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Two new pro-apoptotic glucopyranosides from *Tulbaghia violacea*

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***Tulbaghia violacea* is a widely known anticancer herb used as traditional medicine in the Southern African region. However, the identities of the chemical compounds responsible for the medicinal properties are still at large. Isolation of the pro-apoptotic compounds from *T. violacea* was carried out using apoptosis induction-guided fractionation. The chemical structures of the pro-apoptotic molecules were elucidated on the basis of extensive spectroscopic analysis, including 1D and 2D nuclear magnetic resonance (NMR), infrared (IR) and elemental analysis. Pro-apoptotic glucopyranosides D-fructofuranose-β(2→6)-methyl-α-D-glucoyranoside and β-D-fructofuranosyl-(2→6)-α-D-glucopyranoside were uncovered from aqueous whole plant extracts of *T. violacea* using bioactivity-guided purification. Glucopyranosides D-fructofuranose-β(2→6)-methyl-α-D-glucoyranoside and β-D-fructofuranosyl-(2→6)-α-D-glucopyranoside have structural similarity with the earlier reported methyl α-D-glucopyranoside from the same herb, which selectively kills cancer cells through apoptosis mechanisms. The discovery of these molecules could contribute towards the development of new anticancer drugs.**

Key words: Bioactivity-guided purification, glucopyranoside, pro-apoptotic, *Tulbaghia violacea*.

INTRODUCTION

Tulbaghia violacea is an African herb traditionally used to treat fever, colds, asthma, tuberculosis, stomach-ache and cancer of the oesophagus (van Wyk et al., 2000). It has testicular effects suggesting it might increase testosterone levels explaining why it is used as an aphrodisiac in certain African cultures (Ebrahim and Pool, 2010). Additionally, *T. violaceae* concoctions have been reported to possess anti-diabetic (van Huyssteen et al., 2011) as well as anti-microbial properties (Bull et al., 2005; Ncube et al., 2012) while it has the ability to reduce both systolic and diastolic blood pressure (Raji et al., 2012).

Saccharides are the first products formed in photosynthesis, and are the products from which plants synthesize their own reserves, structural components of cell walls as well as a variety of conjugates including glycosides, glycoproteins, proteoglycans and glycolipids, which then become the foodstuffs of other organisms (Velíšek and Cejpek, 2005). The main pathways of saccharide biosynthesis and degradation comprise an important component of primary metabolism that is essential for all living organisms. Secondary metabolites are ultimately derived from the metabolism of saccharides by the acetate, shikimate, mevalonate and 1-deoxy-D-xylulose pathways (Dewick, 2002; Velíšek and Cejpek, 2005).

Like in their close relatives, *Allium* species, volatile sulfur-containing biomolecules are responsible for the characteristic smell and taste of “sweet garlic” (*T. violacea)*. However, unlike *Allium* species in which characterization of secondary metabolites has been extensive, characterization of secondary metabolites in *T.*

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violacea is still not on the same level. Only a few scientific reports on the isolated components from *T. violacea* are available in the literature, including work by Jacobsen et al. (1968), Bate-Smith (1968), Gmelin et al. (1976), Burton (1990), Burton and Kaye (1992), Watson and Dallwitz (2000), Gaidamashvili and van Staden (2002a, b, 2006), Kubec et al. (2002) and Maoela (2005). Most recently, three closely related anticancer fractions (Tv-7, Tv-58 and Tv-62) were isolated from *T. violacea* aqueous extracts, only Tv-7 has its chemical structure been solved as methyl-α-D-glucopyranoside (Lyantagaye, 2011). The present study reports the isolation, purification and chemical structure characterization of the remaining two unidentified pure fractions (Tv-58 and Tv-62).

MATERIALS AND METHODS

Collection and preparation of a crude plant extract

A Cape Town commercial farmer, Mr. Jean Jacques, supplied the *T. violacea* Harv plants during a wet winter. A taxonomist Mr. Franz Weitz identified the plants and a voucher specimen No. 6638 was deposited in the herbarium at the University of the Western Cape. Whole fresh plants were washed with distilled water and cut into pieces of about 5 cm. The plant pieces were blended using a domestic blender (PHILIPS HR 1737, Cucina, Brazil) and extracted 3 × 24 h in a solvent system containing water and methanol (1:1 v/v) at 25°C. New solvent was added between the three extractions, the extracts were pooled together and filtered through Whatman No. 1 filter paper. The filtrate was concentrated using a rotary evaporator (NE-1 EYELA, Tokyo Rikakikai Co, Ltd) under reduced pressure at 37°C to remove the methanol. The remaining aqueous solution (referred to as crude aqueous extracts) was freeze-dried and stored in desiccators until further analysis.

Bio-activity (apoptosis induction): Guided purification

The cell lines used were Chinese hamster ovary cells (CHO). Cell culture, treatment and the apoptosis induction-guided purification were done as previously described in Lyantagaye (2013).

Aqueous organic solvent fractionation

Freeze-dried powdered plant material was reconstituted in double distilled water. Successive steps of extraction from the aqueous solution was done using the same volume of organic solvent in the order of n-hexane, ethyl acetate, chloroform, dichloromethane and n-butyl alcohol. The organic fractions were dried using a rotary evaporator. Using different types of chromatography further purification was performed.

Liquid chromatography

Reverse-phase high-pressure liquid chromatography (RP-HPLC) was used**.** 100 to 500 µl of 50 mg/ml crude aqueous extract was analysed using a semi-preparative HAISIL 100 C-18 250 × 10 mm 5 µm (Higgins Analytical) on Beckman HPLC system with management system Gold (Gold V 310) software, supplied by Beckman. Gradient elution with methanol and water containing 0.01% acetic acid was used at 2 ml/min, and the analytes were detected at 254 nm ultraviolet (UV). Fractions were collected after every 1 min using FOXY JR 202F20077 Model fraction collector for 70 min.

Then, the active fractions were analysed starting with adsorption chromatography (AC), size exclusion chromatography (SEC) and last ion exchange chromatography (IEC) to remove co-eluting contaminants.

AC was performed using silica gel$_{254}$ 60 as stationary phase and hexane/chloroform (1:1) solvent system, then the sample was eluted by isocratic methanol/ethyl acetate (1:1) solvent system, pH was adjusted to range between 3.0 and 6.5 using acetic acid. SEC was performed using Sephadex LH-20 as stationary phase and the sample was eluted using isocratic solvent system; methanol: 0.01% acetic acid in water (1:1), at the rate of 2 ml/min for 120 min. Both AC and SEC were run at 2 ml/min for 120 min, and fractions were collected at 10 min intervals. The fractions were dried under reduced pressure using a rotary evaporator (Tokyo Rikakikai Co, Ltd) at 37 to 40°C, and were tested for induction of apoptosis. For IEC, the sample was re-dissolved in deionised water and passed over the Amberlite XAD-2 beads in a column. The column was then washed with deionised water and the aqueous wash solution was collected at 10 min interval fractions for 120 min at the rate of 2 ml/min, freeze-dried and tested for induction apoptosis. The procedure was repeated using several volumes of methanol to elute the compounds that might have bound to the column matrix from the sample. The methanol eluent was collected and dried using rotary evaporator and tested for apoptosis induction.

Analytical thin layer chromatography (TLC) was used to monitor the purification process from the crude extract to the final stage. It was done using silica gel 60 F_{254} analytical TLC aluminium plates, and three different combinations of mobile phase; chloroform:ethyl acetate:methanol:water (2:2:4:1), chloroform:methanol:water (3:3:1) and methanol:chloroform:water (5:2:1). The developed TLC plates were visualized under UV at 254 nm and were sprayed with spraying reagents, whenever it was necessary, sometimes followed by heating at 105°C for 1 to 3 min. The relative retention factor (RR_f) values were used to compare the compounds present in different apoptotic fraction from the plant extracts.

Spectroscopy

Fourier transform infrared (FTIR) spectroscopy

Dried purified sample was crushed with an oily mulling agent (Nujol) in a marble, with a pestle. A thin film of the mull was smeared onto salt plates and measured. Dried purified samples were homogenized in Nujol mull as a medium, scanned for 25 scans and the infrared (IR) spectra between 4000 and 400 cm^{-1} for both Nujol and sample were recorded in NaCl cells in Perkin-Elmer, paragon 1000 PC FTIR spectrophotometer. The sample positive signals were identified as the peaks on the sample spectra that do not appear on the Nujol spectra (the baseline).

Elemental analysis

The samples were sent to the University of Cape Town (UCT) for elemental analysis. The analysis was performed using FISON elemental analyzer 1108 (Thermo Scientific). The result print outs were received from UCT and were recorded.

Nuclear magnetic resonance (NMR) spectroscopy

1D and 2D NMR spectra were recorded on a 600 MHz Varian Unity Inova spectrometer equipped with an Oxford magnet (14.09 T) and a 5 mm indirect detection PFG probe. Software used to run the

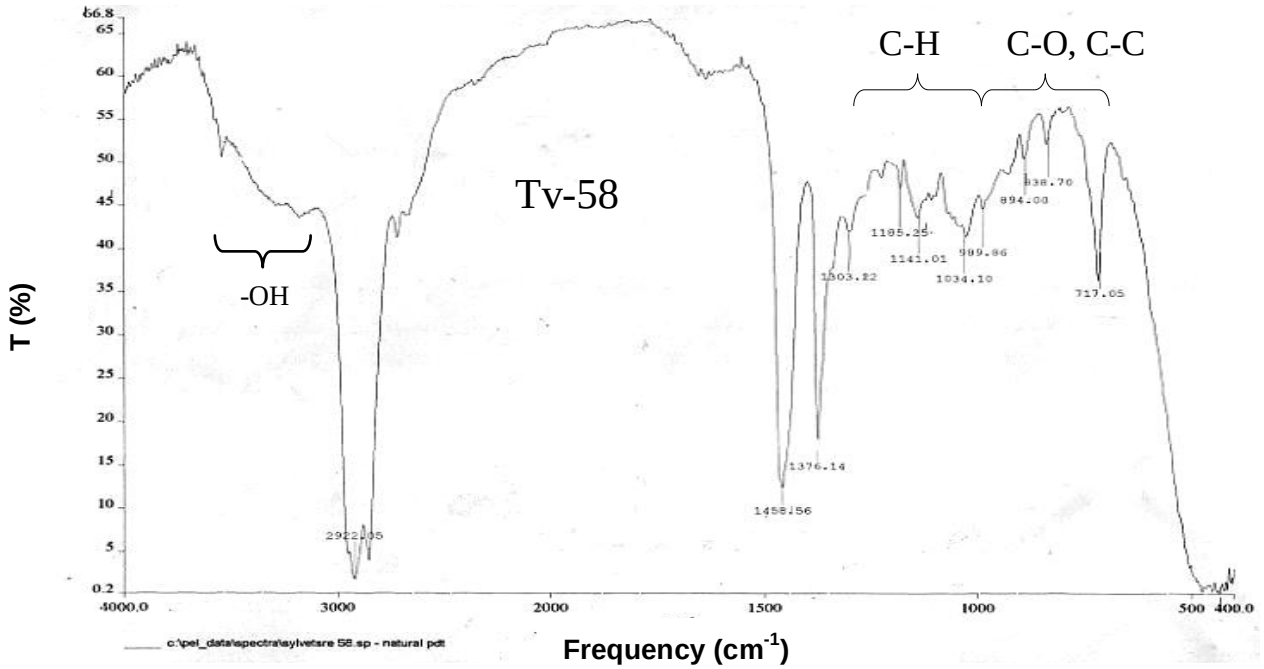

Figure 1. IR (Nujol, cm^{-1}) for Tv-58 sample. ν(OH, C-C, C-O): 3545–3196 cm^{-1} (OH stretchings), 1450-1200 cm^{-1} (C-H bendings), 1100-900 cm^{-1} (C-O and C-C stretching frequencies).

instrument and generate the spectra is VNMR 6.1C. Purified samples were freeze-dried from H_2O and then freeze-dried two times from D_2O. The correlated spectroscopy (COSY), distortion less enhancement by polarization transfer (DEPT), gradient selected heteronuclear single quantum correlation (GHSQC) and gradient selected heteronuclear multiple quantum correlation (GHMQC) NMR spectra were collected using 10 mg of the purified sample dissolved in D_2O (1 ml), the solvent peak being saturated. ^1H chemical shifts (δ_H) were referenced to the residual signals of the protons of solvent and were quoted in ppm downfield from tetramethylsilane (TMS).

RESULTS AND DISCUSSION

The apoptosis activity-guided fractionation with RP-HPLC showed that three HPLC fractions were active, two of which eluted at 58 min (Tv-58) and at 62 min (Tv-62) of the run. The Tv-58 sample was a water-soluble white powder, and the activity was stable at room temperature. The freeze-dried powder of Tv-62 sample was faint white and soluble in water too.

The elemental analysis of Tv-58 revealed: C, 43.03%; H, 8.15%; N, 0%; S, 0%; and calculated O, 48.82%. The elemental analysis of the Tv-62 sample was found to be: C, 30.78%; H, 5.84%; N, 0%; S, 0%; and calculated O, 63.38%. The absence of N and S atoms eliminates the possibility of the sample of being amino acids, proteins or other compounds consisting of the same elements. The presence of substances composed of C, H, and O atoms with the OH as the only functional group was a strong indication of the presence of carbohydrates. On the other hand, the absence of N and S atoms ruled out the presence of N and S containing compounds such as alkaloids, amino acids and proteins.

Both Tv-58 and Tv-62 IR spectra (Figures 1 and 2, respectively) showed similar patterns. The absorption in the regions 3545 to 3196 cm^{-1} (OH stretching), 1450 to 1200 cm^{-1} (OH bending), as well as the C-O and C-C stretching frequencies (1100 to 900 cm^{-1}) may support the presence of saccharide. The absence of absorption in the 2800 to 1500 cm^{-1} region excludes a structure containing a carbonyl group and acyclic form of carbohydrate. The absence of unsaturated bonds (C=C, C≡C, C=O), as determined by the IR, ruled out the presence of acyclic forms of carbohydrates, aromatic compounds, flavonoids, terpenoids, steroids saponins and cardiac glycosides. These results strongly suggested the presence of cyclic forms of carbohydrates for both Tv-58 and Tv-62.

Using 1D and 2D NMR, each of the Tv-58 and Tv-62 molecules showed to contain mainly β-D-fructose and α-D-glucose, the two subunits of sucrose. Carbohydrates are easily identified by ^1H and ^{13}C NMR chemical shifts in the spectral regions δ_H 3 to 5 and δ_c 55 to120 ppm (Moyna and Zauhar, 1999; Yu et al., 2003; Mao and Schmidt-Rohr, 2004). However, both Tv-58 and Tv-62 samples lacked the α-1, β-2-glycosidic linkage that makes D-fructofuranosyl-β(2→1)-α-D-glucopyranoside) from β-D-fructose and α-D-glucose (Velíšek and Cejpek, 2005) (Figure 3).

The assignment of the ^1H and ^{13}C chemical shifts for

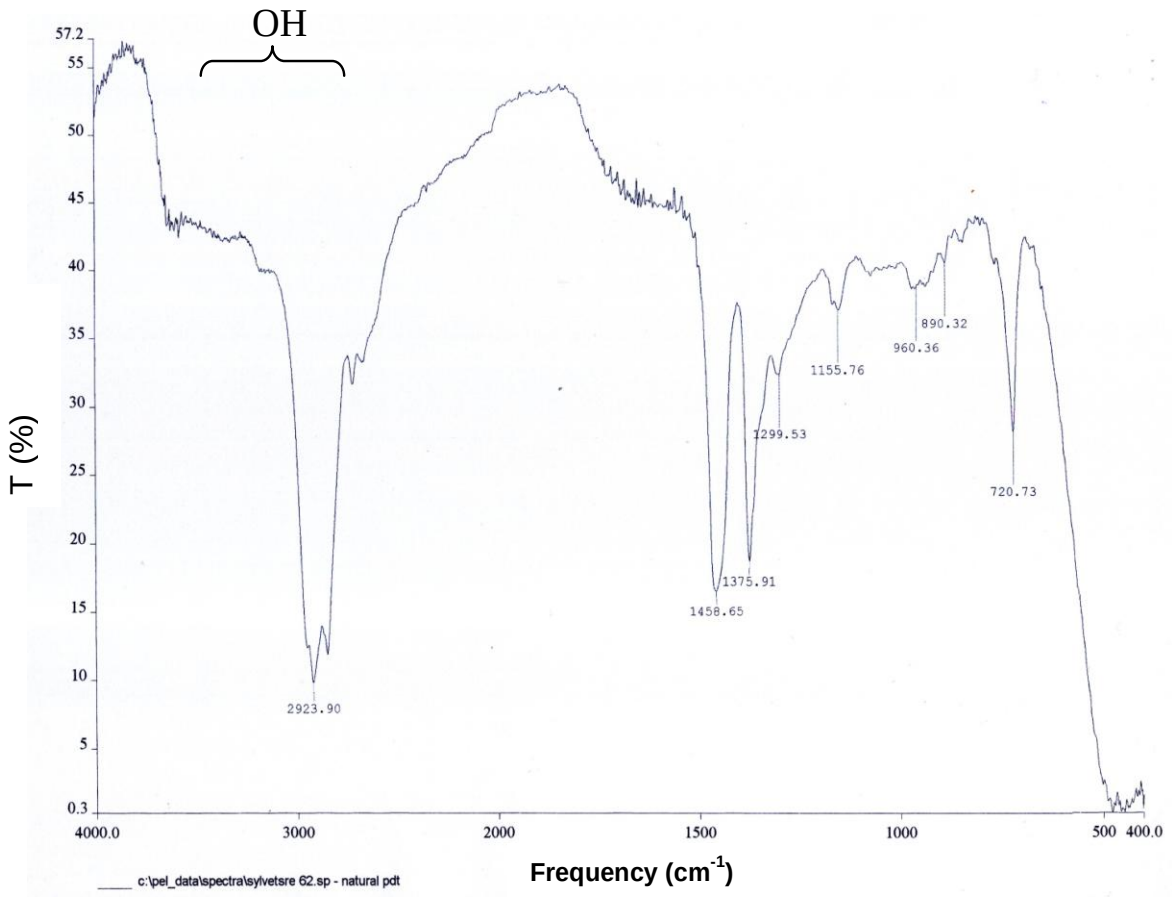

Figure 2. IR (Nujol, cm^{-1}) for Tv-62 sample. ν(OH, C-H, C-C, C-O): 3545–3195 cm^{-1} (OH stretchings), 1450-1200 cm^{-1} (C-H bendings), as well as the C-O and C-C stretching frequencies (1200-900 cm^{-1}).

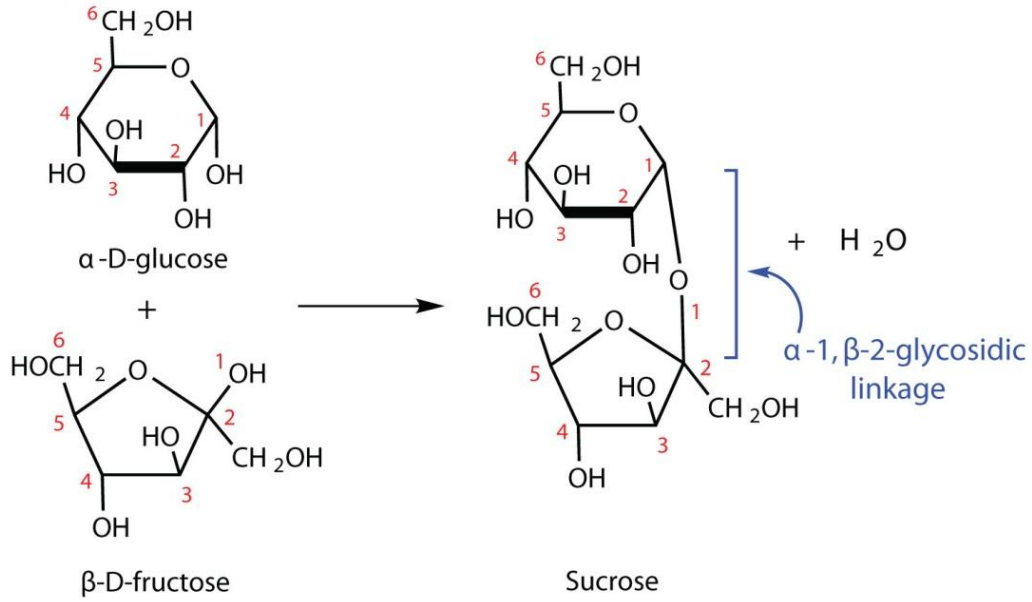

Figure 3. Formation of sucrose (D-fructofuranosyl-β(2→1)- α-D-glucopyranoside) (Velíšek and Cejpek, 2005).

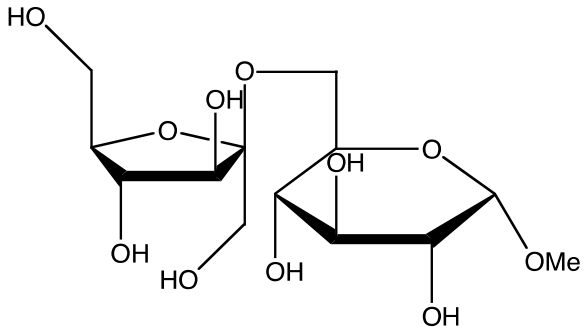

Figure 4. D-fructofuranosyl-β(2→6)-methyl-α-D-glucopyranoside (DFMDG).

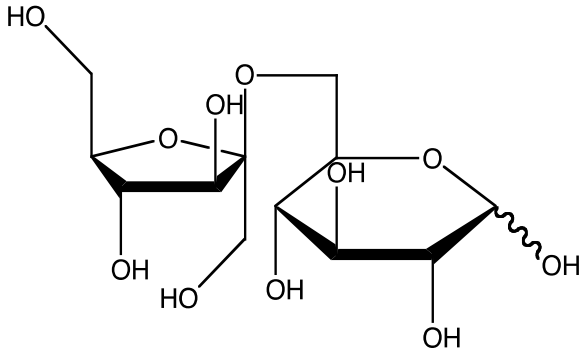

Figure 5. D-fructofuranosyl-β(2→6)-α-D-glucopyranoside (DFDG).

Tv-58 showed that the most downfield doublet signal at 4.76 ppm corresponded to that of an anomeric proton (H-1), and it exhibited weak coupling constant ($^3J_{1,2}$ 3.8) characteristic of an 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 (Table 1) showed the presence of D-fructofuranose-β(2→6)-methyl-α-D-glucoyranoside. The identification of signals in the anomeric atom resonances, and the ^2J (^{13}C-^1H) correlation analysis by DEPT were very useful in characterizing the Fru*f* β(2→6)-methyl-α-Glc as a disaccharide. The observed value of $^3J_{1,2}$ = 3.8 Hz (Table 1) agrees with the Karplus equation of coupling constants (Karplus, 1959) to be characteristic of α conformation whereas the β conformation would have been $^3J_{1,2} \approx 7.4$ Hz (Bhattacharjee et al., 2004). The deshielded terminal CH_3 large singlet ^1H signal at δ_H 3.37 ppm correlated to carbon shift at δ_C 55.66 ppm, observed in the Tv-58 sample analysis, corresponded to that of methoxyl (OMe). The ^3J correlation of the signal of the OMe linked this group to the C-1 of the Glc moiety. The ^{13}C NMR of Tv-58 showed 13 signals, suggesting the presence of a disaccharide. The deshielded shift at δ_c 104.84 ppm, was indicative of a C-2-linked non-reducing D-fructofuranose linked to the Me-Glc moiety to form Fru*f* β(2→6)-methyl-α-Glc (Voll et al., 1990; Lee et al., 1995). The results suggest the presence in Tv-58 of D-fructofuranose-β(2→6)-methyl-α-D-glucoyranoside (Figure 4).

Tv-62 ^{13}C and ^1H NMR showed overlapping resonance signals indicative of the presence of impurity traces. However, chemical shifts representing the glucopyranoside D-fructofuranosyl-β(2→6)-α-D-glucopyranoside were assigned successfully (Table 2), D-glucopyranose present as reducing end as there was no substitution of H observed at the –OH of the C-1 position. Using the same methodologies used for the characterization of D-fructofuranosyl-β(2→6)-methyl-α-D-glucopyranoside earlier, 1D and 2D NMR analyses of Tv-62 in D_2O showed ^1H and ^{13}C resonance signals consistent with that of a glucopyranoside Fru*f* β(2→6)-α-Glc (Table 2), with the α-Glc present as the reducing end rather than methyl-α-D-glucoyranoside. DEPT spectrum showed no cross signal at 104.84 ppm to a proton could be identified. This observation is consistent with the C-2-linked non-reducing β-Fru moiety. The resonance signal at 92.72 ppm is consistent with the C-1 of a reducing α-Glc (Bock and Pedersen, 1983; Bock et al., 1984). The signal at 92.72 ppm correlated (^2J) with the H-1 signal at 5.19 ppm in the ^{13}C-^1H gHSQC NMR spectrum, confirming the anomeric carbon signal (C-1) of the reducing α-Glc. These facts confirmed the presence in Tv-62 sample of a β-D-fructofuranosyl-(2→6)-α-D-glucopyranoside (Figure 5). The power of NMR to solve chemical structures led to a complete chemical structure of Tv58 and Tv62. The use of NMR to identify and characterize natural products has been continually growing for the last 50 years (Breton and Reynolds, 2013).

Conclusions

The pro-apoptosis of glucopyranosides D-fructofuranose-β(2→6)-methyl-α-D-glucoyranoside and β-D-fructofuranosyl-(2→6)-α-D-glucopyranoside from *T. violaceae* extract is being reported for the first time and contributes new knowledge to the biochemistry of the plant *T. violaceae*. The findings provide an opportunity for further study of these compounds towards better understanding of their mechanisms of action. The discovery of these molecules could contribute towards the development of new anticancer drugs.

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Table 1. Assignments of the NMR chemical shifts for ^{13}C (δ_C) at 150 MHz and ^{1}H (δ_H) at 600 MHz for Tv-58 sample.

δ_C (ppm	δ_H (ppm)	Integral	Multiplicity	$J_{H,H}$ (Hz)	^{13}C-^{1}H correlat
-(2→6)-methyl-α-Glc					
99.91	4.76	H	d	$^{3}J_{1,2}$ 3.8	C-1H-1
71.86	3.52	H	dd	$^{3}J_{2,1}$ 3.8, $^{3}J_{2,3}$ 9.5	C-2H-2
73.73	3.62	H	T	$^{3}J_{3,2}$ 9.5, $^{3}J_{3,4}$ 10	C-3H-3
70.20	3.35	H	dd	$^{3}J_{4,5}$ 10, $^{3}J_{4,3}$ 0.7	C-4 H-4
72.22	3.60	H	ddd	$^{3}J_{5,6"}$ 2.3, $^{3}J_{5,6'}$ 5.0, $^{3}J_{5,4}$ 10	C-5H-5
61.17	3.75	H	dd	$^{3}J_{6',5}$ 5.0, $^{2}J_{6',6"}$ 9.8	C-6H-6'
-	3.88	H	dd	$^{3}J_{6",5}$ 2.7, $^{2}J_{6"6'}$ 10.0	H-6"
55.65	3.37	3H	s	-	O-CH_3 (OMe)
β-Fru*f*(2→6)-					
60.55	3.63	H'	s	-	C-1 H-1'
-	3.73	H"	s	-	H-1"
104.84	-	-	-	-	C-2 (quatern)
76.95	4.15	H	d	$^{3}J_{3,4}$ 7.9	C-3 H-3
75.84	4.05	H	T	$^{3}J_{4,5}$ 7.9, $^{3}J_{4,3}$ 7.9	C-4 H-4
80.93	3.91	H	dt	$^{3}J_{5,6'}$ 2,9, $^{3}J_{5,4}$ 7.9	C-5 H-5
64.03	3.50	H'	T	$^{3}J_{6',5}$ 3.0, $^{2}J_{6'6"}$ 8.1	C-6 H-6'
-	3.85	H"	T	$^{2}J_{6",6'}$ 8.1, $^{3}J_{6',5}$ 3.0	H-6"

s=singlet, d=doublet, dd=doublet of doublets, ddd=doublet of doublets of doublets, dt=doublet of triplets, t=triplets.

Table 2. Assignments of the NMR chemical shifts for ^{13}C (δ_C) at 150 MHz and ^{1}H (δ_H) at 600 MHz for Tv-62 sample.

δ_C(ppm)	δ_H (ppm)	Integr	Multiplicity	$J_{H,H}$ (Hz)	^{13}C-^{1}H correlat
-(2→6)-α-Glc					
92.72	5.19	H	d	$^{3}J_{1,2}$ 3.9	C-1H-1
70.24	3.90	H	dd	$^{3}J_{2,1}$ 3.8, $^{3}J_{2,3}$ 9.5	C-2H-2
70.31	3.19	H	t	$^{3}J_{3,2}$ 9.5, $^{3}J_{3,4}$ 9.7	C-3H-3
60.83	3.74	H	Overlapping	-	C-4 H-4
70.24	3.85	H	Overlapping	-	C-5H-5
61.39	3.85	H'	Overlapping	-	C-6H-6'
-	3.64	H"	Overlapping	-	H-6"
β-Fru*f*(2→6)-					
60.52	3.64	H'	Overlapping	-	C-1 H-1'
-	3.72	H"	Overlapping	-	H-1"
104.84	-	-	-	-	C-2 (quaterna)
76.95	4.15	H	d	$^{3}J_{3,4}$ 7.9	C-3 H-3
75.84	4.05	H	t	$^{3}J_{4,5}$ 7.9, $^{3}J_{4,3}$ 7.9	C-4 H-4
80.92	3.91	H	dt	$^{3}J_{5,6'}$ 2,9, $^{3}J_{5,4}$ 7.9	C-5 H-5
63.99	3.50	H'	Overlapping	-	C-6 H-6'
-	3.85	H"	Overlapping	-	H-6"

d=doublet, dd=doublet of doublets, dt=doublet of triplets, t=triplets.

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