Antiviral Activity of *Solanum paniculatum* Extract and Constituents

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Z. Naturforsch. **64c**, 813–818 (2009); received June 24/August 4, 2009

Solanum species are traditionally employed as antiherpes and anticancer agents in different countries. *S. paniculatum* has widespread ethnomedical uses in Brazil, including the treatment of viral infections. This paper reports on the isolation of neotigogenin (**1**) and the new compound $\Delta^{25(27)}$-tigogenin-3-*O*-β-D-glucopyranoside (**2**), obtained as a mixture of *R* and *S* diastereoisomers at C22 from an ethanol extract of *S. paniculatum* leaves, along with the determination of their cytotoxicity against Vero cells and antiviral effect against human herpes virus type 1 (HHV-1), murine encephalomyocarditis virus (EMCv), and vaccinia virus strain Western Reserve (VACV-WR). The extract of *S. paniculatum* inhibited HHV-1 replication [EC_{50} = (298.0 ± 11.2) μg/ml] and showed no effect on EMCv and VACV-WR. On its turn, **1** was inactive against the assayed strains but presented high cytotoxicity [CC_{50} = (2.03 ± 0.03) μg/ml], whereas **2** exhibited significant antiherpes [EC_{50} = (170.8 ± 1.7) μg/ml] and antivaccinia virus effects [EC_{50} = (177.0 ± 3.3) μg/ml], with low cytotoxicity (CC_{50} > 400 μg/ml). The results corroborate *Solanum paniculatum* as a source of cytotoxic and antiviral compounds.

Key words: Solanum paniculatum, $\Delta^{25(27)}$-Tigogenin-3-*O*-β-D-glucopyranoside, Antiviral Activity

Introduction

Several *Solanum* species are traditionally employed as anticancer and antiherpes agents around the world (Nakamura *et al*., 1996). The antiviral effect of steroidal glycosides obtained from *Solanum* species has been demonstrated against human herpes virus type 1 (HHV-1) and their cytotoxicity reported on different cell line cultures (Nakamura *et al*., 1996; Ikeda *et al*., 2000, 2003). *Solanum paniculatum* L. (Solanaceae), popularly named *jurubeba*, is a shrub found in the Brazilian savannah (Costa, 1975). The ethnomedical uses of this species include the treatment of bronchitis, cough, arthritis, jaundice, hepatitis, intestinal fevers, and stomach disorders, among others (Pio Corrêa, 1978; Di Stasi and Hiruma-Lima, 2002).

Despite the extensive traditional use of *S. paniculatum* in Brazil, most of the alleged biological activities remain to be investigated. We have previously reported the effect of a multi-herbal preparation commercialized in Brazil, which contains *S. paniculatum* in its composition, on the lipid metabolism by increasing the triacylglycerol-rich lipoprotein uptake in rats fed with high-fat diet (Botion *et al*., 2005). The chemistry of this species has been the purpose of a number of publications, revealing the presence of steroidal alkaloids like paniculidin, jurubine, solanin, solanidin, and solamargin (Schreiber and Ripperger, 1966; Ripperger *et al*., 1967; Blankemeyer *et al*., 1998), along with spirostanic glycosides (Ripperger and Schreiber, 1968) and their genins neochlorogenin and paniculogenin (Ripperger *et al*., 1967).

Viral infections are a current problem in industrialized and developing countries, accounting for severe damages in human health and economic losses in livestock. The limited number of antiviral drugs in clinical use justifies the search for new templates, and the plant chemical diversity

might represent a source of novelty (Chattopadhyay and Naik, 2007). Within this context, the aim of the present study was to evaluate *in vitro* the cytotoxic and antiviral activity of *S. paniculatum* extract and constituents against HHV-1, murine encephalomyocarditis virus (EMCv), and vaccinia virus (Western Reserve strain, VACV-WR).

Material and Methods

Plant material

The leaves of *S. paniculatum* were collected in the municipality of Divinópolis, state of Minas Gerais, Brazil, in August 2005. The species was identified by Dr. J. R. Stehmann, Department of Botany, Institute of Biological Sciences, UFMG, Belo Horizonte, Brazil, where a voucher specimen is deposited under the code BHCB 69902.

Extraction and isolation

After drying at 40 °C for 72 h, the plant material (1.510 g) was ground and percolated with 96% EtOH at room temperature. The solvent was removed in a rotary evaporator under reduced pressure at 50 ºC, leaving a dark residue (SPE, 254 g), which was kept in a desiccator until constant weight. A portion of SPE (231 g) was subjected to filtration over silica gel employing *n*-hexane, CH_2Cl_2, EtOAc, EtOAc/MeOH (1:1) and MeOH as eluents. The CH_2Cl_2 fraction (20 g) was chromatographed on a silica gel column eluted with a gradient of CH_2Cl_2 and EtOAc, giving 17 fractions (G1–G17). Fraction G4 (2.4 g), eluted with CH_2Cl_2, was further submitted to silica gel chromatography using *n*-hexane/CH_2Cl_2 (12.5:87.5) as eluent, affording compound **1** (120 mg). A portion of SPE (25 g) was also extracted by sonication with aqueous 0.5 M HCl (200 ml). The pH value was adjusted to 10–11 with NH_4OH, following partition with CH_2Cl_2 (3 × 200 ml). The organic solvent was removed and the resulting residue (1.73 g) was chromatographed on a silica gel column, employing mixtures of CH_2Cl_2/EtOAc and CH_2Cl_2/MeOH as eluents. The fraction eluted with CH_2Cl_2/EtOAc (7.5:2.5) (109.6 mg) was further purified by silica gel column chromatography employing CH_2Cl_2/MeOH (7.5:2.5) as eluent and afforded compound **2** (46.8 mg).

Structure determination

Structure determination was accomplished by spectral analysis and comparison with literature data. ^{1}H NMR, ^{13}C NMR, NOESY, TOCSY, HMQC, and HMBC spectra were were obtained in $CDCl_3$ with TMS as internal standard and were recorded on Bruker Advance DRX200 and DPX400 equipments. Chemical shifts are given as δ (ppm). HRMS were performed on a quadrupole-time of flight (TOF) hybrid spectrometer QSTAR XL from Applied Biosystems.

$\Delta^{25(27)}$-*Tigogenin-3-O-β*-D-*glucopyranoside* (**2**): White needles. – IR: ν_{max} = 3400, 2750, 1445, 1100–1000 cm^{-1}. – ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.78 (1H, s, H27a), 4.74 (1H, s, H27b), 4.42 (1H, m, H16), 4.30 (1H, d, J = 12.0 Hz, H26ax), 4.36 (1H, d, J = 8.0 Hz, H1'), 3.96 (1H, broad s, H3), 3.87 (1H, d, J = 12.0 Hz, H26eq), 3.84 (1H, d, J = 12.0 Hz, H6'a), 3.80 (1H, d, J = 12.0 Hz, H6'b), 3.64 (1H, t apparent, J = 8.0 Hz, H4'), 3.56 (1H, t apparent, J = 8.0 Hz, H3'), 3.41 (1H, m, H2'), 3.29 (1H, d, J = 8.0 Hz, H5'), 1.89 (1H, m, H5), 1.78 (1H, m, H17), 0.94 (3H, d, J = 6.0 Hz, H21), 0.79 (6H, s, H18 and H19), 0.76 (3H, d, J = 6.0 Hz, H21). – ^{13}C NMR (100 MHz, $CDCl_3$): δ = 34.50 (C1), 25.47 (C2), 74.37 (C3), 32.41 (C4), 41.81 (C5), 28.71 (C6), 31.58 (C7), 35.31 (C8), 54.27 (C9), 40.77 (C10), 20.84 (C11), 40.29 (C12), 40.77 (C13), 56.65 (C14), 32.14 (C15), 81.37/81.06 (C16), 62.46/62.36 (C17), 16.70 (C18), 11.54 (C19), 41.81/41.71 (C20), 14.51/17.36 (C21), 109.53/109.46 (C22), 29.01/28.71 (C23), 32.65/32.41 (C24), 143.92 (C25), 65.11 (C26), 108.78 (C27), 101.04 (C1'), 73.56 (C2'), 76.63 (C3'), 69.76 (C4'), 75.71 (C5'), 61.75 (C6'). – HRMS: found m/z = 599.3699 [M+Na]; calcd. for $C_{33}H_{52}O_8$ m/z = 576.766.

Cell culture and virus

Kidney cells of the African green monkey *Cercopthecus aeothiops* (Vero cell line ATCC CCL-81) were used in all experiments. Cells were grown in Dulbecco's modified Eagle medium (DMEM), containing 5% fetal bovine serum, 50 μg/ml gentamicin, 100 U/ml penicillin, and 5 μg/ml fungizone. The following strains were used in the assays: a clinical isolate of HHV-1 obtained in the Laboratory of Virus, Institute of Biological Sciences, UFMG, Belo Horizonte, Brazil, EMCv, and Vaccinia Virus strain Western Reserve (VACV-WR), which were kindly donated by Dr. I. Kerr (Cancer Research UK, London Research

Institute, London, United Kingdom) and Dr. C. Jungwirth (University of Würzburg, Germany), respectively.

Cytotoxicity assay

Vero cell monolayers were trypsinized, washed with culture medium and plated in a 96-well flat-bottomed plate with $6 \cdot 10^4$ cells per well. After 24 h of incubation, the diluted extract and compounds **1** and **2** (800–0.125 μg/ml) were added to the wells, and the plates were further incubated for 48 h at 37 °C in a humidified incubator with 5% CO_2. The supernatants were removed from the wells and 28 μl of MTT (Merck) solution (2 mg/ml in PBS) were added to each well. The plates were incubated for 1.5 h at 37 °C, and DMSO (130 μl) was added to the wells to dissolve the formazan crystals. The plates were placed on a shaker for 15 min, and the optical density was determined at 492 nm (OD_{492}) on a multi-well spectrophotometer (Stat Fax 2100) (Kumar and Das, 1996). The results were obtained from four replicates with at least four concentrations for each sample. Cytotoxicity was calculated using the equation $(A - B)/A \cdot 100$, where A and B are the OD_{492} values of untreated and treated cells, respectively.

The 50% cytotoxic concentration (CC_{50}) of the assayed samples is defined as the concentration that reduces the OD_{492} value of treated uninfected cells to 50% of that of untreated uninfected cells.

Antiviral assays

The viral samples were titrated using the TCID microculture assay, and the titre was expressed as the virus dilution which causes 100% cytopathic effect in a cell monolayer after 48 h of incubation for HHV-1 and EMCv and 72 h for VACV-WR (Rodriguez *et al.*, 1990). The determined titres were $2.5 \cdot 10^6$, $1.0 \cdot 10^6$ and $1.0 \cdot 10^6$ $TCID_{100}$/ml, respectively, for HHV-1, EMCv, and VACV-WR.

The antiviral activities of the crude extract, compounds **1** and **2** were evaluated by the MTT colorimetric assay (Betancur-Galvis *et al.*, 1999). Vero cell monolayers were grown in 96-well microtiter plates. Dilutions of the crude extract and compound **1** in non-cytotoxic concentrations were added to the wells after viral infection. The plates were incubated at 37 °C in humidified 5% CO_2 atmosphere for a period of 48 and/or 72 h. Controls consisted of untreated infected, treated non-infected, and untreated non-infected cells. Positive controls [acyclovir (Calbiochem, USA); α-2a interferon (Bergamo, Brazil)] were also employed in each assay. Cell viability was evaluated by the MTT colorimetric method as described above for the cytotoxicity assay.

The 50% antiviral effective concentration (EC_{50}) is expressed as the concentration that achieves 50% protection of treated infected cells from virus cytophatic effect. The percentage of protection is estimated by the equation $[(A - B)/(C - B)] \cdot 100$, where A, B and C are the OD_{496} values of treated infected, untreated infected and untreated non-infected cells, respectively.

Data analysis

The CC_{50} and EC_{50} values for each sample were obtained from dose-effect curves. The CC_{50} and EC_{50} values are the average of four assays carried out with four different concentrations within the inhibitory range of the samples. The therapeutic index (*i.e.* selective index, SI) is defined as CC_{50}/EC_{50}.

Results and Discussion

Fractionation of the *S. paniculatum* crude extract and sequential purification by silica gel column chromatography afforded two solids. Compound **1** was identified as neotigogenin based on ^1H and ^{13}C NMR data and by comparison with literature records for a saponin of this genin (Bernardo *et al.*, 1996; Agrawal *et al.*, 1985). Although derivatives of spirostanic steroidal sapogenins have been already described for *S. paniculatum* (Ripperger *et al.*, 1967; Ripperger and Schreiber, 1968), this is the first report on neotigogenin in this species.

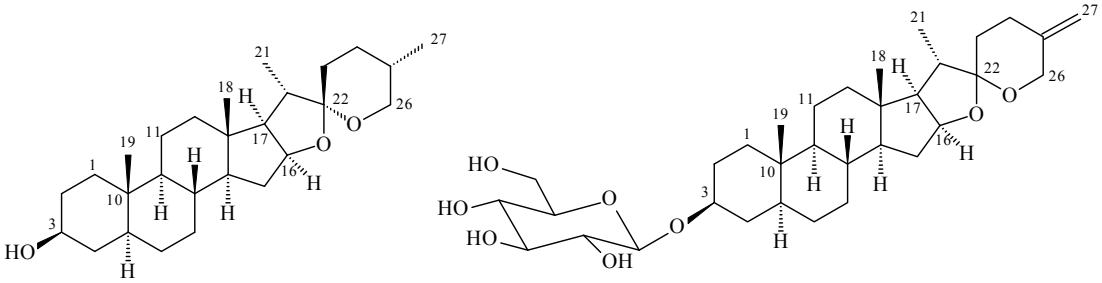
Compound **2** gave a positive Liebermann-Burchard test, and comparison of NMR spectral data of **1** and **2** obtained from 1D and 2D experiments showed similar chemical shifts for most signals in both molecules, except those of ring F in the aglycone of **2**. ^1H NMR data of **2** suggested a glycoside of the $\Delta^{25(27)}$-spirostan steroidal type skeleton, disclosed by the olefinic hydrogen signals at C27 of the aglycone at δ 4.74 (s) and 4.78 (s), along with signals of the sugar residue between δ 3.2 and 4.4. Characteristic A/B *trans*-ring fusion, indicating that **2** is a 5*R*-steroidal spirostanol deriva-

tive, was inferred from ^{13}C NMR data with signals at δ 41.81 (C5), 54.27 (C9) and 11.54 (C19) (Acharya *et al*., 2009; Zamilpa *et al*., 2002; Bernardo *et al*., 1996). Further evidence of the *trans* stereochemistry between A/B rings was accomplished by NOESY data, which showed correlations between H3 (δ 3.96) and H5 (δ 1.89, m). The aglycone of **2** was identified as 5α-spirost-25(27)-ene-3β-ol ($\Delta^{25(27)}$-tigogenin) by comparison of ^{13}C and ^{1}H NMR spectroscopic data of **2** obtained by 2D-NMR spectroscopy with those reported in the literature for borivilanoside F, a saponin isolated from *Chlorophytum borivilianum* which contains this aglycone (Acharya *et al*., 2009).

The positive TOF mass spectrum gave the quasi-molecular ion [M+Na] at *m/z* 599.3699, which allowed deducing the molecular formula $C_{33}H_{52}O_8$ for compound **2**, compatible with a monoglucoside of $\Delta^{25(27)}$-tigogenin. The chemical shift of the C3 signal (δ 74.37) indicated that the sugar moiety is linked at this position. Glucose was identified by comparison of ^{1}H and ^{13}C NMR data with reference glycosides from the literature (Bernardo *et al*., 1996) and by HSQC-TOCSY and COSY experiments, which pointed out the whole connection between hydrogen atoms of the glucose unit, starting from the anomeric carbon signal (δ 101.04). The anomeric hydrogen signal appeared in the ^{1}H NMR spectrum as a doublet centered at δ 4.36 (1H, J = 8.0 Hz, H1' of Glu), thus characterizing a β-glucoside. Therefore, the structure of **2** was identified as $\Delta^{25(27)}$-tigogenin-3-*O*-β-D-glucopyranoside (Fig. 1). The duplicity of some carbon signals in the ^{13}C NMR spectrum, notably those of the spirostane ring such as C16 (δ 81.37/81.06), C17 (δ 62.46/62.36), C20 (δ 41.81/41.71), C21 (δ 14.51/17.36), C22 (δ 109.43/109.53), C23 (δ 29.01/28.71) and C24 (δ 32.65/32.41), indicates that compound **2** was obtained as a mixture of *R* and *S* diastereoisomers at position C22. Moreover, the two doublets attributed to C21 methyl hydrogen atoms at δ 0.94 and 0.76 (3H each, J = 6.0 Hz), showing cross peaks with δ 14.51 (C21) and 17.36 (C21) in the HSQC spectrum, confirmed the epimeric mixture. As far as we know, this is the first report on the occurrence of **2** with any of the configurations at C22. On its turn, $\Delta^{25(27)}$-tigogenin has been reported to have the *R* configuration at C22 (Brunengo *et al.*, 1988).

As stated in Material and Methods, the mixture of stereoisomers **2** was isolated from a fraction obtained by a classical procedure of alkaloid extraction, namely aqueous acid extraction followed by alkalinization and partitioning with dichloromethane. Although analysis of the organic layer revealed several Draggendorf-positive spots in TLC, no alkaloid could be further purified from this fraction. The explanation for obtaining saponin **2** as an epimeric mixture could rely on ketal hydrolysis of the putative natural 22*R* compound under the acid conditions employed for extraction what would result in the open-chain δ-hydroxy ketone and its spontaneous recyclization to afford **2** as a mixture of *R* and *S* diastereoisomers. The *cis* stereochemistry between rings D and E after recyclization was confirmed by NOESY data, which showed cross peaks between H16 (δ 4.42, m) and H17 (δ 1.78, m).

The antiviral activity of *S. paniculatum* extract, compounds **1** and **2** was assayed against three virus strains and the results are depicted in Table I. The extract exhibited *in vitro* antiherpes activity with cytotoxic (CC_{50}) and antiviral (EC_{50}) values of (428.9 ± 19.2) and (298.0 ± 11.2) μg/ml, respec-



1 **2**

Fig. 1. Chemical structures of neotigogenin (**1**) and $\Delta^{25(27)}$-tigogenin-3-*O*-β-D-glucopyranoside (**2**).

Table I. *In vitro* cytotoxic (CC_{50}) and antiviral (EC_{50}) activity of *S. paniculatum* extract and constituents.

Sample	CC_{50} [μg/ml]	EC_{50} [μg/ml]				
	Vero cells	HHV-1[a]	SI[b]	EMCv[c]	VACV-WR[d]	SI[b]
Extract	428.9 ± 19.2	298.0 ± 11.2	1.4	NA[e]	NA[e]	
1	2.03 ± 0.03	NA[e]		NA[e]	NA[e]	
2	> 400	170.8 ± 1.7	> 2.3	NA[e]	177.0 ± 3.3	> 2.2
Acyclovir		40[f]				
α-2a Interferon				$1.5 \cdot 10^{6}$[fg]	$2.5 \cdot 10^{6}$[fg]	

[a] Viral titre $2.5 \cdot 10^{6}$ $TCID_{100}$/ml in 48 h. [b] SI, selective index. [c] Viral titre $1.0 \cdot 10^{6}$ $TCID_{100}$/ml in 48 h. [d]Viral titre $1.0 \cdot 10^{6}$ $TCID_{100}$/ml in 72 h. [e] NA, no activity at the assayed concentrations. [f] 80 to 100% inhibition of cytopathic effect. [g] Concentration in UI/ml.

tively. Neotigogenin (**1**) showed no activity against the assayed viral strains, but high toxicity against Vero cells [CC_{50} = (2.03 ± 0.03) μg/ml], suggesting a potential as anticancer drug. However, this compound was assayed *in vivo* against tumour models of P388 leukemia, Lewis lung carcinoma and B16 melanoma cells, as well as *in vitro* on yeast strains with defined genetic alterations measured as a screen for potential anticancer activity in the NCI anticancer drug programme, and regarded inactive in all models (Developmental Therapeutics Program NCI/HIH, available at http://dtp.nci.nih.gov/index.html. Accessed on November 25, 2008). On its turn, the 3-*O*-glucosyl derivative **2** of $\Delta^{25(27)}$-tigogenin showed significant antiherpes [EC_{50} = (170.8 ± 1.7) μg/ml] and antivaccinia virus effects [EC_{50} = (177.0 ± 3.3) μg/ml], with low cytotoxicity (CC_{50} > 400 μg/ml).

In conclusion, the results reported point out the extract of *S. paniculatum* leaves as a promising source of antiherpes and antivaccinia virus derivatives. The new $\Delta^{25(27)}$-tigogenin-3-*O*-β-D-glucopyranoside accounts for extract activity against vaccinia virus and HHV-1. Finally, our finding is in line with the traditional use of *Solanum* plants as antiherpes agents in different countries (Nakamura *et al.*, 1996).

Acknowledgements

This work was supported by funds from FAPEMIG – Fundação de Amparo à Pesquisa de Minas Gerais (Brazil). CAPES (Brazil) and CNPq (Brazil) are also acknowledged for PhD (to Y. M. V. and G. C. B.) and research (to A. B. O., E. G. K. and F. C. B.) fellowships.

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