Artigo

PHYTOCHEMICAL ANALYSIS OF HYDROETHANOLIC EXTRACTS FROM POWDERED ROOTS OF *Panax ginseng* C. A. Meyer AND *Heteropterys tomentosa* A. Juss AND EVALUATION OF THEIR EFFECTS ON ASTROCYTE CELL DEATH

Andréia Gomes Bezerra[a],*, Giuseppina Negri[a,d], Joaquim Maurício Duarte-Almeida[b,d], Soraya Soubhi Smaili[c] and Elisaldo Araújo Carlini[d]

[a]Departamento de Psicobiologia, Universidade Federal de São Paulo, 04023-062 São Paulo – SP, Brasil
[b]Laboratório de Farmacognosia e Produtos Naturais, Universidade Federal de São João del-Rei, 35501-296 Divinópolis – MG, Brasil
[c]Departamento de Farmacologia, Universidade Federal de São Paulo, 04044-020 São Paulo – SP, Brasil
[d]Departamento de Medicina Preventiva, Universidade Federal de São Paulo, 04023-062 São Paulo – SP, Brasil

The medicinal plants *Panax ginseng* C. A. Meyer (Araliaceae) and *Heteropterys tomentosa* A. Juss (Malpighiaceae) are widely and separately used by the Brazilian population as phytotherapeutics for the same medicinal purposes as tonics and to improve cognition. A chemical analysis was carried out on hydroethanolic extracts of powdered roots from *P. ginseng* and *H. tomentosa* using HPLC-DAD-ESI-MS/MS (High Performance Liquid Chromatography coupled to Diode-Array Detector and Electrospray Ionization - Mass Spectrum/Mass Spectrum). The ginsenosides Rg1, Rf, mRg and mRf were the main constituents in a hydroethanolic extract from *P. ginseng*, while in the hydroethanolic extract from *H. tomentosa*, caffeoylquinic acid derivatives and astilbin isomers were the main constituents. Concentration-time-effect curves were generated in cultures of astrocytes that were incubated with hydroethanolic extracts of these species to elucidate their toxicities. The *P. ginseng* extract was nontoxic at all of the tested times and concentrations. The hydroethanolic extract from *H. tomentosa* demonstrated toxicity at a concentration of 1000 µg/mL. *P. ginseng* extract had no protective effect against staurosporine. Many studies have demonstrated the neuroprotective effect of ginsenosides, caffeoylquinic derivatives and flavonoids.

Keywords: neuroprotection; toxicity; ginsenosides; caffeoylquinic acid derivatives; astilbin isomers.

INTRODUCTION

The *Panax* species that are most widely used as medicinal plants are *Panax ginseng* (Korean or Asian ginseng) and *Panax quinquefolius* (American ginseng). The root of *Panax ginseng* C. A. Meyer (Araliaceae) has been used for hundreds of years in Far Eastern countries as a tonic to enhance bodily functions against various ailments without apparent adverse effects.[1-3] The main bioactive constituents in *P. ginseng* are triterpenoidal saponins, known as ginsenosides. The aglycones of ginsenosides are classified into dammarane types, such as protopanaxadiol (sugar substituted at positions C-3 and/or C-20), protopanaxatriol (sugar substituted at positions C-6 and/or C-20) and oleanolic acid.[1-3] Biotransformation methods, such as hydrolysis, steaming, heating, and enzymatic and microbial transformations, produce less polar ginsenosides, which possess fewer than two sugar chains and exhibit more potent pharmacological activities than more polar ginsenosides.[4-6]

Ginsenosides exhibit a wide range of pharmacological and therapeutic properties, including immune system modulation, brain function improvement, anti-stress activity, anti-cancer activity, anti-aging activity, anti-obesity activity and anti-hyperglycemic activity.[7-9] There is an increasing evidence of the beneficial effects of ginsenosides on the central nervous system that has been attributed to the pharmacological actions of ginsenosides on cerebral metabolism, oxidative stress and radical formation, neurotransmitter imbalance, membrane-stabilizing effects, and even antiapoptotic effects.[2,3,10] *P. ginseng* exhibited a neuroprotective effect against cerebral ischemia-induced injury in rat brain by decreasing lipid peroxides and increasing the expression of superoxide dismutase and glutathione peroxidase.[11]

Heteropterys tomentosa A. Juss (Malpighiaceae) syn. *Heteropterys aphrodisiaca* is a native plant from Mato Grosso, Goiás and São Paulo States (Brazil). The roots of this species are used by the Brazilian population to improve the central nervous system and for its protective effects against a wide range of medical conditions, due to 'aphrodisiac', 'tonic' or 'stimulant' effects.[12] Treatment with hydroethanolic extract from the roots of *H. tomentosa* improves learning and memory.[13,14] Flavonoid glycosides, cardiac glycosides, saponins, and hydrolyzable and condensed tannins and astilbin isomers were detected in the hydroethanolic extract of this species.[13,14] The hydroethanolic extracts of the roots (the most commonly used portion), branches and leaves of this species did not show evidence of adaptogenic activity. Catechin, chlorogenic acid, rutin and taxifolin were the main constituents of these extracts.[15]

Apoptosis is a major form of cell death in cerebral ischemia/reperfusion pathogenesis.[16] The brain damage that occurs through cerebral ischemia is one of the major causes of disability in the western world. This process is a cascade of events that begins before the cell has lost its membrane integrity, which is induced by diverse stimuli, such as common signaling mediators and oxidative stress, among others.[16] Transient global cerebral ischemia that results from a lack of blood supply to the whole brain as a consequence of cardiac arrest leads to the selective and delayed death of certain populations of neurons.[17] The neuroprotective effects of ginsenosides, caffeoylquinic acid derivatives and flavonoids have been demonstrated in many studies.

Astrocytes are the most abundant cell type in the central nervous system (CNS); they provide structural and nutritive support to the neuronal cells and maintain CNS homeostasis.[16] The phytotherapeutic use of medicinal plants in infusion, decoction or maceration should be limited because these plants could cause serious damage to cells

*e-mail: andreia.bzr@gmail.com

when used improperly and because of the toxicological effects of their constituents on the central nervous system.

The present study evaluated the phytochemical composition of hydroethanolic extracts from powdered roots of *P. ginseng* and *H. tomentosa* in order to analyze these two different species. The roots of these species have been used in folk medicine primarily to increase resistance to physical, chemical and biological stress, to take advantage of its beneficial effects on central nervous system and to boost general vitality.[1-3,18,19] While many studies have investigated *P. ginseng*, which had been used for thousands of years in Traditional Chinese Medicine, only a few recent studies of *H. tomentosa*, which is used in Traditional Brazilian Medicine as an aphrodisiac, had been reported.[18,19] The effects of these plants on the viability and protection of astrocytes using *in vitro* assays were also evaluated.

EXPERIMENTAL

Plant material

The powdered roots of *Panax ginseng* (batch GINP04/02, valid until June 2008) and *Heteropterys tomentosa* (batch NOC06/02, valid until August 2008) were obtained commercially from Santos Flora®. The company provided the proof of botanical identification of species.[20]

Drugs and preparation of extracts

Taxifolin, rosmarinic acid and chlorogenic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Solutions of these standards (100 μg/mL in ethanol) were prepared and analyzed by HPLC-DAD (High Performance Liquid Chromatography coupled to Diode-Array Detector) to carry out the optimization of chromatographic conditions. HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). HPLC-grade water was prepared from distilled water using a Milli-Q system (Millipore, Waters, Milford, MA, USA). Prior to lyophilization, 100 g of each powdered species was extracted with 1 L of hydroethanolic solution (50%, v/v) by turbolysis as described by Bezerra *et al.*[21] The solutions were filtered, concentrated under reduced pressure in a rotary evaporator, lyophilized and stored in amber flasks in a freezer at 5 °C.

HPLC-DAD-ESI-MS/MS (High Performance Liquid Chromatography coupled to Diode-Array Detector and Electrospray Ionization - Mass Spectrum/Mass Spectrum) analysis of the hydroethanolic extracts

The lyophilized extracts (10 mg) were dissolved in 3 mL of methanol:water (20:80, v/v) and filtered through a 0.45-μm filter (German Sciences, Tokyo, Japan). Then, a 31.2-μL aliquot of each hydroethanolic extract was injected into the following HPLC system: DADSPD-M10AVP Shimadzu system equipped with a photodiode array detector coupled to Esquire 3000 Plus, Bruker Daltonics, consisting of two LC-20AD pumps, an SPD-20A diode array detector, a CTO-20A column oven and an SIL 20AC autoinjector (Shimadzu Corporation, Kyoto, Japan). The mass detector was a quadrupole ion trap equipped with an atmospheric pressure ionization source through an electrospray ionization interface that was operated in the full-scan MS/MS mode. All of the operations, acquisition and data analysis were controlled by CBM-20A software. All of the solvents were of HPLC grade and were filtered using a solvent filtration apparatus. For the separation of constituents, a reverse-phase Phenomenex Gemini C-18 (250 × 4.6 mm × 5 μm) connected to a guard column was used. The mobile phases consisted of A (0.1% aq. formic acid)

and B (methanol), with the following elution: 0 min – 20% B in A; 10 min – 30% B in A, 20 min – 50% B in A; 30 min – 70% B in A; 40 min – 90% B in A; 45 min – 40% B in A; and 50 min – 20% B in A. The flow rate was kept constant at 0.5 mL/min, and the temperature of the column was maintained at 28 °C. Double in-line detection was carried out in the DAD using 270 nm and 340 nm as the preferred wavelengths, and in-line UV spectra were recorded in the range of 200-400 nm. MS analysis was carried out using electrospray ionization at atmospheric pressure using acquisition mode scan. The ionization conditions were adjusted as follows: ion source electrospray voltage of -40 V, capillary voltage of 4500 V and capillary temperature of 325 °C. Helium was used as the collision gas and nitrogen as the nebulizing gas. Nebulization was aided with a coaxial nitrogen sheath gas at a pressure of 27 psi. Desolvation was assisted using a counter-current nitrogen flow set at a flux of 7.0 L/min. Full-scan mass acquisitions were performed in both negative and positive ion modes by scanning the *m/z* range from 100 to 1000. Collision-induced dissociation (CID) spectra were obtained in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.5 to 1.3 V. The structures of the constituents that were found in these hydroethanolic extracts were suggested mainly based on the MS spectral data conjugated with the UV-DAD spectra, reference compounds and available mass spectra data recorded in the literature. The structures of ginsenosides were based on their occurrences in *Panax* species and also through a comparison with MS spectral data of ginsenosides in the online chemical database Scifinder® (http://www.scifinder.org), Reaxys® (http://www.reaxys.com), and Riken MSn Spectral database for Phytochemicals (Respect) (http://spectra.psc.riken.jp/). The following databases were used for the identification of constituents in hydroethanolic extract from *Heteropterys tomentosa*: Riken MSn spectral database for phytochemicals (ReSpect) (http://spectra.psc.riken.jp/), Phenol-Explorer (www.phenol-explorer.eu), Chem. Spider (http://www.chemspider.com) and HMDB (www.hmdb.ca).

Culture of rat cortex astrocytes

A culture of astrocytes was obtained from the cortex of 3-day-old rats as described previously by Smaili and Russell.[22] The animals were obtained from INFAR, UNIFESP. The project was approved by the Ethics Committee of UNIFESP (0464/05).

The cells were grown in low-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 0.1% fungizone, 1% penicillin/streptomycin, 1 mM sodium pyruvate and 4 mM L-glutamine in 5% CO_2/95% air. The medium was replaced daily until confluency (after 8-14 days).

Evaluation of cell death using Hoechst 33342

Cell death was characterized by staining. Cell morphology was evaluated by fluorescence microscopy following Hoechst 33342 to verify the ability of the extracts to protect cells against toxic stimuli. The plant extracts were added to the cells at initial concentrations of 10, 100 and 1000 μg/mL and were evaluated after 6, 24 and 48 hours of incubation to assess the toxicity of the extracts. Following a separate incubation, the cells were washed with buffer and stained with Hoechst 33342 (1 μg/mL) for 15 min at room temperature in the dark. Then, the cells were washed twice with PBS, examined and photographed under a fluorescence microscope. The cells were identified by characteristic death features, such as nuclear condensation, formation of membrane blebs and apoptotic bodies. Fragmented nuclei or nuclei with condensed chromatin were counted, and the percentage of these nuclei relative to the total nuclei that were observed in each field was calculated. The results obtained for each concentration and incubation

time were determined by at least four independent experiments that were performed in triplicate.[23]

Evaluation of protective effects of the Panax ginseng extract using Hoechst 33342

To evaluate the protective effects of *P. ginseng* against a well-known apoptotic agent, cells were incubated with the extract at 100 µg/mL for 24 hours. This concentration was chosen due to the lack of toxicity observed in previous experiments. In the last 6 hours of incubation, an agent known to induce cell death was added: staurosporine (250 nmol L⁻¹), adapted from Hirata *et al.*[24] Staurosporine is a drug that exerts multiple mechanisms of action, but it acts on mitochondria and endoplasmic reticulum to increase the concentration of Ca^{2+} in the cytoplasm, which, in turn, increases the level of apoptosis.[24]

The same procedure described above for labeling with Hoechst 33342 was performed in this experiment.

Statistical analysis

Data analysis was performed using a one-way ANOVA followed by Duncan's test, when appropriate. The results are expressed as the mean ± standard error (SE) of the mean, and a significance level of 5% ($P<0.05$) was adopted.

RESULTS AND DISCUSSION

HPLC-DAD-ESI-MS/MS analyses of hydroethanolic extracts

Table 1 (*P. ginseng* and *H. tomentosa*) lists the retention times (Rt), MS spectral data and wavelengths of maximal absorption (λ_{max}) for the chemical constituents in hydroethanolic extracts. The MS spectra of all compounds are provided as supplementary information. The MS analysis did not provide information about sugar moieties that possess the same molecular formula, such as galactose ($C_6H_{12}O_6$), glucose ($C_6H_{12}O_6$), arabinofuranose ($C_5H_{10}O_5$), arabinopyranose ($C_5H_{10}O_5$), and xylose ($C_5H_{10}O_5$), or locations (C-3 or C-20 for ginsenosides). The assignment of the linkage position for attached sugars and the characterization of constituents were achieved through the positive identification of the constituents in these plants or other plant materials using literature data. For ginsenosides, the work of Zhu *et al.*[25] demonstrated that the acid hydrolyzes of ginsenosides, and the NMR experiments showed the presence of D-glucose and L-arabinose.

Ginsenosides in hydroethanolic extract from powdered roots of *Panax ginseng*

Ginsenosides are classified according to their corresponding aglycones into the following groups: protopanaxadiol (type I), protopanaxatriol (type II), ocotillol and oleanolic acid types.[2-6,25-28] For ginsenosides, the full-scan MS in positive ion mode generated sodiated molecules [M + Na]⁺ as the most intense peaks, while the negative ion mode afforded [M − H + HCOOH]⁻ adduct ions due to the use of formic acid, as well as a deprotonated molecule [M − H]⁻. Malonyl-ginsenosides, acetyl-ginsenosides and oleanolic acid ginsenosides do not form adduct ions [M − H + HCOOH]⁻.[25-28]

Mixtures of ginsenosides in extracts are difficult to analyze due to their similar polarity and existing isomers. In our study, ginsenoside isomer ions were identified through ESI-MS based on the [M − H]⁻ and [M − H + HCOOH]⁻ ions in full-scan MS in negative ion mode and on [M + Na]⁺ in positive ion mode based on their occurrence in *Panax* species and also through a comparison with the MS spectral

data of ginsenosides in the online chemical database Scifinder® (http://www.scifinder.org), Reaxys® (http://www.reaxys.com), and Riken MSn Spectral database for Phytochemicals (Respect) (http://spectra.psc.riken.jp/).

The more polar ginsenosides account for more than 80% of the total ginsenosides in wild ginseng.[4] In a study carried out by Yang *et al.*,[4] dry ginseng extract (DGE) was prepared with aqueous ethanol (80%), and a purified extract of ginseng (PEG) was produced from DGE via patented technologies in order to increase the content of less polar ginsenosides. According to some authors,[4-6] changes in the chemical composition of ginsenosides of ginseng species can occur through the hydrolysis of ginsenosides with large molecular mass, the chemical transformation of ginsenosides, a reduction in the quantity of malonyl ginsenosides and the production of 20-(R)-ginsenosides enantiomers. According to Yang *et al.*,[4] the biotransformation of ginsenosides occurs via dehydration and deglycosylation under acidic or enzymatic conditions to produce less polar ginsenosides (non-hydrophilic), which are rarely present in wild ginseng. The 22 less polar ginsenosides that were isolated from PEG were used for the identification of ginsenosides in DGE. The sugar chains at C-20 were eliminated more easily than those at C-3. Ginsenosides with sugar chains at C-20 were not isolated from the purified extract of ginseng (PGE).[4]

In our study, the ginseng extract was prepared using a mixture of aqueous ethanol (50%). Ginsenosides **1-7** in this hydroethanolic extract predominantly contain the type II 20(S)-protopanaxatriol aglycone skeleton. The types of sugar substitution were identified as hexose (glucose), deoxyhexose (rhamnose) and pentose (arabinose) based on the study of Zhu *et al.*[25] The ginsenosides Rg1, Rf and 24(R)-pseudoginsenoside F11 are isomers.[2-8,25-29] Ginsenoside Rf is found only in Asian ginseng (*P. ginseng*), while 24(R)-pseudoginsenoside F11 is present only in American ginseng (*P. quinquefolius*).[2-8,25-29]

The main ginsenosides, compounds **3** and **6**, are isomers that exhibited [M + Na]⁺ at *m/z* 823, [M − H]⁻ at *m/z* 799 and [M + HCOOH − H]⁻ at *m/z* 845 in the ESI-MS spectra (Table 1). For both ginsenosides, the molecular mass of the aglycone corresponds to 476 - type II [20(S)-protopanaxatriol], in which the sugar chains are linked to a hydroxyl moiety at C-6 and/or C-20. According to Wu *et al.*,[5] ginsenoside isomers can be grouped as [M − H]⁻ ions, as for example, *m/z* 799 (Rg1, Rf), which exhibited a different retention time. Ginsenoside Rg1 (6-*O*-glucosyl-20-*O*-glucosyl-20(S)-protopanaxatriol), molecular formula $C_{42}H_{72}O_{14}$ and molecular mass 800, contains 20(S)-protopanaxatriol as an aglycone, and the saccharide (two glucoside) moieties are linked to the aglycone through an ether linkage at C-6 and C-20.[2-8,25-29] In contrast, ginsenoside Rf (6-O-glucosyl(1-2)glucosyl-20(S)-protopanaxatriol), also with molecular formula $C_{42}H_{72}O_{14}$ and molecular mass 800, contains 20(S)-protopanaxatriol as an aglycone, and the disaccharide (diglucoside) moiety is linked to the aglycone through an ether linkage at C-6.[2-8,25-29] According to Xu *et al.*,[3] ginsenoside Rg1 is more polar and consequently exhibited a shorter retention time on reversed-phase HPLC columns than its isomer Rf. Compound **3** was identified as Rg1 and compound **6** as Rf. The presence of ginsenosides Rg1 and Rf in this hydroethanolic extract may indicate that these ginsenosides were produced by the decarboxylation of the malonylated ginsenosides, according to some studies.[4,5] The ginsenosides Rg1 or Rf and malonyl-ginsenosides were identified in the dry extract of ginseng (DGE) that was prepared using an aqueous solution with 80% methanol, but not in the purified extract of ginseng (PEG).[4] Ginsenosides Rg1, Rf, mRg1 and mRf were identified from white ginseng and processed ginseng.[5] The ginsenosides Rg1 and Rf were also identified in Ginseng Radix and are used as multi-components in traditional complex herbal medicines.[6]

The ESI-MS spectrum of compound **1** showed [M + Na]$^+$ at *m/z* 985, suggesting that this ginsenoside possesses a molecular mass of 962 mass units and 20(S)-protopanaxatriol as an aglycone linked to three hexosyl moieties. Three ginsenoside isomers (Re1, Re2 and Re3) with 962 mass units that possess the molecular formula $C_{48}H_{82}O_{19}$, 20(S)-protopanaxatriol as an aglycone and three glucosyl moieties were reported by Zhu *et al.*[25] The glucosyl moieties were determined through acid hydrolysis and ^{2}DNMR experiments.[25] Therefore, compound **1** was tentatively characterized as a protopanaxatriol triglucoside.

For compound **2**, the ESI-MS spectra showed [M + Na]$^+$ at *m/z* 955, [M – H]$^-$ at *m/z* 931 and [M – H + HCOOH]$^-$ at *m/z* 977 (Table 1), indicating that ginsenoside **2** possesses 20(S)-protopanaxatriol as an aglycone and three sugar moieties (two hexoses, most likely glucoses, and one pentose, probably arabinose). Ginsenoside Re4, with a molecular mass of 932 and the molecular formula $C_{47}H_{80}O_{18}$, was isolated from *P. ginseng* by Zhu *et al.*[25] For Re4, glucosyl and arabinosyl moieties were determined through acid hydrolysis that yielded D-glucose and L-arabinose and by ^{2}DNMR experiments.[25] Therefore, compound **2** was suggested to be a protopanaxatriol arabinosyl diglucoside or Re4.

The ESI-MS spectra of compound **7** showed [M + Na]$^+$ at *m/z* 807, [M – H]$^-$ at *m/z* 783 and [M – H + HCOOH – H]$^-$ at *m/z* 829. Ginsenoside 20(S)-Rg2 possesses a molecular mass of 784 and is formed from 20(S)-protopanaxatriol aglycone linked to a disaccharide (glucosyl rhamnoside) moiety through an ether linkage at C-6.[4-6,27-30] In addition, other ginsenoside isomers with [M – H]$^-$ at *m/z* 783 could be 20(R)-Rg2, 20(S)-Rg3, 20(R)-Rg3 and F2. 20(S)-Ginsenosides eluted before 20(R)-ginsenosides.[4,5] In addition, 20(R)-ginsenosides are rare in natural crude ginseng and can be generated by the addition reaction with the hydroxyl moiety at C-20 after the elimination of the sugar chains.[4,5] The 20(R)-ginsenoside enantiomers 20(R)-Rg2

and 20(R)-Rg3 are produced during the manufacture of ginseng preparations.[4,5] Ginsenoside **7** was characterized as the protopanaxatriol glucosyl rhamnoside or ginsenoside 20(S)-Rg2 - (6)-(alpha--L-rhamnopyranosyl(1-rham-2-glu)-beta-D-glucopyranosyl)-20(S)--protopanaxatriol.[4-6,27-31] Red ginseng exhibited more anti-cancer activity compared to white ginseng due to the ginsenosides that were generated from processing, such as ginsenoside Rg3.[4,5] Ginsenosides 20(S)-Rg2, 20(R)-Rg2, 20(S)-Rg3, 20(R)-Rg3 and F2 were isolated in purified extract of ginseng.[4]

The ESI-MS spectra of compounds **4** and **5** showed [M + Na]$^+$ at *m/z* 909 and [M – H]$^-$ at *m/z* 885. No adduct ion [M – H + HCOOH]$^-$ was observed, indicating that these compounds may be malonyl ginsenosides.[2,7,29-31] Ginsenosides **4** and **5** display an identical molecular mass of 886, differing solely in reversed-phase retention times, as shown in Table 1. Ginsenosides **4** and **5** were suggested as malonyl-Rg1 and malonyl-Rf, respectively. Malonyl ginsenosides are an important form of ginsenosides in white ginseng[28] that can be de-malonylated and transformed to their corresponding ginsenosides upon processing.[4,5] Thus, the high content of ginsenosides Rg1 and Rf could indicate that these ginsenosides were probably produced through de-malonylated malonyl-Rg1 and malonyl-Rf, respectively.

Hydroxycinnamic acid and flavonoids in hydroethanolic extracts from the roots of *Heteropterys tomentosa*

Little is known about the chemical composition of *H. tomentosa*.[32] The following databases were used for the identification of constituents: Riken MSn spectral database for phytochemicals (ReSpect) (http://spectra.psc.riken.jp/), Phenol-Explorer (www.phenol-explorer.eu), Chem. Spider (http://www.chemspider.com) and HMDB (www.hmdb.ca). The ESI-MS spectrum of compound **8** revealed a deprotonated molecular ion at *m/z* 683, which, after MS/MS fragmentation,

Table 1. Retention times (Rt) and MS spectral data for the chemical constituents **1-7** found in hydroethanolic extracts from powdered roots *Panax ginseng* and for the chemical constituents **8-18** found in hydroethanolic extracts from powdered roots *Heteropterys tomentosa*

Peak	Rt min		[M + Na]$^+$ *m/z*	[M + HCOOH – H]$^-$ *m/z*	[M – H]$^-$ *m/z*	Suggested structure
1	27.1		985	-	-	protopanaxatriol triglucoside
2	33.8		955	977	931	protopanaxatriol arabinosyl diglucoside or Re4
3	35.1		823	845	799	ginsenoside Rg1
4	35.9		909	-	885	malonyl-Rg1
5	36.5		909	-	885	malonyl-Rf
6	40.3		823	845	799	ginsenoside Rf
7	41.3		807	829	783	20(S)-Rg2

Peak	Rt min	λ max (nm)	[M + H]$^+$ *m/z*	(-)ESI-MS/MS *m/z* (%base peak)	[M – H]$^-$ *m/z*	Suggested structure
8	5.7	310		521, 341 (100), 179 (50)	683	rosmarinic acid diglucoside
9	9.7	ND		-	191	quinic acid
10	17.2	280		567 (80), 375 (100)	729	3,5-dihydroxy-7,3',4',5'-tetramethoxy flavanonol hydroxyferuloyl glucoside
11	21.4	330, 300		353, 191	571	3-*O*-caffeoylquinic acid malonyl pentoside
12	22.9	280		587	739	taxifolin digalloyl pentoside
13	24.2	280		375	537	3,5-dihydroxy-7,3',4',5'-tetramethoxy flavanonol glucoside
14	25.3	330		353	515	3,4-dicaffeoylquinic acid
15	27.0	280	451	303, 285, 177, 151	449	taxifolin rhamnoside
16	29.6	330		353	515	3,5-dicaffeoyl quinic acid
17	30.4	330, 300		359	651	rosmarinic acid dirhamnoside
18	32.5	330, 300		515	677	3,4,5-tricaffeoylquinic acid

*Suggested structure is tentative only, based on mass spectral/UV data.

produced fragment ions at *m/z* 521, attributed to the loss of a glucose moiety, and an abundant fragment ion at *m/z* 341, indicating the presence of a caffeoyl hexoside moiety. The MS/MS/MS spectrum of the precursor ion at *m/z* 341 produced an abundant fragment ion at *m/z* 179 (deprotonated caffeic acid) (Table 1). Rosmarinic acid is composed of one caffeic acid unit and one danshensu unit conjugated via an ester. According to MS spectral data reported by Gouveia and Castilho,[33] compound **8** was suggested to be rosmarinic acid diglucoside. Compound **9** exhibited a deprotonated molecule at *m/z* 191 and was suggested to be quinic acid.

Chlorogenic acids are a family of esters formed between quinic acid and *trans*-cinnamic acids, such as, caffeic, *p*-coumaric and ferulic acid, which can be esterified at one or more of the hydroxyls at positions 1, 3, 4, and 5 of quinic acid to generate a series of positional isomers. In RPHPLC, the isomers of the monocaffeoylquinic acids elute in the order of 3-, 5-, and 4-isomers. For dicaffeoylquinic acid, the elution order is 3,4-isomer, 3,5-isomer, and 4,5-isomer.[34]

Compounds **11**, **14**, **16**, **17** and **18** showed a UV/vis absorption typical of caffeoylquinic acid derivatives, with maximum absorption between 305 and 330 nm (band I) and a shoulder between 290 and 300 nm (band II).[34,35] The ESI-MS spectra of compound **11** exhibited a deprotonated molecular ion at *m/z* 571, which, after MS/MS fragmentation, produced an abundant fragment ion at *m/z* 353 due to the loss of 218 mass units (pentoside – 132 mass units more than malonyl – 86 mass units moieties) and a fragment ion at *m/z* 191 (for quinic acid). According to Gouveia and Castilho,[33] compound **11** was suggested to be 3-*O*-caffeoylquinic acid malonyl pentoside.

The ESI-MS spectra of compounds **14** and **16** showed protonated and deprotonated molecules at *m/z* 517 and *m/z* 515, respectively, suggesting di-*O*-(*E*)-caffeoylquinic acid positional isomers. The MS/MS spectrum of the precursor ion at *m/z* 515 showed a fragment ion at *m/z* 353. Based on the retention time,[34] compounds **14** and **16** were suggested to be 3,4-di-*O*-(*E*)-caffeoylquinic acid and 3,5-di-*O*-(*E*)-caffeoylquinic acid, respectively.

The ESI-MS spectrum of compound **18** displayed a deprotonated molecule at *m/z* 677, which readily lost a caffeoyl moiety (162 mass units) to produce an abundant fragment ion at *m/z* 515. This compound could be either 1,3,4-tricaffeoylquinic acid or 3,4,5-tricaffeoylquinic acid. Caffeoylquinic acids with a larger number of free equatorial hydroxyl groups in the quinic acid residue are generally more hydrophilic than those with a larger number of free axial hydroxyl groups.[36] Compound **30** was suggested to be 1,3,4-tricaffeoylquinic acid.

For compound **17,** the ESI-MS spectrum exhibited a deprotonated molecule at *m/z* 651, which, after MS/MS experiments, revealed a fragment ion at *m/z* 359 (deprotonated rosmarinic acid), attributed to the loss of dirhamnosyl moiety. Compound **17** was suggested to be rosmarinic acid dirhamnoside.

Compounds **10**, **12**, **13** and **15** exhibited UV spectra with a maximum absorption at 280 nm, which is typical of flavanonols. The ESI-MS spectra of compounds **10** and **13** exhibited deprotonated molecules at *m/z* 729 and *m/z* 537, respectively. The molecular mass of compound **10** is 192 mass units (hydroxyferuloyl moiety) more than that of compound **13**, with which it shares a similar fragmentation pattern. The MS/MS spectra for both compounds showed fragments ions attributed to the loss of glucose (162 mass units) at *m/z* 567 for compound **10** and at *m/z* 375 for compound **13**. Both compounds exhibited a fragment ion at *m/z* 375 (Table 1) attributed to aglycone 3,5-dihydroxy-7,3',4',5'-tetramethoxy flavanonol. Compound **13** was tentatively assigned as 3,5-dihydroxy-7,3',4',5'-tetramethoxy flavanonol glucoside and compound **10** as 3,5-dihydroxy-7,3',4',5'-tetramethoxy flavanonol hydroxyferuloyl glucoside.

The ESI-MS spectra of compound **15** exhibited sodiated, protonated and deprotonated molecules at *m/z* 473, 451 and 449, respectively. The MS/MS spectrum in negative ion mode produced an abundant fragment ion at *m/z* 303, corresponding to the loss of 146 mass units (rhamnose moiety), indicating taxifolin, also known as dihydroquercetin, as an aglycone. The fragments ions at *m/z* 285, 177 and 151 were attributed to taxifolin and are produced via a retro Diels-Alder reaction. These fragments provide information on the number and type of substituents in the A- and B-rings of flavonoids.[33] Three isomers are known for taxifolin rhamnoside: astilbin (absolute configuration 2R, 3R), isoastilbin (absolute configuration 2R, 3S) and neoastilbin (absolute configuration 2S, 3S) (Table 1). The presence of these three isomers in this species was reported by Galvão *et al.*[14] Compound **15** was suggested to be taxifolin rhamnoside.

The ESI-MS spectrum of compound **12** showed a deprotonated molecule at *m/z* 739, which, after MS/MS experiments, produced a fragment ion at *m/z* 587, attributed to the loss of galloyl moiety (152 mass units).[37] Compound **12** was suggested to be taxifolin digalloyl pentoside. The exact location of the galloyl group on the glycosidic moiety was difficult to determine based on mass spectra.

Properties affecting the central nervous system, such as anxiolytic, antidepressant, learning and memory improvement, antioxidant effects and the protective activities of reproductive male organs, are the most important biological activities that have been reported for species of *Heteropterys*. Flavonoids are the main constituents in *Heteropterys* species, followed by hydroxycinnamic acids.[18] The roots of *H. tomentosa* are used as aphrodisiacs, tonics or stimulants, as well as for nervous debility, vasodilatation properties and antiulcer activity in Brazil. The qualitative analysis of the plant extract from *H. tomentosa* roots revealed the presence of glycosides, polyphenols, tannins, alkaloids, saponins, triterpenoids and anthracenes and detected the flavonoids catequin and taxifolin derivatives.[13-15,19]

In vitro experiments - evaluation of cell death using Hoechst 33342

As seen in Figure 1A, a one-way ANOVA performed through the calculation of the percentages of dead cells did not show differences between cells that were incubated with the *P. ginseng* hydroethanolic extract and the control group cells. The *P. ginseng* extract did not increase cell death; therefore, no toxicity was demonstrated in the three doses of the extract or in the three incubation periods that were assessed.

The *H. tomentosa* hydroethanolic extract exhibited toxicity when incubated with cells for 24 hours (Figure 1B). An ANOVA detected a significant effect [$F(3.32)= 21.83$; $P<0.05$] on the percentage of cell death. Duncan's test showed that a concentration of 1000 µg/mL caused an increase in the percentage of cell death relative to that of all of the other groups ($P<0.01$). Concentrations of 10 and 100 µg/mL of hydroethanolic extract showed similar effects after 6 hours of incubation, as was observed in the control group, which underscores the low cellular toxicity of these doses during this period.

It was expected that after 48 hours of incubation, 1000 µg/mL concentrations of the *H. tomentosa* extracts would further decrease the viability of astrocytes, but the results indicated that the cells did not differ from the control. This result may have occurred because the control cells began to run out of nutrients after 48 hours in unchanged medium, leading to a loss of adherence and natural death. The loss of adherence hinders the visualization of cells when stained with Hoechst 33342, making difficult the visualization of dead cells after 48 hours of incubation, which was consequently not carried out adequately.
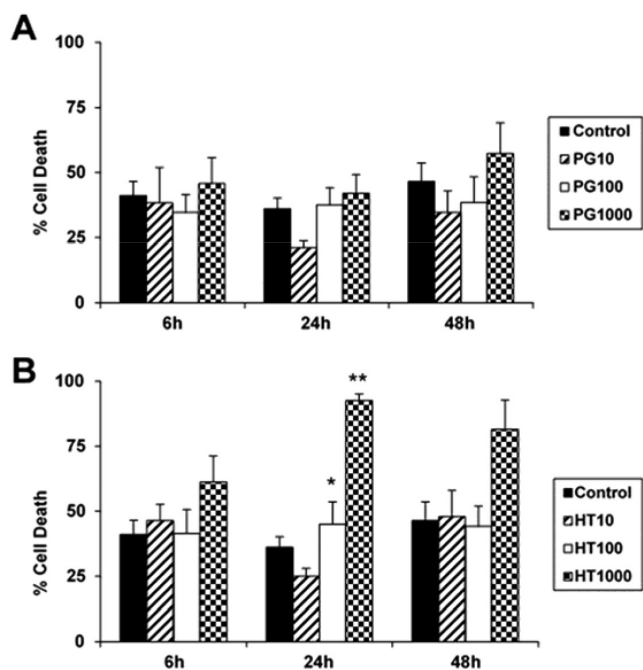
*Figure 1. Cells stained with Hoechst 33342. Percentage of dead cells after incubation for 6, 24 and 48 hours at doses of 10, 100 and 1000 µg/mL. The data represent the mean ± SEM of four independent experiments that were performed in triplicate. (A) Extract of P. ginseng (PG) ANOVA, NS. (B) Extract of H. tomentosa (HT) ANOVA / Duncan: * P<0.05 (compared to group HT10 at 24 hours); ** P<0.05 (compared with all other groups at 24 hours)*

Evaluation of protective effects of the *Panax ginseng* extract using Hoechst 33342

Cells that were incubated with staurosporine showed a statistically significant increase in death compared to the control group and the *Panax ginseng* group (PG) [Figure 2]. Preincubation with the *P. ginseng* extract did not prevent apoptosis induced by staurosporine.
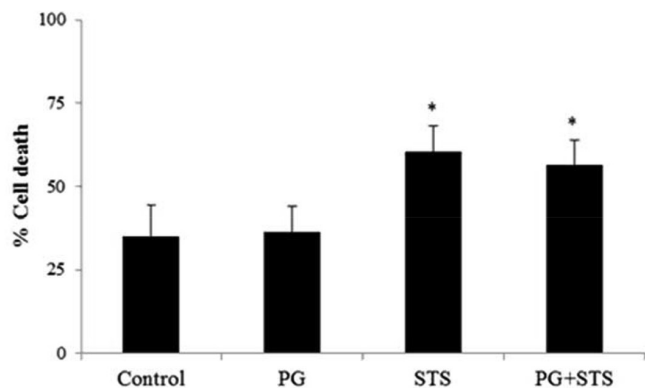

*Figure 2. Cells stained with Hoechst 33342. Effect of the extract of P. ginseng (PG - 100 µg/mL) against staurosporine (STS - 250 nM), an apoptotic agent. The percentage of cell death of STS was compared to that of the control group. The data represent the mean ± SEM of four independent experiments that were performed in triplicate. ANOVA / Duncan: * P<0.05*

In a model of apoptosis induced by oxidative stress generated by H_2O_2, an aqueous extract of *P. ginseng* maintained the antioxidant enzyme activity of astrocytes and reduced cell death.[38] In SK-N-MC neuronal cells, the treatment that was carried out with an extract of *P. ginseng* decreased apoptosis induced by a neurotoxic pollutant. In addition, very high concentrations decreased cell viability,

demonstrating the importance of such research in determining the optimal concentrations for cytoprotection.[39]

Central nervous system protection of ginsenosides, caffeoyl quinic derivatives and taxifolin derivatives

The main ginsenosides in hydroethanolic extract from powdered roots of *P. ginseng* were Rg1 and Rf, which could be produced through de-malonylated malonyl-Rg1 and malonyl-Rf, respectively. Ginseng total saponins improved the neurological deficits after focal cerebral ischemia in rats by inducing endogenous neural stem cell activation and enhancing adult central nervous system regeneration.[40] A reduction of the secondary injury was observed after traumatic brain injury only in rats that were treated with ginseng total saponins. This decrease occurred through the inhibition of oxidative and nitrative stress, attenuation of inflammatory response and reduction of apoptotic cell death.[41]

Ginsenoside Rg1 protected cells by antagonizing apoptosis[42] and exhibited neuroprotective effects;[43] in addition, Rg1 is an antiapoptotic molecule that is capable of blocking the caspase-dependent signaling cascade in Jurkat cells.[44] Ginsenoside Rg1 exhibited attenuated methamphetamine-induced dopaminergic degeneration *in vivo* through the inhibition of impaired enzymatic antioxidant systems, mitochondrial oxidative stress and apoptosis,[43] while ginsenoside Rb1 prevented apoptosis induced by amyloid.[44,45]

The main constituents in hydroethanolic extract from powdered roots of *H. tomentosa* were caffeoylquinic derivatives and taxifolin derivatives (astilbin). 3-*O*-Caffeoylquinic acid, 5-*O*-feruloylquinic acid, 3,5-*O*-dicaffeoylquinic acid and 3,4-*O*-dicaffeoylquinic acid are potent antioxidant compounds that quench radical species and inhibit H_2O_2-induced apoptosis via suppressing mitochondrial membrane depolarization and caspase-9 activation.[46] Chlorogenic acid induced the expression of NADPH:quinine oxidoreductase 1 in neuronal cells, protecting neurons from H_2O_2-induced apoptosis by the up-regulation of this antioxidant enzyme.[10] Caffeoyl quinic acid exhibited a neuroprotective effect on mouse brain[47] and alleviated oxidative stress.[48] A hydroethanolic extract from *H. tomentosa* containing astilbin, isoastilbin and neoastilbin exhibited a positive effect on learning and memory.[14]

Flavonoids, due to their antioxidant activities, can prevent oxidative stress, one of the causes of disorders that affect the central nervous system. In addition, as multi-target botanical therapeutics, these compounds can also modulate both enzyme and receptor activities.[49] Taxifolin exerted cardioprotective effects against diabetic cardiomyopathy through the inhibition of oxidative stress and cardiac myocyte apoptosis[50] and exhibited a protective effect against glutamate induced oxidative injury in HT22 cells.[51] Diosmin, a flavonoid glycoside, possesses an anti-apoptotic effect in the treatment of varicose veins and renal injury and protects against cerebral ischemia/reperfusion injury.[46] However, flavonoids can act as pro-oxidants at certain concentrations, depleting the nuclear antioxidant defense systems and leading to oxidative DNA damage.[52]

CONCLUSIONS

In the hydroethanolic extract from *P. ginseng*, the ginsenosides Rg1 and Rf are the main ginsenosides and are considered more active than more polar ginsenosides. Some studies have demonstrated that these ginsenosides are produced by the decarboxylation of the malonylated ginsenosides malonyl-Rg1 and malonyl-Rf. In the hydroethanolic extract from *H. tomentosa*, the main constituents are taxifolin derivatives and caffeoylquinic acids. These species are used as phytotherapeutics in folk medicine, and their pharmacological

properties have been attributed to the presence of ginsenosides in the roots of *P. ginseng* and flavanonols in the roots of *H. tomentosa*. Many studies have demonstrated the beneficial effects of flavonoids and ginsenosides on central nervous system regeneration.

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