s), 1.08 (3H, s), 1.14 (3H, s) and 1.16 (3H, s).

Compound **2a** and **2b** were obtained as amorphous powder, with m.p. 177-180 °C. This was identical to that published for α-amyrin and β-amyrin [24].

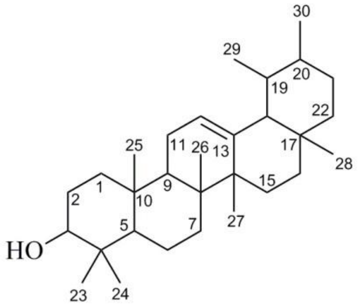
α-Amyrin (2a) & β-Amyrin (2b); Amorphous powder, m.p. 177-180 °C [lit.[13] 179-181 °C]; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.22 (1H, m, H-3), 5.19 (1H, t, *J* = 4.0 Hz, H-12), 0.80 (3H, s, H-23), 0.79 (3H, s, H-24), 1.00 (3H, s, H-25), 1.01 (3H, s, H-26), 1.08 (3H, s, H-27), 0.96 (3H, s, H-28), 0.88 (3H, br. s, H-29), 0.91 (3H, br. s, H-30) for α-amyrin (**2a**) and δ 3.22 (1H, m, H-3), 5.13 (1H, t, *J* = 4.0 Hz, H-12), 0.80 (3H, s, H-23), 0.79 (3H, s, H-24), 1.00 (3H, s, H-25), 1.01 (3H, s, H-26), 1.07 (3H, s, H-27), 0.96 (3H, s, H-28), 0.91 (3H, s, H-29), 0.88 (3H, s, H-30) for β-amyrin (**2b**).

Stigmasterol (3); Needle shaped crystals , m.p. 167-169°C [lit.[14] 170-171°C]; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.52 (m, H-3), 5.35 (1H, m, H-6), 0.67 (3H, s, Me-13), 1.01 (3H, s, Me-10), 0.91 (3H, d, *J* = 6.8 Hz, Me-20), 5.27 (1H, dd, *J* = 15.2, 8.0 Hz, H-22), 5.18 (1H, dd, *J* = 15.2, 7.2 Hz, H-23), 0.83 (3H, d, *J* = 6.0 Hz, Me-25), 0.85 (3H, d, *J* = 6.0 Hz, Me-25) and 0.82 (3H, t, *J* = 6.5 Hz, Me-28).

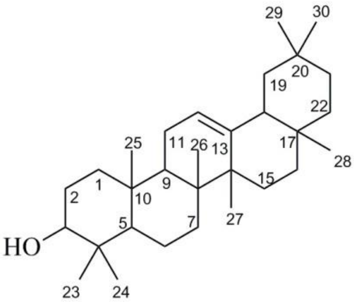
The ^{1}H NMR spectrum this sample exhibited two triplets at δ 5.17 ppm and δ 5.13 ppm (*J* = 4.0 Hz each) characteristic of olefinic proton (H-12) of α- and β-amyrin, respectively. These spectral features demonstrated that it was a mixture of two closely related triterpenoids. Apart from these, eight singlets and two broad singlets of -CH_3 protons were identified in the range δ 1.20 – 0.70 ppm. The singlet at δ 0.96 ppm (6H) indicated the presence of CH_3-28. That means that the analysed component was not an acid with the carboxylic group at C-17. The peaks of the methyl groups of α-amyrin were identified at δ 1.08 ppm (3H, s, CH_3-27), δ 0.91 ppm (3H, br. s; CH_3-30) and δ 0.88 ppm (3H, br. s; CH_3-29). The protons of CH_3-27, CH_3-29 and CH_3-30 groups of β-amyrin had peaks at δ 1.07 ppm (3H, s, CH_3-27), δ 0.88 ppm (3H, s, CH_3-30) and δ 0.91 ppm (3H, s, CH_3-29). The other signals were identical for both amyrins: δ 1.01 (6H, s, CH_3-26), δ 1.00 (6H, s, CH_3-25), δ 0.96 (6H, s, CH_3-28), δ 0.80 (6H, s, CH_3-23), δ 0.79 (6H, s, CH_3-24). On the basis of the above spectral data, compound **2a** and **2b** were characterized as a mixture of α-amyrin and β-amyrin. The identity of these compounds as a mixture of α-amyrin (**2a**) and β-amyrin (**2b**) was further confirmed by comparison of its spectral data with reported values [24].



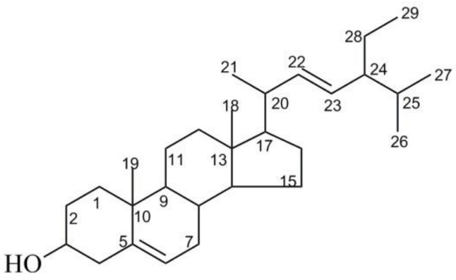
1



2a



2b



3

Compound **3** was obtained as needle shaped crystals, which melted at 167-169°C, which was identified to that reported for stigmasterol [14]. The ^{1}H NMR spectrum of **3** revealed a one proton multiplet at δ 3.52, the position and multiplicity of which was indicative of H-3 of the steroidal nucleus. The typical signal for the olefinic H-6 of the steroidal skeleton was evident from a dublet at δ 5.35 (d, *J* = 8.2 Hz, H-6) integrating one proton. The olefinic protons (H-22 and H-23) appeared as characteristic downfield signals at 5.27 (1H, dd, *J* = 15.2, 8.0 Hz) and 5.18 (1H, dd, *J* = 15.2, 7.2 Hz), respectively in the ^{1}H NMR spectrum due to couplings with the neighboring olefinic and methine protons. The spectrum further revealed signals at δ 0.67 and δ 1.01 (3H each) assignable to two tertiary methyl groups at C-13 and C-10, respectively. The ^{1}H NMR spectrum also showed a doublet of three proton intensity centered at δ 0.91 (*J* = 6.8 Hz) was demonstrative of a methyl group at C-20. The above spectral features were in close agreement to those observed for stigmasterol (**3**) [14,25].

The methanolic extract of *B. daigremontianum* was subjected to free radical scavenging activity by using *tert*-butyl-1-hydroxytoluene (BHT) as reference standard. In this investigation, the aqueous soluble partitionate of *B. daigremontianum* showed the highest free radical scavenging activity with IC_{50} 95.00 µg/mL. The crude methanol extract of *B. daigremontianum* also revealed moderate free radical scavenging activity (IC_{50}=145.0 µg/mL).

The methanolic extract of *B. daigremontianum* was tested for total phenolic content. The total phenolic content of the extract was found to be 4.96 mg of GAE /gm of sample. On the other hand, in the hypotonic solution induced hemolysis of rat RBC, the methanolic extract of *B. daigremontianum* at 1.0 mg/mL inhibited 67.1% haemolysis as compared to 71.9 % revealed by acetyl salicylic acid (0.10 mg/mL) on the other hand during heat induced condition, the methanolic extracts of *B. daigremontianum* showed 79% inhibition and that for ASA was 42.20% inhibition.

As a part of discovery of cardio protective drugs from natural sources, the methanolic extract of *B. daigremontianum* was also assessed for thrombolytic activity. Addition of 100 µL streptokinase, a positive control (30,000 I.U.), to the clots in rat's blood and subsequent incubation for 90 minutes at 37°C, showed 66.77% lysis of clot. On the other hand, distilled water was treated as negative control which exhibited negligible percentages of lysis of clot (3.80%). The mean difference in the clot lysis percentage between positive and negative control was statistically significant. In this study, the methanolic extract of *B. daigremontianum* exhibited thrombolytic activity by 6.54%.

CONCLUSION

It is clearly evident that the extract of *B. daigremontianum* exhibited some biactivities (*i.e.*, free radical scavenging activity, membrane stabilizing activity, thrombolytic activity) which are in accordance with the folk uses of these plants in various diseases mentioned earlier. This plant can be further investigated in order to isolate the bioactive molecules.

ACKNOWLEDGEMENTS

The authors wish to thank The School of Pharmacy, University of London, UK for assisting with the NMR studies. One of the authors (M.A.R.) is grateful to the University of Dhaka for partial financial support to conduct the project entitled “Antihypertensive principles from *Kalanchoe pinnata* (Lam) and *Bryophyllum daigremontianum* (Raym)”.

REFERENCES

1. Kulka RG. Cytokinin inhibit epiphyllous plantlet development on leaves of *Bryophyllum marnierianum*. Exp Bot 2006; 17: 122 – 125.
2. Nahar K, Khan MGU, Rahman MS, Hasan CM, Rashid MA. Secondary metabolites from *Bryophyllum daigremontianum*. Dhaka Univ J Pharm Sci 2008; **7**: 141-144.
3. Supratman UT, Fujita K, Akiyama H, Hayashi A, Murakami H, Sakai K, Ohigashi H. Anti-tumor promoting activity of bufadienolides from *Kalanchoe pinnata* and *K. daigremontiana* x *tubiflora*. Biosci Biotechnol Biochem 2001; 65: 947 – 949.
4. Ojewole JA. Antinociceptive, anti-inflammatory and antidiabetic effects of *Bryophyllum pinnatum* (Crassulaceae) leaf aqueous extract. J Ethnopharmacol 2005; 99: 13 – 19.
5. Akinpelu DA. Antimicrobial activity of *Bryophyllum pinnatum* leaves. Fitoterapia 2000; 71: 193 – 194.
6. Nahar K, Khan MGU, Rahman MS, Hasan CM, Rashid MA. Antimicrobial and cytotoxic activities of *Bryophyllum daigremontianum*. Dhaka Univ J Pharm Sci 2008; 7: 99-101.
7. Jolchine G. The metabolism of acetic acid in the leaves of *Bryophyllum daigremontianum* Berger. The formation of organic acids by different condensation reactions involving the acety radical in the chloroplasts and in the non-chlorplast cellular fraction. Bull Soc Chim Biol (Paris) 1962; 44: 337-64.
8. Stevens JF, Hart Ht, Van Ham RCHJ, Elema ET, Van Den Ent MMVX, Wildeboer M, Zwaving JH. Distribution of alkaloids and tannins in the Crassulaceae. Biochem Sys Ecol 1995; 23, 157 – 165.
9. Kaisar MA, Rahman MS, Rahman MZ, Hasan CM, Rashid MA. A review on phytochemicals from some medicinal plants of Bangladesh. J Pharm Nutri Sci 2011; 1: 87-95.

10. Kabir S, Rahman MS, Chowdhury AM, Hasan CM, Rashid MA. An unusual bisnor-clerodane diterpenoid from *Polyalthia simiarum*. Nat Prod Commun (2010; 5, 1543 – 1546.
11. Vanwagenen BC, Larsen R, Cardellina JHII, Randazzo D, Lidert ZC, Swithenbank C. Ulosantoin, a potent insecticide from the sponge *Ulosa ruetzleri*. J Org Chem 1993; 58: 335-337.
12. Yadava RN, Chakravarti N. New antifungal triterpenoid saponin from *Launaea pinnatifida* Cass. Indian J Chem 2009; 48B: 83-87.
13. Gislei FA, Carneiroa LMV, Juniora APF, Bandeirab PN, Lemosb TLG, Viana GSDB. Evidence for excitatory and inhibitory amino acids participation in the neuropharmacological activity of alpha- and beta-amyrin acetate. The Open Pharmaco J 2009; 3: 9-16.
14. Abiodun F, Ali S, Quadir IM, Choudhary IMI. Phytochemical and biological investigation of chloroform and ethylacetate fractions of *Euphorbia heterophylla* leaf (Euphorbiaceae). J Med Plants Res 2008; 2: 365 – 369.
15. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT - Food Sci Tech 1995; 28: 25 – 30.
16. Skerget M, Kotnik P, Hadolin M, Hras AR, Simoni M, Knez J. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chem 2005; 89: 191 – 198.
17. Majhenic L, Skerget M, Knez Z. Antioxidant and antimicrobial activity of guarana seed extracts. Food Chem 2007; 104: 1258 – 1268.
18. Harbertson JF, Spayd S. From the ASEV 2005 Phenolics Symposium - Measuring Phenolics in the Winery. Am J Enol Vitic 2006; 57: 280 – 288.
19. Shinde UA, Phadke AS, Nair AM, Mungantiwar AA, Dikshit VJ, Saraf MN. Membrane stabilizing activity - a possible mechanism of action for the anti-inflammatory activity of *Cedrus deodara* wood oil. Fitoterapia 1999; 70: 251 – 257.
20. Sikder MA, Kuddus MR, Kaisar MA, Karna S, Rashid MA. . *In vitro* membrane stabilizing activity, total phenolic content, free radical scavenging and cytotoxic properties of *Aphanamixis polystachya* (Wall.). Bangladesh Pharm J 2010; 13: 55 – 59.
21. Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM, Daginawala HF. Development of an *in vitro* model to study clot lysis activity of thrombolytic drugs. Thrombosis J 2006; 4: 14.

22. Sikder MA, Siddique AB, Hossian AKMN, Miah MK, Kaisar MA, Rashid MA. Evaluation of thrombolytic activity of four Bangladeshi medicinal plants, as a possible renewable source for thrombolytic compounds. J Pharm Nutri Sci 2011; 1: 4 – 8.
23. Ferous AJ, Mamun MA, Hasan CM. Glut-5(6)-en-3-beta-ol from the aerial parts of *Scoparia dulcis*. Fitoterapia 1992; LXIV(5): 469.
24. Migas P, Cisowski W, Dembińska-Migas W. Isoprene derivatives from the leaves and callus cultures of *Vaccinium corymbosum var. Bluecrop*. Acta Poloniae Pharmaceutica-Drug Res 2005; 62: 45 – 51.
25. Pateh UU, Haruna AK, Garba M, Iliya I, Sule IM, Abubakar MS, Ambi AA. Isolation of stigmasterol, β-sitosterol and 2-Hydroxyhexadecanoic acid methyl ester from the rhizomes of *Stylochiton lancifolius* Pyer and Kotchy (Aeaceae). Nig J Pharm Sci 2008; 7: 19 – 25.