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Methyl-α-D-glucopyranoside from *Tulbaghia violacea* extract induces apoptosis *in vitro* in cancer cells

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Abstract

Pro-apoptotic activity of *Tulbghia violacea* extracts was evaluated in order to understand the mechanisms of action that might be related to its traditional use as anticancer medicine. Apoptosis-guided purification was used to isolate active compound whose chemical structure was solved by using spectroscopic, microanalysis and X-ray crystallography techniques. *T. violacea* extract kills Chinese hamster ovary cells, MCF7, and HeLa cells through the induction of apoptosis Methyl-α-D-glucopyranoside was identified as one of the main pro-apoptotic compounds present in *T. violacea* extract. This is the first time ever demonstration that *T. violacea* contains methyl-α-D-glucopyranoside, which selectively kills cancer cells through apoptosis mechanisms.

Introduction

Plants are known to be important sources of chemotherapeutic agents (Moraes et al., 2000; Mann, 2002). Sweet garlic, *Tulbaghia violacea* (Alliaceae), is indigenous to Southern Africa region (Lyantagaye, 2011) and is used in traditional medicine to treat a variety of disease conditions including cancer of the esophagus, fever, colds, asthma, tuberculosis, and stomach problems (van Wyk et al., 2000). However, the mechanisms of action against these diseases, and the constituents of the plant responsible for the activity are yet to be reported. Few chemical compounds that have been isolated and reported so far from *T. violacea* have been exhaustively reviewed in Lyantagaye (2011), none of the compounds has ever been scientifically linked to any of the above diseases. Many chemotherapeutic agents of plant origin kill the cancerous cells by inducing apoptosis (Ledezma et al., 2004; Oommen et al., 2004). This implies that compounds or mixture of compounds that have the ability to induce apoptosis in mammalian cells are potential chemotherapeutic agents and can be used as leads towards the development of new anti-cancer drugs.

Apoptosis, the cell's intrinsic death program, plays a critical role in many processes such as elimination of damaged or cancerous or infected cells, and their development, immune responses, and aging (Reed, 2000). Cancer development is associated with accumulation of neoplastic cells due to enhanced cell proliferation, diminished cell turnover or combination of both processes. It is commonly accepted that inhibition of apoptosis plays an important role in the carcinogenic process (Evan et al., 1992). Many anti-apoptotic processes lead to abnormal activation of proliferative pathway causing cancer (Allsop, 1993; Reed, 1994). Killing of cancer cells by cytotoxic therapies, such as chemotherapy, g-irradiation or ligation of death receptors is predominantly mediated by triggering apoptosis (cell suicide) in target cells (Fulda and Debatin, 2003).

The present study aimed at contributing to the scientific knowledge of the traditional therapeutic use of the *T. violacea* against cancer (Lyantagaye, 2011). The strategy of the study involved testing of the *T. violacea* crude aqueous extracts for the ability to induce apoptosis, followed by the activity-guided fractionation for apoptosis, purification and chemical structure

characterization. The induction of apoptosis in cultured Chinese hamster ovary cells, estrogen-dependent human breast tumor 'adenocarcinoma' (MCF7) cells, human cervix epitheloid carcinoma (HeLa) cells treated with the aqueous extract from *T. violacea*, has been demonstrated as characterized by the shrinkage of the cells, phosphatidylserine translocation, depolarization of mitochondrial membrane potential, activation of caspase-3, and fragmentation of the genomic DNA. Apoptosis activity-guided purification of the crude extract concentrated the activity in the fractions eluted at 7th, 58th and 62nd min. Elemental analysis, IR, NMR, and XRD crystallography techniques led the identification of methyl-α-D-glucopyranoside as the compound responsible for the activity in the extract eluted at the 7th min. Although methyl-α-D-glucopyranoside is not novel, the revelation of its ability to induce apoptosis in cancer cells is novel and presents an opportunity for development of a novel anti-cancer therapy. Solving of the molecular structures of the active compounds contained in the fractions eluted at 58th min and 62nd min is underway.

Materials and Methods

Preparation of plant extracts

Blender crushed fresh plant material was homogenized in water:methanol (1:1) v/v solvent system 3 x 24 hours at room temperature. The extracts were pooled together and filtered through a Whatman No. 1 filter paper. The filtrate was concentrated to about half the original volume using a rotary evaporator under reduced pressure at 37°C to remove the methanol. The remaining aqueous solution (referred to as aqueous extract) was freeze-dried.

Purification of the active compounds

The crude aqueous extract was fractionated using organic solvents in the order of increasing polarity: *n*-Hexane, ethyl acetate, chloroform, dichloromethane and *n*-butyl alcohol. The fractions were tested for the induction of apoptosis in Chinese hamster ovary cell using the annexin V binding assay. Then, followed by purification on RP-HPLC gradient elution with methanol (0-100%) in aqueous for 90 min at 2 mL/min using semi-preparative HAISIL 100 C-18 (Higgins Analytical) on a Beckman HPLC system. Then, size exclusion chromatography using sephadex LH-20 (Sigma), ion exchange using Amberlite XAD-2 (SUPELCO), and crystallization techniques were used.

Chemical structure determination of the active compound

Elemental analysis was performed using FISON elemental analyzer 1108. Infrared analysis was done on a Perkin-Elmer paragon 1000 PC FT-IR spectrophotometer from NaCl cells. 1D and 2D ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) analyses were done on a 600 MHz Varian unity inova spectrometer equipped with an Oxford magnet (14.09 T) and a 5 mm indirect detection PFG probe. Single-crystal X-ray diffraction (XRD) was recorded on a Nonius Kappa-CCD diffractometer, and structure was solved by direct method using SHELXL-86 (Sheldrick, 1985) and refined employing full-matrix least-squares with SHELXL-97 (Sheldrick, 1997) refining on F^2.

Cell culture and treatment

The cell lines used were Chinese hamster ovary cells, estrogen-dependent human breast tumor 'adenocarcinoma' (MCF7) cells, human cervix epitheloid carcinoma (HeLa) cells, human osteosarcoma (MG63) cells and human squamous cell carcinoma (H157) cells. 5 mL of 2.4×10^4 cells/mL in 25 cm^2 flasks cells were cultured in nutrient mixture F-12 (Ham) + L-glutamine supplemented with 0.2% penicillin-streptomycin + 5% fetal calf serum) medium, or RPMI 1640 medium with glutamax-1 supplemented with 0.2% penicillin-streptomycin + 10% fetal calf serum. Cell counting was performed using a Neubauer hemocytometer cell counting chamber. After 24-48 hours incubation at 37°C in a 5%CO_2 humidified incubator, the cells were subcultured in 6-well plates at 2 mL of 2.5×10^4 cells/mL/well, or in 96-well plates at 100 µL of 0.25×10^5 cells/mL/well, and were incubated at the same conditions for 24 hours. Where necessary, one sterilized cover slip was placed in each well before introducing the cell suspension, for microscopic analysis purposes. Then, the cells were or were not treated with known concentration of the plant extract or staurosporine (positive control for apoptosis induction) diluted in the culture media and incubated at the same conditions for different times ranging from 0 to 72 hours.

Microscopy

A Nikon TMS (Japan) inverted light microscope was used routinely to monitor the cell culture development and for cell counting. Cells grown on cover slips were washed twice with PBS and observed under the Nikon inverted light microscope. Cells were observed at a magnification of 20 x eyepiece. Photo-graphs were taken by using a Nikon Nikkormat (Japan) photo camera.

Determination of the translocation of the cell membrane lipids phosphatidylserine

Phosphatidylserine externalization is a down-stream event of interleukin-1β–converting enzyme family protease activation during apoptosis. Since externalization of phosphatidylserine occurs in the earlier stages of apoptosis, APOPercentage ™ apoptosis assay or annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA

fragmentation. Phosphatidylserine externalization was determined using APOPercentage ™ apoptosis assay kit from Biocolor Ltd. The assays employs a red dye (APOPercentage dye), which enters the cells during the phosphatidylserine translocation in cells undergoing apoptosis, after which no more dye gets into or out of the cells. The dye inside the apoptotic cells can be qualitatively and quantitatively analyzed using FACS (Biocolor, 2004). Cells were treated with the inducer made medium containing 5% of the APOPercentage ™ dye. The treated cells were incubated for 1 hour. Then the cells were washed twice with PBS to remove excess dye. Following dye uptake, the level of apoptosis was quantified using Becton–Dickinson, USA FACScan flow cytometer.

For annexin V staining, the cells were cultured in a 6-well plate at a density of 2.5 x 10^4 cells/mL/well for 24 hours. The cells were treated for 1 hour with the inducer made in the respective medium; untreated cells were set for control. Then the cells were washed with cold PBS, trypsinized and re-suspended in 1 x binding buffer at a concentration of 1 x 10^6 cells/mL. 100 µL of the cell suspension (1 x 10^5 cells/mL) was transferred to a 5 mL tube and was stained with annexin V-FITC/or annexin V-PE according to the manufacture's instructions (Becton Dickinson). Cells with lost integrity of the plasma membrane (necrotic and late apoptotic cells) were detected by a second dye staining with 7-amino-actinomycin D (7-AAD). The stained samples were analyzed using FACS (Becton Dickinson) using FL-1, FL -2 and FL-3 channels for, respectively, Annexin V-FITC, Annexin V-PE and 7-AAD. A minimum of 10,000 cells per sample was acquired and analyzed using CELL-Quest PRO software (Becton Dickinson).

Analysis of caspase-3 activation

Activation of ICE-family proteases/caspases initiates apoptosis in mammalian cells, and regulation of caspase-3 activation is one of the focuses in discovery for anti-cancer drugs. The purpose was to determine the increased enzymatic activity of the caspase-3 class of proteases in apoptotic cells. Caspase activity was measured as per CaspACE™ assay system colorimetric (Promega) manual. Cells were treated with a known concentration of inducer made in medium. After the treatment, cells were trypsinized and the total protein was extracted from the cells. The protein samples were quantified using Bradford assay to make uniform amounts for each sample (Bradford dye: 100 mg coomassie brilliant blue G 250, 50%concentrated phosphoric acid and 25% ethanol. The bradford dye was diluted 5 times in deionised water before use). To measure caspase activity in the cell protein extract, replicate wells containing blank, untreated cells control, staurosporine induced cells positive control, plant extract induced cells, and plant extract treated cells inhibited by Z-VAD-FMK samples were prepared as shown in the manufacturer's manual. 2 µL of the DEVD-pNA substrate (10 mM stock) was added to all wells. The plate was covered and incubated with shaking at 37°C for 2 hours. The absorbance was measured using microtitre plate reader at 405 nm. There is a direct correlation between the absorbance and the amount of caspase activity.

Or, the treated cells were washed with PBS, trypsinized and the cells pellet was incubated with anti-active caspase-3 monoclonal antibody, provided in the Active Caspase-3 PE Mab Apoptosis Kit (BD Biosciences), as per the active caspase-3 PE Mab assay manual (BD Biosciences). The cells were harvested by centrifugation for 2 min at 200 x g and re-suspended in 500 µL Perm/ Wash™ Buffer and analyzed by using FL2 channel on a FACScan (Becton– Dickinson, Franklin Lakes, NJ, USA). A minimum of 10,000 cells per sample were acquired and analyzed using CELLQuest PRO software (BD Biosciences).

Analysis of genomic DNA fragmentation

Treated cells were washed with PBS, trypsinized, and were transferred into 1.5 mL eppendorf tubes. The cells pellet was re-suspended in 0.5 mL TTE, to which 0.1 mL ice-cold 5M NaCl and 0.7 mL ice-cold isopropanol was added. The samples were vortexed and placed at 20°C overnight to precipitate the DNA. Then the samples were centrifuged at 10,000 x g for 10 min, at 4°C. The pellets were washed with 0.5 mL 70% ethanol and centrifuged at 10,000x g for 10 min, at 4°C. The supernatants were removed and the tubes containing DNA pellets were left open to dry by air. The pellets were re-dissolved in 30 µL TE (10 mM Tris-HCl and 1 mM EDTA, pH 7.4) and the DNA was size fractionated by electrophoresis on 2%agarose gels containing 0.5 µg/ mL ethidium bromide. The DNA was electrophoresed at 10 V/cm in 1 x TBE electrophoresis buffer (10 X TBE: 0.9 M Tris, 0.89 M Boric acid and 25 mM EDTA, pH 8.3; 1 x TBE: 10 x TBE diluted 10 folds). After electrophoresis the DNA was visualized on an UV transilluminator. Gel images were recorded with a UVP (Ultra Violet Products) image capture system.

Results

The crude aqueous extract of *T. violacea* kills Chinese hamster ovary cells and cancer cell lines MCF7 and HeLa through the induction of cell shrinkage and translocation of phosphatidylserine from the outer to the inner side of the plasma membrane (Figure 1 and 2), activation of caspace-3 (Figure 3) and fragmentation of genomic DNA (Figure 4). These events are the major indicators of the occurrence of apoptosis.

The investigation into the apoptotic effect of *T. violacea* crude aqueous extract in mammalian cancer cell lines demonstrated that 0.5 mg/mL of the crude aqueous

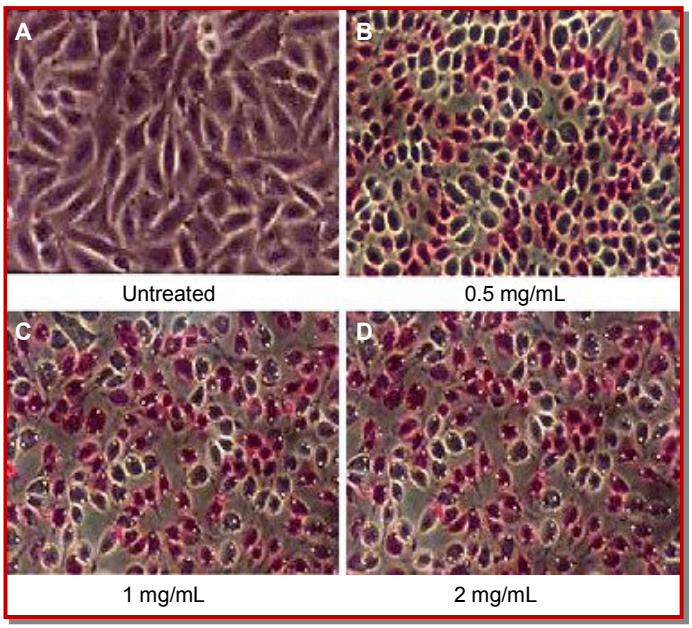

Figure 1: The APOPercentage ™ apoptosis assay showing CHO cells treated with *T. violacea* crude aqueous extract stain positive for apoptosis. (A) shows the untreated control cells which did not stain the purple-red, (B, C and D) show cells that have been treated with 0.5, 1 and 2 mg/mL respectively of *T. violacea* crude aqueous extract and stain purple-red

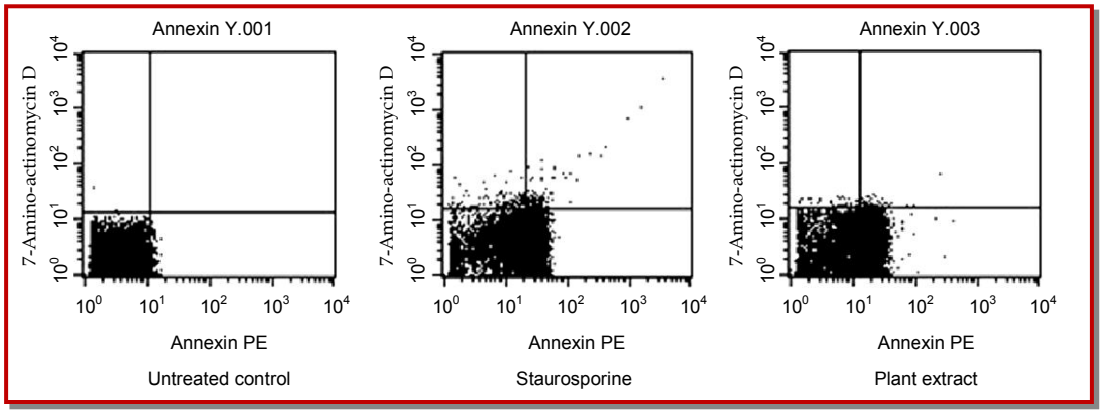

Figure 2: Annexin V-PE apoptosis assay of CHO cells treated with *T. violacea* aqueous crude extract (0.5 mg/mL) for 1 hour, 1 µM staurosporine was used as a positive control for apoptosis. In the untreated control, the cell population is confined within the first quadrant boundaries. For the positive control and the plant extract, the dot plot shows that there was an increase in the Annexin V -PE staining (horizontal axis). The absence of the 7-AAD staining (vertical axis) means no necrosis

extract of the *T. violacea* is able to induce apoptosis in MCF7 (59%) and HeLa (64%) but not in H157 (<10%) and MG63 (<10%) (Figure 5).

Out of the *n*-hexane, ethyl acetate, chloroform, dichloromethane and *n*-butyl alcohol extract fractions tested for apoptosis induction, most activity was confined in *n*-butanol and ethyl acetate fractions (Figure 6). Further fractionation of the *n*-butanol and ethyl acetate fractions using RP-HPL, revealed that the most activity of the *n*-butanol fraction was concentrated in sub-fraction eluted at 7th min of the run (Tv-7), and most activity of the ethyl acetate fraction was concentrated in 2 sub-fractions eluted at 58th min (Tv-58) and at 62nd min (Tv-62). The ability to activate caspase-3 in Chinese hamster ovary cells as shown in Figure 7 was used to demonstrate the ability of Tv-7, Tv-58 and Tv-62 samples to induce apoptosis.

The freeze-dried powder of Tv-7 was glistening white, readily soluble in water, stable for more than 24 months under the laboratory conditions. Has Rf value of 0.8 against the mobile phase from on a silica gel 60 F_{254} (Merck) TLC plate and a faint white spot when sprayed with vanillin-H_2SO_4 reagent, followed by heating at 105°C for 1-2 min. The elemental analysis found: C, 43.5%; H, 8.0%; N, 0%; S, 0%; calculated O, 48.5%, predicting the formula $(CH_2O)_n$. IR spectra data showed ν^{Nujol} cm^{-1} : 3196 (–OH), 1200–900 (C-C, C-O). The ^{1}H NMR spectra data are summarized in Table I.

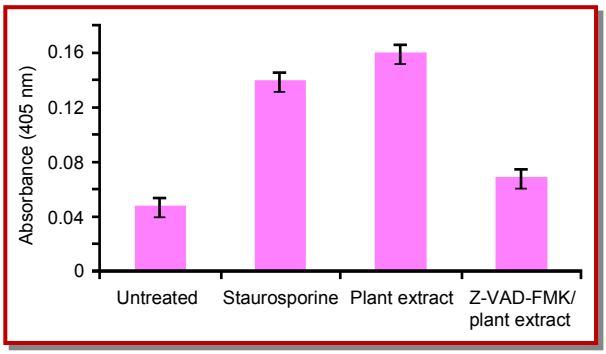

Figure 3: Measurement of caspase-3 activity in CHO cells treated with *T. violacea* crude aqueous extract CHO cells were treated with 1 µM staurosporine (positive control), 0.5 mg/mL of the plant extract and the plant extract plus Z-VAD-FMK inhibitor or HAM's F-12 medium (negative control). Cell extracts were tested for caspase-3 activity according to the manufactures instructions. The absorbance is direct related to caspase-3 activity. The experiment was repeated three times and averages were plotted on the graphs, the plant extract induced the most caspase-3 activity

The most downfield doublet signal at 4.76 p.p.m corresponded to that of an anomeric proton (H-1), and it exhibited weak coupling constant ($^3J_{1,2}$ 3.8) characteristic of α-linked glucopyranose ring. A methoxyl (OMe)

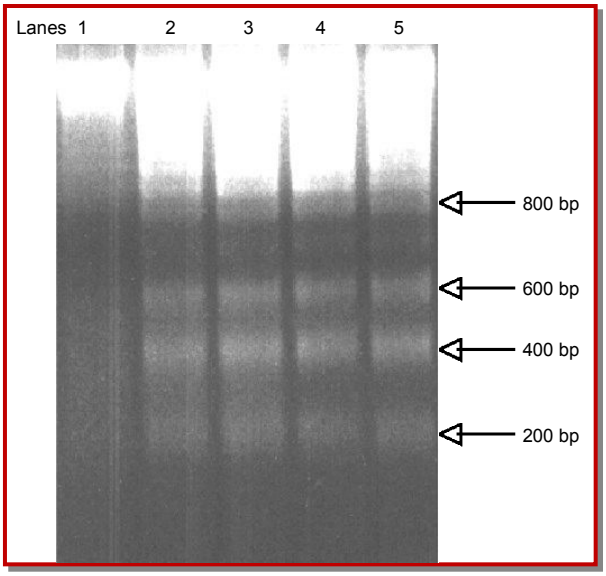

Figure 4: Genomic DNA fragmentation in CHO cells treated with *T. violacea* aqueous crude extract. Lanes 1 represents untreated control; Lane 2 represents 1 µM staurosporine treated cells as positive control of apoptosis; Lanes 3, 4 and 5 represent 0.5, 1 and 2 mg/mL of the *T. violacea* crude aqueous extract. The ladder like pattern of bands indicated the genomic DNA fragments

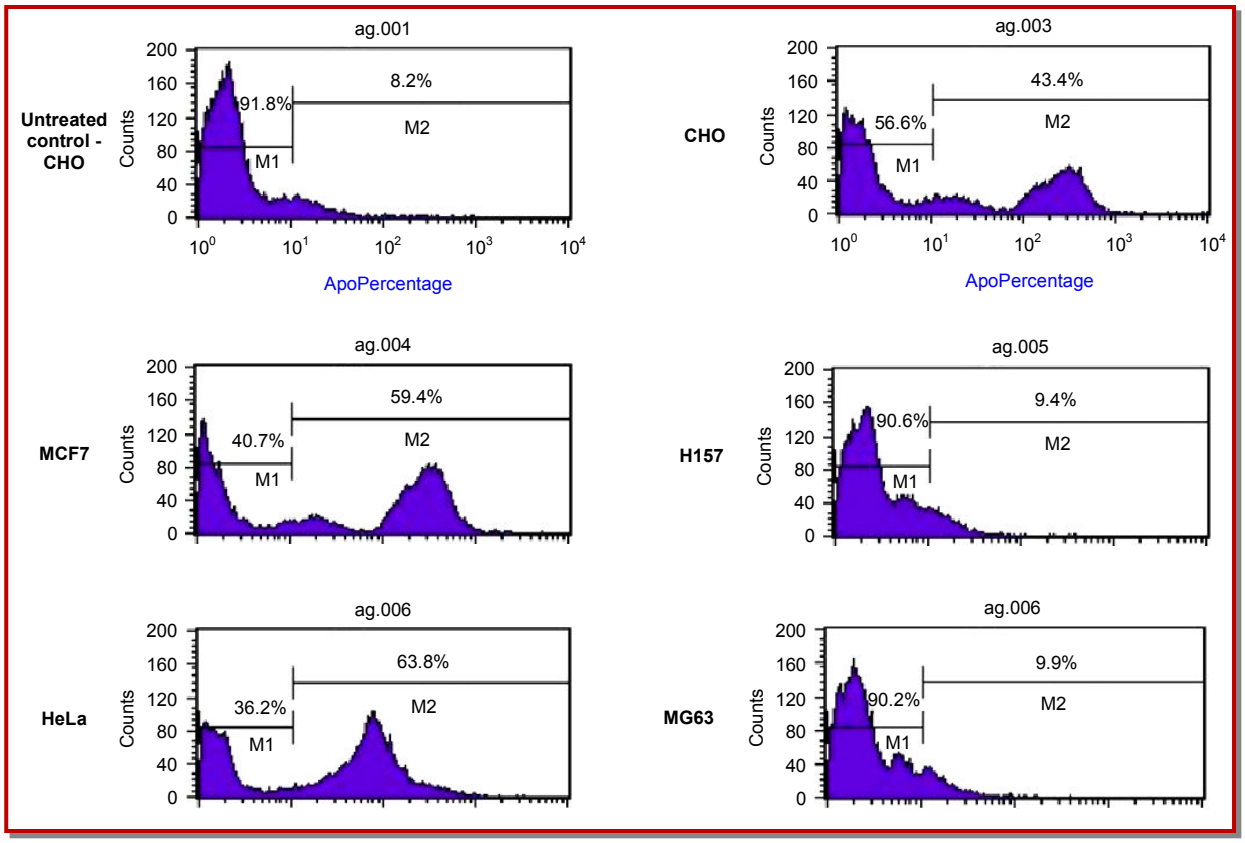

Figure 5: APOPercentage™ apoptosis assay on CHO, MCF7, H157, HeLa and MG63 cell lines treated for 1 hour with 0.5 mg/mL *T. violacea* crude aqueous extract. The M1 and M2 regions of the histograms represent the viable cell and apoptotic cell populations, respectively

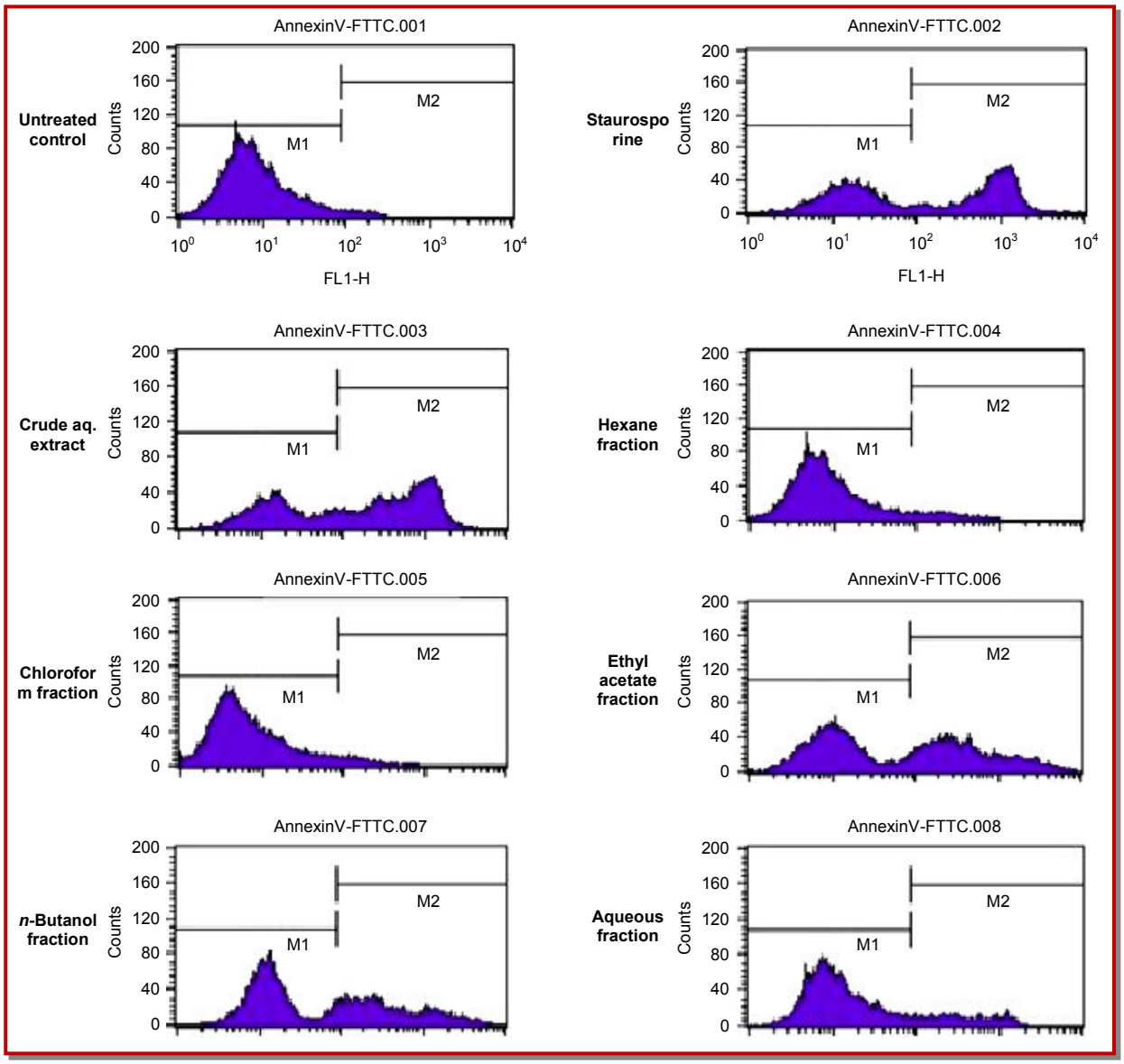

Figure 6: Annexin V-FITC binding on CHO cells treated with different organic solvent fractions, and analyzed by FACScan flow cytometer

proton signal was observed as a singlet integrating for three protons at 3.37 ppm, suggesting a methyl-*O*-glucopyranoside. The remaining ^{1}H resonances for this spin system were assigned by using the ^{1}H-^{1}H 2D NMR COSY spectrum. The assignment of the remaining ^{13}C resonances was achieved by using the 2D gHSQC experiment (^{2}J), supported by DEPT and gHMQC experiments. The ^{3}J correlation of the signal of the OMe protons to the anomeric carbon shown in the 2D gHMQC NMR spectrum is a strong evidence for the location a methyl group at C-1. The data show consistence with that of α-D-glucopyranose, and thus confirming a methyl α-D-glucopyranoside. The data is supported by the XRD data confirming the structure of the methyl-α-D-glucopyranoside in Tv-7 sample (Figure 8).

Discussion

The present study has demonstrated that *T. violacea* extract contains compounds that have the ability to induce apoptosis *in vitro* in Chinese hamster ovary cells, estrogen-dependent human breast tumor 'adeno-carcinoma' (MCF7) cells, and human cervix epitheloid carcinoma (HeLa) cells. The observation agrees with the work by Bungu et al. (2006), which showed that *T. violacea* inhibits growth and induces apoptosis in MCF7 and HeLa cells *in vitro*. On the other hand, the present study has also found that *T. violacea* has no significant effect on human osteosarcoma (MG63) cells and human squamous cell carcinoma (H157) cells, meaning that the activity is selective on certain cancer cells. The 9% cell death for the H157 and MG63 cells is not significant and

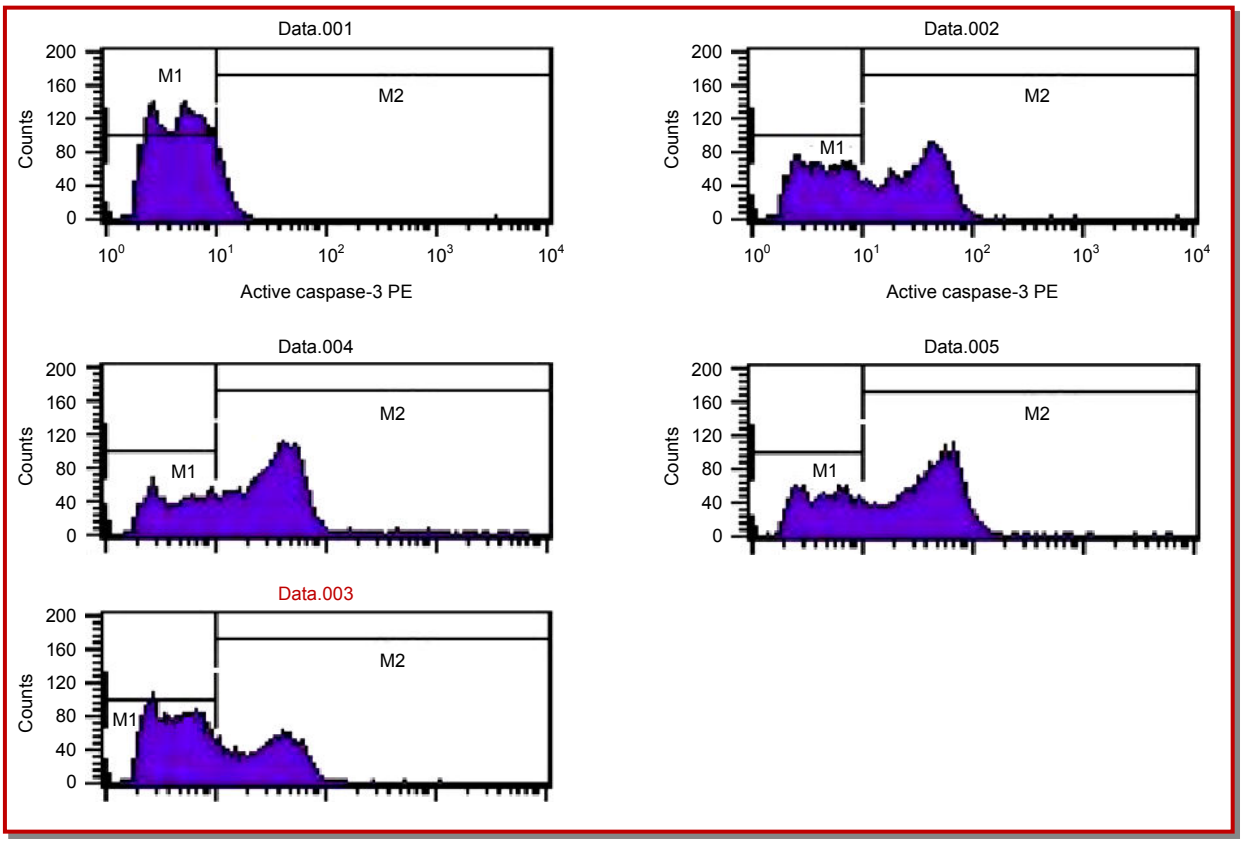

Figure 7: The induction of apoptosis by Tv-7, Tv-58 and Tv-62 in CHO cells. Flow cytometric analysis of populations of untreated control cells (Data.001) and cells induced for 2 hours with Tv-7 (Data.004), Tv-58 (Data.003), Tv-62 (Data.002) and staurosporine (Data.005) using anti-active caspase-3-PE antibody. M1 represents cell population, which were negative for the presence of active caspase-3, whereas M2 represents cell populations, which were positive for active caspase-3-PE staining

is comparable to that of the untreated control, which can be assumed to be the background for the experiment. The results suggest that the apoptotic signaling pathways for H157 and MG63 cells are different from the apoptotic signaling pathways for the Chinese hamster ovary, MCF7 and HeLa cells. The molecular explanation for this difference is not clear yet, but one can speculate that it may be due to different mutations in the genes involved in their apoptosis pathways, or genetic factors leading to different patterns of expression of pro-apoptotic genes, which render some cell lines sensitive to a particular inducing agent and others resistant to the same agent.

The staining of the *T. violacea* extract treated cells with the APOPercentage ™ dye while the untreated control of the cell culture did not stain the dye, cells becoming round in shape, shrunk and detached from neighbou-

Table I

Assignments of the NMR chemical shifts for ^{13}C (d_C) at 150 MHz and ^{1}H (d_H) at 600 MHz for Tv-7 sample. s=singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublets of doublets, t = triplets

d_C (p.p.m)	d_H (p.p.m)	Integral	Multiplicity	$J_{H,H}$ (Hz)	Assignment
99.9	4.8	H	D	$^{3}J_{1,2}$ 3.8	C-1 H-1
71.9	3.5	H	Dd	$^{3}J_{2,1}$ 3.8, $^{3}J_{2,3}$ 9.5	C-2 H-2
73.7	3.6	H	T	$^{3}J_{3,2}$ 9.5, $^{3}J_{3,4}$ 10	C-3 H-3
70.2	3.4	H	Dd	$^{3}J_{4,5}$ 10, $^{3}J_{4,3}$ 0.7	C-4 H-4
72.2	3.6	H	Ddd	$^{3}J_{5,6''}$ 2.3, $^{3}J_{5,6'}$ 5.5, $^{3}J_{5,4}$ 10	C-5 H-5
61.2	3.7	H	Dd	$^{3}J_{6',5}$ 5.5, $^{2}J_{6',6''}$ 12.2	C-6 H-6′
	3.8	H	Dd	$^{3}J_{6'',5}$ 2.3, $^{2}J_{6''6'}$ 12.2	C-6 H-6″
55.7	3.4	3H	S		O-CH_3

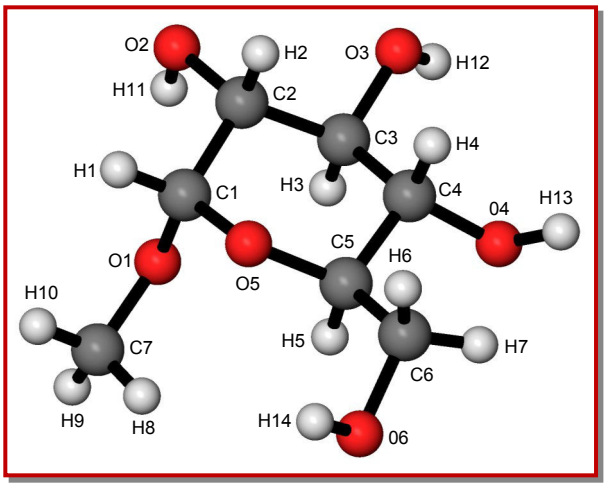

Figure 8: XRD crystal structure of methyl a-D-glucopyranoside (MDG) from Tv-7 sample of *T. violacea* extract

ring cells are important morphological features of apoptotic cells (Saikumar et al., 1999). The *T. violacea* extract also induced caspase-3 activity and genomic DNA fragmentation in the treated cells; these features are hallmarks of apoptosis (Kerr et al., 1972; Savil and Fadok, 2000).

The apoptosis-guided fractionation of the crude extract concentrated the activity in the polar fractions eluted at 7th, 58th and 62nd min of the HPLC analysis. By using liquid-liquid fractionation of the crude extract with organic solvents, the activity was confined in *n*-butanol and ethyl acetate fractions. The results suggested that the active compounds present in the crude extract were polar compounds and could be separated from non-polar compounds.

The elemental analysis of the fraction eluted at 7th min of the RP-HPLC predict the formula close to $(CH_2O)_n$, suggesting that the primary component would be a saccharide. A broad strong IR absorption band characteristic of bonded–OH group was observed at 3196 cm^{-1}. The absence of absorption in the 2800–1500 cm^{-1} region excluded unsaturated compounds, carbonyl group and acyclic form of carbohydrate.

^1H NMR spectrum contained ^1H signals at the 3.3-4.8 ppm region, which is the resonance position consistent with protons on carbons attached to oxygen. The most downfield doublet signal at 4.8 ppm corresponded to that of an anomeric proton (H-1), and it exhibited weak coupling constant ($^3J_{1,2}$ 3.8) characteristic of α-linked glucopyranose ring. A methoxyl (OMe) proton signal was observed as a singlet integrating for three protons at 3.37 ppm, suggesting a methyl-*O*-glucopyranoside. The remaining ^1H and ^3C resonances suggested the presence of glucose linked to OMe. The ^3J correlation of the signal of the OMe protons to the anomeric carbon shown by 2D gHMQC NMR experiment was a strong evidence for the location a methyl group at C-1. The data showed consistence with that of methyl-α-D-glucopyranoside.

The crystals grown from the same sample were singly analyzed on an X-ray diffractometer and confirmed the presence of methyl-α-D-glucopyranoside, the structure has been reported before by Berman and Kim (1968). Therefore, methyl-α-D-glucopyranoside was confirmed to be responsible for the apoptotic activity of the fraction eluted at the 7th min. Methyl-α-D-glucopyranosides are very common in plants although no study had ever before been interested in the determination of the presence of this molecules in *T. violacea* (Lyantagaye, 2011). To our knowledge, this is the first time ever methyl-α-D-glucopyranoside is demonstrated to possess pro-apoptotic activity. Further study is underway to determine the mechanisms and molecular pathways of the observed activity.

Plants are known to be one of the most important sources of chemotherapeutic agents (Moraes et al., 2000; Mann, 2002). Many chemotherapeutic agents of plant origin kill the cancerous cells by inducing apoptosis (Brantley-Finley et al., 2003; Ledezma et al., 2004; Oommen et al., 2004). This implies that compounds or mixture of compounds that have the ability to induce apoptosis in mammalian cells are potential chemotherapeutic agents and can be used as leads towards the development of new anticancer drugs.

Conclusion

The present study has demonstrated for the first time that *T. violacea* contains methyl-α-D-glucopyranoside, which selectively induce apoptosis in Chinese hamster ovary cells, estrogen-dependent human breast tumor 'adenocarcinoma' (MCF7) cells, and human cervix epitheloid carcinoma (HeLa) cells.

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