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Research Article
Specific Cytotoxicity of a Novel Arbutin Derivative from *Myrothamnus flabellifolius* Against Human Leukemia Cells

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Abstract

Background: *Myrothamnus flabellifolius* is a South African medicinal plant which is known to be used traditionally in the treatment of cough, cold, gingivitis and kidney problems. Phytochemicals from the leaves of this plant contain anti-oxidant and anti-inflammatory compounds. In a previous study, the methanolic extract from the leaves and stems of this plant showed anti-cancer activity against human leukemia cells (HL-60) without affecting the normal lymphocytes (TK6). **Materials and Methods:** For this, the methanolic extract was fractionated by HPLC into thirty fractions. The fractions were tested against HL-60, TK-6 and the fraction that showed selective death of leukemia cells were further fractionated. Compounds in this fraction were identified using mass spectrometry and nuclear magnetic resonance. **Results:** Thirteen fractions were selected and tested against HL-60 and TK6. Out of the 13 tested only one was the most active fraction and therefore, it was selected for further fractionation using HPLC. Using mass spectrometry and nuclear magnetic resonance, a novel arbutin derivative (compound 1) [(3R,6S)-3,4,5-Trihydroxy-6-(p-hydroxyphenoxy)tetrahydro-2H-pyran-2-yl]methyl 2-(E)-methyl-4-(2-methyl-3-vinyl-2-cyclohexen-1-yl)-2-butenoate was isolated from the active fraction. This compound has selective *in vitro* cytotoxic activity with IC_{50} 0.53 μM against HL-60 with negligible effects on TK6. **Conclusion:** The data from this study has led in establishing a novel arbutin derivative to be specifically cytotoxic to acute myeloid leukemia cells. This compound can be chemically modified and developed as a potential anti-leukemia drug.

Key words: *Myrothamnus flabellifolius*, arbutin derivative, anticancer, HL-60, TK6, acute myeloid leukemia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Current chemotherapeutics for treatment of leukemia include chemotherapy and radiation which are proven to be toxic to normal cells[1,2]. Due to this, the current cancer treatment practices cause severe side effects to patients. The challenge in cancer treatment research is to find novel drugs that can selectively destroy the cancer cells without harming the normal cells[3]. In search of finding new anti-cancer compounds, the present study aimed to identify and characterize the anti-cancer compounds in the *Myrothamnus flabellifolia*. In the African folk medicine, *M. flabellifolius* (MF) is prepared as infusions and decoctions to treat colds, hypertension, strokes and kidney problems[4]. The decoctions can also be used to alleviate menstrual pain, hemorrhoids and backaches. The whole plant is also reported to be used as inhalant to treat asthma and other respiratory conditions. In traditional folklore the MF smog has been documented to treat uterine pain. Moreover, the leaves can be used locally for wound healing and skin irritation[5]. Chemical investigation of MF extracts proved the presences of essential oils, alkaloids, phenolics, flavanoids, tannins, saponins, gums, steroids, amino acids and reducing sugars[6]. Hydrodistillation of the plant produced many essential oils, the major constituents of which were trans-pinocarveol and pinocarvone (19.57 and 11.13%, respectively)[6]. A study showed that these essential oils have anti-oxidant and anti-microbial activity[7]. The aqueous extract of MF was evaluated for its free radical scavenging activity using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH). The results showed that 25 μg mL^{-1} of the water extract exhibits good scavenging ability against free radicals (66%) compared to combination of *M. flabellifolius* and *Ozoroa paniculosa*[7,8] (91%). This anti-oxidant activity may explain their beneficial effect on diabetes, hypertension and cancer[8].

Anti-microbial effects of *Myrothamnus* extract have been previously studied. For example, the ethanol-water extract containing proanthocyanidines, condensed tannins and flavan-3-ols have shown activity against Gram-negative bacteria *Porphyromonas gingivalis*, the primary cause of periodontitis[9]. The *Myrothamnus* plant extract (100 mg mL^{-1}) is shown to inhibit the bacterial adhesion to the surface of human oral epidermal carcinoma KB cells by 50%. This anti-adhesive effect is likely due to inhibition of *P. gingivalis* proteases activity involved in the pathogenesis of periodontitis. These results show the potential of *Myrothamnus* extract to prevent and treat *P. gingivalis* associated oral diseases[9]. In 2011, two separate studies were conducted to determine the anti-viral activity. The first study by Gescher *et al.*[10] in 2011 showed that the inactivation of herpes simplex virus type 1 (HSV-1) was attributed to proanthocyanidin from acetone-water extract. Proanthocyanidin inhibits the virus adsorption and penetration into the host cells with inhibitory concentration IC_{50} 0.4 μg mL^{-1} and cytotoxic concentration CC_{50}50 μg mL^{-1} against Vero cells[10]. The second study evaluated the anti-viral activity against human immunodeficiency virus type1 (HIV-1) and moloney murine leukemia virus (M-MLV). The MF was shown to inhibit viral reverse transcriptase with IC_{50} 34 μM for HIV-1 and IC_{50} for 0.5 μM for M-MLV. The anti-viral activity was attributed to 3, 4, 5 tri-O-galloylquinic acid and a hydrolysable tannin[11]. Recently, these galloyl quinic acids isolated from other plants were also shown to have anti-leishmania activity by inhibiting arginase and an essential enzyme for the protozoa viability[12]. In addition, these polyphenol compounds have anti-tumor activity by targeting DNA polymerase which is the primary mechanism of inhibition of viral activity[11]. These compounds have the ability to inhibit bronchial hyper-reactivity and allergic reactions[4].

Overall, this plant is reported to have anti-microbial, anti-fungal, anti-viral, anti-inflammatory, anti-diabetic and anti-asthmatic and anti-oxidant properties. These beneficial activities are likely to be due to the presence of numerous polyphenols including phenolic acids, tannins and flavonoids[13-15]. The anti-cancer properties of *M. flabellifolia* were observed in Dhillon *et al.*[16] study which demonstrated its selective anti-cancer activity against leukemia cells HL-60 with minimal effects on normal lymphocytes TK6. The methanol extract of the plant exhibited IC_{50} value of 62.5 μg mL^{-1} for HL-60 cells and 248 μg mL^{-1} for TK6 cells[16]. The goal of this study was to identify the anti-cancer compounds present in the methanolic extract that contributed to the specific toxicity of the cancer cells with negligible effects on normal cells.

MATERIALS AND METHODS

Plant extraction: The air-dried plant material which included stem and leaves was ground to a fine powder. For 0.5 g of plant powder, 5 mL MeOH was added and incubated at 4°C overnight. The sample was centrifuged for 5 min and the supernatant was collected. The residue was re-extracted in a similar way. The combined supernatant (total volume of 10 mL) was then dried to 3 mL final volume using SpeedVac.

HPLC fractionation: Samples were fractionated using an Agilent 1100 series HPLC. Mixtures of solutions A (0.1% of

formic acid in H_2O) and B (0.1% of formic acid in acetonitrile) were prepared as mobile phases. Fractionation of the MeOH extract was performed on Phenomenex HPLC Aqua C18 reverse phase column, 125 A° (150×4.6 mm, 3 µm particle size). The amount of solution B was linearly increased from 5-90% during the first 25 min followed by an immediate increase to 100% B. The composition of the mobile phase was kept constant at 100% B for 2 min to washout the column returned to the initial conditions and then equilibrated for 3 min before the next injection. Samples were analyzed with an injection volume of 100 µL. The extract was divided and collected as thirty fractions. There was about 300 µL per fraction that was collected. The total time per extract was 30 min. To collect enough volume of fractions that could be utilized for structure elucidation and cell bioassay approximately 300 HPLC runs were performed. The fraction that showed activity against HL-60 cells was further fractionated using a Spherisorb C8 column (250×4.6 mm, 5 µm particle size). The run started with 30% B for 5 min then solvent B was linearly increased from 30-90% over 21 min. The composition of solvent B was kept constant at 100% B for 10 min and finally returned to the initial conditions (30% B) for 9 min. The fractions were collected at 30 sec intervals. About 100 injections onto the HPLC column were performed cell bioassays and for structure elucidation of compound 1. The column temperature was maintained at 40°C and the flow rate was 0.3 mL min^{-1} for both experiments. A determined UV spectrum was scanned from 200-400 nm and absorbance at 280 nm.

Cell lines and cell culture: Human leukemia cells HL-60 (ATCC® CCL-240™) and normal lymphocytes TK6 (ATCC® CCL-8015™) cell lines were purchased from ATCC (Manassas, VA). The HL-60 and TK6 cells were cultured in RPMI-1640 (ATCC, Manassas, VA) with 10 and 20% Fetal Bovine Serum (FBS), respectively. The FBS was purchased from Atlanta Biologicals. Both cell lines were maintained at 37°C in an incubator with 5% CO_2. Cell counts were obtained by using a hemocytometer and trypan blue exclusion method to calculate cell densities prior treatment. Both leukemia and normal cells utilized for assays possessed viability above 94%. The DMSO (Fischer Scientific Co.) was used to dissolve the plant fractions of the MeOH extract.

Anti-cancer bioassay: Out of 30 fractions, 13 fractions were evaporated and dissolved in DMSO tested against human leukemia cells (HL-60) and normal lymphocytes (TK6). Out of 30 fractions, 13 fractions were selected for a cell viability assay. The fractions were prepared at different concentrations (500, 250, 125, 62.5 and 31.25 µg mL^{-1}). The assay is carried out in 96-well black clear-bottom culture plates. Each well contained 100 µL of each fraction and 100 µL of cell suspensions (10^5 cells mL^{-1}). After 24 h incubation in a 5% CO_2 incubator at 37°C, half of the treated and control wells were killed with 100 µL of 70% ethanol. All cells were stained with 100 µL of Propidium Iodide (PI) (0.01 mg mL^{-1}) and incubated for 15 min at 4°C. Finally, the supernatant containing the PI was discarded after centrifugation and cells were resuspended in cell culture media. The fluorescence was measured using fluorometer[16] at an excitation of 485/20 nm and an emission of 590/35 nm.

The cell's viability was calculated using the following equation:

$$\frac{(\text{Extract treated cells} - \text{Background control})}{(\text{70\% Ethanol killed cells} - \text{Background control})} \times 100$$

where, the background is the culture media (RPMI-1640+10% FBS) in which the HL60 cells were suspended and (RPMI-1640+20% FBS) in which the TK6 cells were suspended. Controls included DMSO at the same concentration used to dissolve the fractions (0.6%) and culture media.

NMR and mass spectrometry: The following types of NMR analyses were recorded in a Bruker Avance III 400 MHz NMR spectrometer (Bruker, Karlsruhe, Germany); ^1H, COSY, HMQC and HMBC. The ESI-QTOF-MS measurement of compound 1 was performed on a Mass Spec Bruker Maxis 4G ESI-QTOF instrument.

RESULTS AND DISCUSSION

The methanolic extract was separated in 30 fractions obtained from HPLC and these were collected in tubes. The dry weights of the obtained pellets of the fractions after Speed Vac drying the solvents were used to calculate concentrations. The fraction that had substantial weight to perform cell bio assay, NMR and MS-MS were chosen. Out of the 30 fractions, 13 were tested and one of them showed showed 70% cell death in human leukemia HL-60 cells with least effects on normal lymphocytes at 500 µg mL^{-1}. This fraction was then separated again using HPLC and the major peak was collected for identification. The chromatogram (Fig. 1) shows the fraction that is separated on HPLC. The major peak 'a' was collected and identified as compound 1.

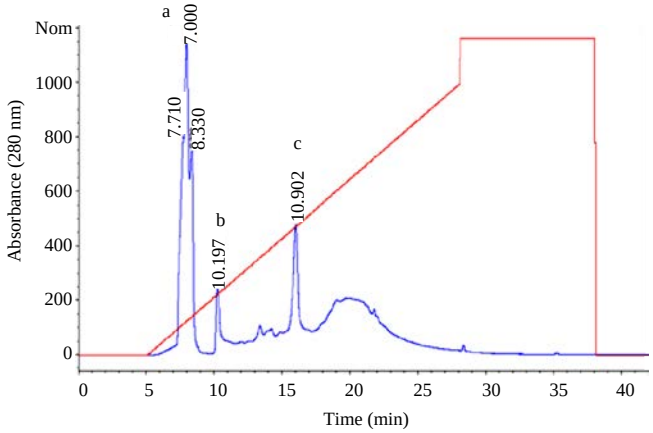

Fig. 1: HPLC chromatogram of the fraction that showed 70% leukemic cell death with>99% cell viability in normal lymphoblast cells and peak a was collected and identified as compound 1

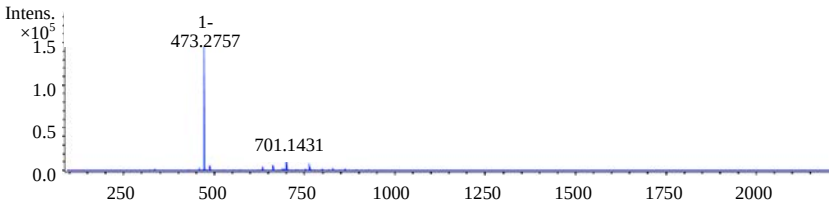

Fig. 2: ESI-QTOF-MS spectrum of the compound 1

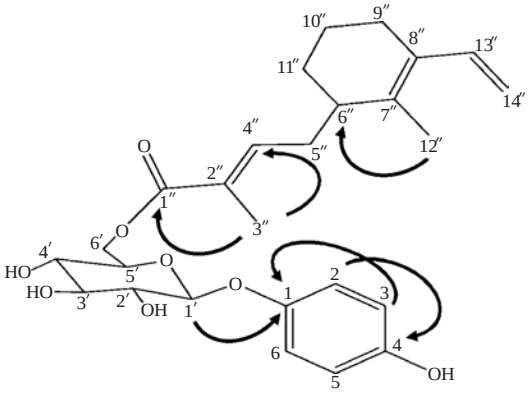

Fig. 3: Structure of compound 1. The chemical name of compound is [(3R,6S)-3,4,5-Trihydroxy-6-(p-hydroxyphenoxy)tetrahydro-2H-pyran-2-yl]methyl 2-(E)-methyl-4-(2-methyl-3-vinyl-2-cyclohexen-1-yl)-2-butenoate and arrow indicate HMBC correlarion

The ESI QTOF-MS analysis of compound 1 showed a $[M-H]^-$ ion at m/z 473.27 (Fig. 2), which is corresponded to C26H34O8 (calcd 474.22). The ESI-QTOF-MS and NMR analysis confirmed the structure of compound 1.

Table 1 summarizes the results obtained from 1D ^{1}H NMR, 2D NMR spectra, COSY and HMBC.

The one dimensional 1D ^{1}H NMR spectrum and two-dimensional 2D NMR spectra results shown in Table 1 were important to assist in structure identification by simplifying the complexity arising from overlapping of peaks in 1D NMR. In the 2D NMR experiment, the cross peaks were located at the off-diagonal positions of the spectrum. This gives information on the coupling between sets of ^{1}H and ^{1}H nuclei or between sets of ^{1}H and ^{13}C nuclei depending on the 2D NMR experiment. Examples of 2D NMR experiments applied in this study are homonuclear correlation spectroscopy (COSY), Heteronuclear Multiple Quantum Correlation (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) (information provided in supplement). To determine the conformational analysis of the hexose, the coupling constant of the anomeric proton determined whether the glycosyl is in α- or β-anomeric configuration[12]. The coupling constant for H-1 (J = 7.6 Hz) and the C-1 delta (102.2 ppm) help to confirm the sugar as β-glucose (Fig. 3)[17-19]. The coupling constant between H-1/H-2 for α-glucose is reported to be 3.8 Hz

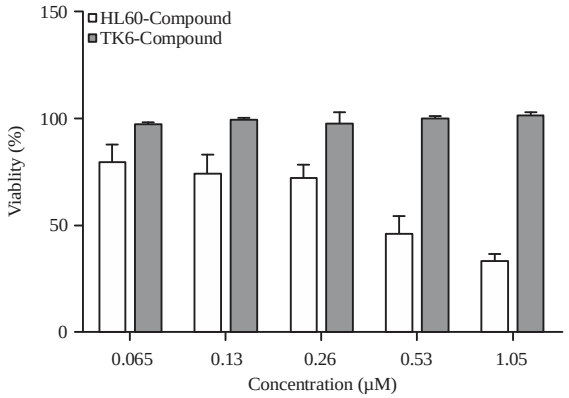

Fig. 4: *In vitro* cytotoxic activity of compound 1 against human leukemia cells HL-60 normal lymphocytes TK6

Table 1: ^{1}H (400 MHz), ^{13}C (400 MHz) NMR, COSY and HMBC (H→C) data for compound 1 (Acetone-d_6, δ, ppm, J/Hz)

C atom	δ_H	δ_C	COSY	HMBC
Glc				
1′	4.81 (1H, d, J=7.6)	102.2	3.47	1
2′	3.47 (1H, dd, J = 8.8, 7.6)	73.7	3.54	
3′	3.54 (1H, dd, J = 8.8, 8.9)	77.0	3.44	
4′	3.44 (1H, dd, J=8.9, 9.1)	70.6	3.73	
5′	3.73 (1H, ddd, J = 9.1, 7.4, 2.0)	74.2	4.55, 4.19	
6′	4.55 (1H, dd, J = 11.8, 2.0)			
	4.19 (1H, dd, J=11.8, 7.4)	63.8		
Aglycone				
1	-	150.9		
2	6.95 (1H, d J = 6.6)	118.7		4
3	6.75 (1H, d J = 6.6)	115.4		1
4	-	152.3		
5	6.75 (1H, d J = 6.6)	115.4		1
6	6.95 (1H, d J = 6.6)	118.7		4
Ester				
1″	-	167.1		
2″	-	126.5		
3″	1.85 (3H)	11.5	6.82	1″, 2″, 4″
4″	6.82 (1H, dt J = 7.6, 1.4)	142.7	2.31, 2.26	
5″	2.31 (1H, m)			
	2.26 (1H, overlap)	23.3	1.63	
6″	1.63 (1H, m)	40.8	1.32, 1.37	
7″	-	145.3		
8″	-	149.9		
9″	1.15 (2H, t)	31.0	1.31	8″
10″	1.31 (2H, overlap)	29.4		
11″	1.37 (1H, m)			
	1.32 (1H, m)	22.7		
12″	1.29 (3H, s)	16.5		6″, 7″
13″	6.01 (1H, J = 17.3, 10.7)	115.2	5.25, 5.03	
14″	5.25 (1H, dd J = 17.3, 1.9)			
	5.03 (1H, dd J = 10.7, 1.9)	110.8		

and the C-1 delta at much lower delta (92 ppm). The HMQC experiment was employed to confirm the correlations between ^{1}H and ^{13}C (that are separated by one bond) of the hexose (Fig. 3).

Arbutin (hydroquinone β-D-glucoside) which is the parent molecule in the proposed structure is a phenolic glycoside compound that is naturally occurring in many plants[20]. Arbutin has been applied topically as skin-lightening agent by inhibiting the tyrosinase activity and preventing melanin production[20]. The 6'-O-Caffeoylarbutin is an arbutin derivative, from *Vaccinium dunalianum* inhibits melanogenesis in zebrafish. It has shown higher anti-melanin activity and less toxicity than arbutin[21,22]. Arbutin also used as anti-infective for urinary system. Arbutin derivatives have been previously isolated from *M. flabellifolius*. The 2, 3-di-O-galloylarbutin was isolated from the acetone-water extract of the air-dried aerial parts from *M. flabellifolius* welw[23]. Arbutin derivatives with other sugar components are responsible for the resistance of *Myrothamnus* to environmental stress such as drought. Arbutin is accumulated in the cell membranes and protects the membrane components during dehydration[24].

Compound 1 was tested against both cell lines HL-60 and TK6 and it showed a selective anti-cancer activity against HL-60 with IC_{50} value 0.53 µM with negligible effects on TK6 in Fig. 4. There are very few studies about arbutin as anti-cancer compounds. For example, a study by Li *et al.*[25] shown that arbutin decreased TCCSUP human bladder cancer cell proliferation via increase in the expression of p21 which involve in cell cycle regulation. The mechanism of arbutin as anti-cancer compound is by inactivating the extracellular signal-regulated kinase (ERK) which is involved in the regulation of cell proliferation[25]. Many naturally derived arbutin derivatives have been isolated from different plant species. Example of hydroxycinnamoyl arbutins are 4′-O-[(E)-p-coumaroyl]arbutin (1), 4′-O-[(E)-caffeoyl]arbutin (2) and 4′-O-[(E)-feruloyl]arbutin (3) (These arbutin derivatives were isolated from the *Casearia multinervosa* (Flacourtiaceae). These derivatives (1), (2) and (3) have been reported to exhibit anti-cancer activity against the P388 mouse lymphocytic leukemia cell line. The LC_{50} values of (1-3) are found to be 543, 179 and 464 mm. Another arbutin derivative with cytotoxic activity is robustasides D. This compound isolated from the leaves and twigs of *Grevillea* "Poorinda Queen"(Proteaceae) showed *in vitro* cytotoxic activity against human embryonic kidney cell HEK-293 with IC_{50} value of 195 and human hepatoma cell line HEP-G2 with IC_{50} value of 221 mm. The proposed structure isolated from *Myrothamnus* is very similar to arbutin derivatives isolated from Xu *et al.*[22] study. Hence, it is proposed that the mechanism of anti-cancer activity could be similar. Hence, this study here warrants further investigation on the mechanism of this compound in preventing cell proliferations and cell death.

CONCLUSION

Screening several fractions of methanolic extract of *M. flabellifolius* led to the identification of the novel compound which is not studied previously to be anti-cancerous to acute myeloid leukemia cells. However, further probe is needed to understand the anti-cancer mechanism of this compound. This study opens new avenues to modifying this compound to increase its efficacy on the leukemia cells while selectively not targeting normal blood cells.

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REFERENCES

1. Monsuez, J.J., J.C. Charniot, N. Vignat and J.Y. Artigou, 2010. Cardiac side-effects of cancer chemotherapy. Int. J. Cardiol., 144: 3-15.
2. Remesh, A., 2012. Toxicities of anticancer drugs and its management. Int. J. Basic Clin. Pharmacol., 1: 2-12.
3. Tallman, M.S., D.G. Gilliland and J.M. Rowe, 2005. Drug therapy for acute myeloid leukemia. Blood, 106: 1154-1163.
4. Moore, J.P., G.G. Lindsey, J.M. Farrant and W.F. Brandt, 2007. An overview of the biology of the desiccation-tolerant resurrection plant *Myrothamnus flabellifolia*. Ann. Bot., 99: 211-217.
5. Viljoen, A.M., M.E. Klepser, E.J. Ernst, D. Keele, E. Roling and S. Van Vuuren *et al*., 2002. The composition and antimicrobial activity of the essential oil of the resurrection plant *Myrothamnus flabellifolius*. South Afr. J. Bot., 68: 100-105.
6. Molefe-Khamanga, D.M., N.A. Mooketsi, M.G. Matsabisa and R.M. Kensley, 2012. Qualitative phytochemical studies of solvent extracts from *Myrothamnus flabellifolius*. Online Int. J. Med. Plant Res., 1: 1-5.
7. Motlhanka, D.M.T. and G. Mathapa, 2012. Antioxidant activities of crude extracts from medicinal plants used by diabetic patients in Eastern Botswana. J. Med. Plants Res., 6: 5460-5463.
8. Pandey, K.B. and S.I. Rizvi, 2009. Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Med. Cell. Longevity, 2: 270-278.
9. Lohr, G., T. Beikler, A. Podbielski, K. Standar, S. Redanz and A. Hensel, 2011. Polyphenols from *Myrothamnus flabellifolia* Welw. inhibit *in vitro* adhesion of *Porphyromonas gingivalis* and exert anti-inflammatory cytoprotective effects in KB cells. J. Clin. Periodontol., 38: 457-469.
10. Gescher, K., J. Kuhn, E. Lorentzen, W. Hafezi, A. Derksen, A. Deters and A. Hensel, 2011. Proanthocyanidin-enriched extract from *Myrothamnus flabellifolia* Welw. exerts antiviral activity against herpes simplex virus type 1 by inhibition of viral adsorption and penetration. J. Ethnopharmacol., 134: 468-474.
11. Kamng'ona, A., J.P. Moore, G. Lindsey and W. Brandt, 2011. Inhibition of HIV-1 and M-MLV reverse transcriptases by a major polyphenol (3, 4, 5 tri-O-galloylquinic acid) present in the leaves of the South African resurrection plant, *Myrothamnus flabellifolia*. J. Enzyme Inhibition Med. Chem., 26: 843-853.
12. De Sousa, L.R., S.D. Ramalho, J.B. Fernandes, M.F.D.G. da Silva and M.R.D.C. Iemma *et al*., 2014. Leishmanicidal galloylquinic acids are noncompetitive inhibitors of arginase. J. Brazil. Chem. Soc., 25: 1832-1838.
13. Moore, J.P., K.L. Westall, N. Ravenscroft, J.M. Farrant, G.G. Lindsey and W.F. Brandt, 2005. The predominant polyphenol in the leaves of the resurrection plant *Myrothamnus flabellifolius*, 3,4,5-tri-O-galloylquinic acid, protects membranes against desiccation and free radical-induced oxidation. Biochem. J., 385: 301-308.
14. Lamoral-Theys, D., L. Pottier, F. Dufrasne, J. Neve and J. Dubois *et al*., 2010. Natural polyphenols that display anticancer properties through inhibition of kinase activity. Curr. Med. Chem., 17: 812-825.
15. Hirano, T., M. Gotoh and K. Oka, 1994. Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells. Life Sci., 55: 1061-1069.
16. Dhillon, J., V. Miller, J. Carter, A. Badiab, C.N. Tang, A. Huynh and B. Peethambaran, 2014. Apoptosis-inducing potential of *Myrothamnus flabellifolius*, an edible medicinal plant, on human myeloid leukemia HL-60 cells. Int. J. Appl. Res. Nat. Prod., 7: 28-32.
17. Markham, K.R. and H. Geiger, 1994. 1H Nuclear Magnetic Resonance Spectroscopy of Flavonoids and their Glycosides in Hexadeuterodimethylsulfoxide. In: The Flavonoids: Advances in Research Since 1986, Harborne, J.B. (Ed.). Chapman and Hall, London, UK., pp: 441-497.
18. Govindaraju, V., K. Young and A.A. Maudsley, 2000. Proton NMR chemical shifts and coupling constants for brain metabolites. NMR Biomed., 13: 129-153.
19. Duus, J.O., C.H. Gotfredsen and K. Bock, 2000. Carbohydrate structural determination by NMR spectroscopy: Modern methods and limitations. Chem. Rev., 100: 4589-4614.

20. Xu, W.H., Q. Liang, Y.J. Zhang and P. Zhao, 2015. Naturally occurring arbutin derivatives and their bioactivities. Chem. Biodiversity, 12: 54-81.
21. Pyka, A., K. Bober and A. Stolarczyk, 2007. Densitometric determination of arbutin in cowberry leaves (*Vaccinium vitis idaeae*). Acta Poloniae Pharm., 64: 395-400.
22. Xu, M., Q.C. Lao, P. Zhao, X.Y. Zhu and H.T. Zhu *et al*., 2014. 6'-O-Caffeoylarbutin inhibits melanogenesis in zebrafish. Nat. Prod. Res., 28: 932-934.
23. Engelhardt, C., F. Petereit, J. Anke and A. Hensel, 2007. A new arbutin derivative from the herb of *Myrothamnus flabellifolia* Welw. Die Pharmazie Int. J. Pharm. Sci., 62: 558-559.
24. Oliver, A.E., D.K. Hincha, N.M. Tsvetkova, L. Vigh and J.H. Crowe, 2001. The effect of arbutin on membrane integrity during drying is mediated by stabilization of the lamellar phase in the presence of nonbilayer-forming lipids. Chem. Phys. Lipids, 111: 37-57.
25. Li, H., Y.M. Jeong, S.Y. Kim, M.K. Kim and D.S. Kim, 2011. Arbutin inhibits TCCSUP human bladder cancer cell proliferation via up-regulation of p21. Die Pharmazie Int. J. Pharm. Sci., 66: 306-309.