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Original Article

Saponins, tannins and flavonols found in hydroethanolic extract from *Periandra dulcis* roots

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Periandra dulcis Mart. ex Benth. Fabaceae (Syn.: *P. mediterranea* (Vell.) Taub.) is native to the northern and middle parts of Brazil. In Brazilian ethnomedicine, their roots are used as anti inflammatory, expectorant, diuretic and laxative. An HPLC-ESI-MS/MS system was employed to provide a rapid method to make a tentative characterization of the compounds found in the hydroethanolic extract from *P. dulcis* roots. The structures of sixteen compounds found in this hydroethanolic extract were suggested mainly by MS data conjugated with the UV-DAD spectra, reference compounds and available mass spectra data in literature. Saponin derivatives of hederagenin and soyasapogenol E, such as hederagenin-3-*O*-rhamnosyl glucosyl glucuronide, soyasapogenol *E*-3-*O*-rhamnosyl glucosyl glucuronide and periandrin isomers were found as the main constituents, with a minor content of flavonols quercetin and myricetin glycosides derivatives and hydrolysable tannins, such as dihexahydroxydiphenoyl galloyl glucoside and trisgalloyl hexahydroxydiphenoyl glucose. To the best of our knowledge, with exception of periandrins found in the roots, nothing has been published about the chemical composition of *P. dulcis*..

Introduction

Periandra dulcis Mart. ex Benth., Fabaceae (Syn.: *P. mediterranea* (Vell.) Taub.) is native to the northern and middle parts of Brazil (Corrêa, 1926; Cruz, 1965). Their roots are known by their sweet taste and have been used in Brazilian ethnomedicine as anti-inflammatory, expectorant, diuretic and laxative (Suttisri et al., 1993). Saponins, such as periandrins, periandradulcins and several related triterpenes have been reported as constituents of *P. dulcis* roots (Hashimoto et al., 1984a,b; Ikeda et al., 1991; Santos et al., 1997; Suttisri et al., 2003). Saponins consist of a sugar moiety glycosidically linked to a hydrophobic aglycone (sapogenin). Triterpenoid saponins consist of thirty carbon aglycones and are commonly found in plants; whereas steroidal saponins containing 27 carbons in the aglycones are rare in nature, found only in monocotyledons (Chapagain and Wiesman, 2008).

Saponins are effective in inhibiting tumor cell proliferation (Gauthier et al., 2011), lowering blood cholesterol and triacylglycerol (Megalli et al., 2006). They, as well, exhibit antifungal, cytotoxic, anti-platelet aggregation (Huang et al., 2007) and haemolytic activity (Gauthier et al., 2009; Tava et al., 2009). Flavonoids are a class of polyphenolic compounds, usually present in glycosidic form, widely distributed in fruits and vegetables (Zheng et al., 2008), which exert many biological activities (Kaume et al., 2012). Flavonoids *O*-glycosides have

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sugar substituents bound to a hydroxyl group of the aglycones, on the other hand, in *C*-glycosides the sugar substituents are attached to the aglycone through a carbon-carbon bond. For *C*-glycosylflavones, the negative ion mode revealed that the main fragments observed in the MS/MS spectra occur through the cross-ring cleavages in the sugar units (intra glycosides cleavage), with the more extensive fragmentation at the *C*-6 sugar residue, and from loss of water molecules. For *O*-glycosylflavones, the fragmentations involve the heterocyclic cleavage at the glycosidic *O*-linkage (hemiacetal *O*-*C* bond) with a concomitant H-rearrangement, leading to the elimination of the saccharide residue (Ferreres et al., 2008; Figueirinha et al., 2008; Abad-Garcia et al., 2009).

The most frequent sugar found in flavonoid glycosides are glucose, rhamnose, arabinose and glucuronic acid (Vukics and Guttman 2010, Kaume et al., 2012). Two sugar can be attached to the flavonoid aglycone, either at two different positions (di-*O*-glycosides, di-*C*-glycosides, di-*C*,*O*-glycosides) or at the same position (*O*-diglycosides, *C*,*O*-diglycosides) (Barreca et al., 2013; Vukics and Guttman 2010; Kaume et al., 2012).

Hydrolysable tannins are secondary metabolites widespread throughout the plant kingdom. They are characterized by their water solubility and molecular masses between 500 and 5000 u, and are formed by esters of gallic acid, glucose and products of their oxidative reactions. Among the hydrolysable tannins, the gallotannins hydrolyze to yield gallic acid, while ellagitannins hydrolyze to yield ellagic acid. Ellagitannins are the products of oxidation, leading to C-C linkages between suitably orientated galloyl residues of glucogalloyl molecules that form hexahydroxydiphenoyl (HHDP) units (Buzzini et al., 2008; Okuda and Ito, 2011). The distribution of gallotannins is limited in nature, while ellagitannins are widespread in many plant families. Condensed tannins (syn. proanthocyanidins) are derivatives of catechin and esters of gallic acid with quinic acid (Arapitsas, 2012).

The constituents present in the hydroethanolic extract of *P. dulcis* were analyzed using high performance liquid chromatography (HPLC) coupled with diode array (DAD) UV spectroscopy, electrospray ionization mass spectrometry and tandem mass spectrometry. HPLC has improved the analytical ability for the study of complex plant extracts (Zhang and Ye, 2009; Armirotti et al., 2007a,b; Vessecchi et al., 2010, 2011). ESI-MS and ESI-MS/MS analyses are important tools for the characterization and identification of metabolites found in plants (McLuckey and Wells, 2001), peptides (Murray et al., 2013; Vessecchi et al., 2011) and other natural products (Andreo et al., 2012). Also, tandem mass spectrometry (MS/MS) has been successfully applied for the structural characterization of metabolites found in plants (Zhang and Ye, 2009; Rodriguez-Perez et al., 2013).

The purpose of this investigation was to elucidate the structure of the constituents found in the hydroethanolic extract from the roots of *P. dulcis* by combining data obtained by DAD and electrospray ionisation ESI/MS, after the separation of chemical constituents by reversed-phase HPLC. There is very few data about the chemical composition of this species, with exception of periandrins and periandradulcins (saponins) found in the roots.

Material and Methods

Material

Rutin, apigenin, luteolin, quercetin, myricetin, rhamnetin and glycyrrhizic acid were purchased from Sigma-Aldrich Chemical CO. (St. Louis, MO, USA). Solutions of 100 µg/ml of these compounds were prepared in ethanol and analyzed by HPLC-DAD. The purity of standards was confirmed to be no less than 96% through HPLC analysis. 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).

Plant material

The roots of *Periandra dulcis* Mart. ex Benth., Fabaceae (Syn.: *P. mediterranea* (Vell.) Taub.) (Alcaçuz Nacional Pó) from northeastern region of Brazil, were supplied in pieces with a diameter of more than 0.30 mm (product number 2448) by Quimer Ervas & Especiarias S.A. The material was deposited at the University herbarium Prof. Carlos Stellfeld, Laboratório of Farmacognosia of UFPR (accession No. 262.1).

Extraction

The botanical material was grounded and passed through a 30 Mesh screen, it was then stored in sealed glass vials. A lyophilized extract of the roots of *P. dulcis* was prepared using 100 g of the powder of plants, previously extracted with 1 liter of hydroethanolic solution 50% (v/v), by turbo-extraction (Mendes et al., 2002). This process disintegrates a high proportion of the plant cells through shear force using a high-speed mixer (5000-20,000 rpm), facilitating the solubilization of compounds in the extractor liquid. The crude preparation was filtered initially through cotton and then through Whatman filter paper no. 1 and concentrated under reduced pressure in a rotary evaporator to obtain a crude extract, and was lyophilized using a lyophilizator (3 atm of pressure and temperature of –40ºC) for 48 h,and the extract was stored in amber flasks at 5ºC (freezer).

Hydrolysis of flavonoids O-glycosides

The free flavonoid aglycones of the *O*-glycosides were released through acidic hydrolysis as follows: 50 mg of lyophilized extract were dissolved in 4 ml of 10% (v/v) sulfuric acid (H_2SO_4), and heated in boiling water for 1 h. After cooling, the reaction mixture was neutralized using a saturated solution of sodium carbonate and then filtered under reduced pressure. The filtrate was concentrated to approximately 1 ml.

Phytochemical analysis

The lyophilized hydroethanolic extract from the roots of *P. dulcis* was screened by thin layer chromatography (TLC), for phenolic acids, flavonoids, saponins, lignans and tannins according to Stahl (1969) and Wagner and Bladt (1996).

Solutions of rutin, apigenin, luteolin, quercetin, myricetin, rhamnetin and glycyrrhizic acid were prepared at a concentration of 1 mg/ml and spotted onto a silica gel plate (Silica gel coated TLC plates; Merck) in order to tentatively characterize the chemical class of constituents found in this extract. The lyophilized hydroethanolic extract was dissolved in methanol (10 mg/ml) and also spotted onto a silica gel plate. A developing system was adapted for each class of compounds. Plates were visualized under a UV detector at 254 nm and 365 nm before and after spraying with chromogenic agents including anisaldehyde sulfuric acid (saponins) and ferric chloride ($FeCl_3$) (phenolic compounds, including flavonoids and tannins). Glycyrrhizic acid was used for the detection of saponins. The presence of flavonoids was screened by spraying the plates with 5% aluminum chloride ($AlCl_3$) in ethanol.

RPHPLC-DAD-ESI-MS/MS analyses

For the reversed phase high performance liquid chromatography (RPHPLC) analysis, lyophilized extract (10 mg/3 ml) was dissolved in water:methanol (80:20 v/v) and filtered with a 0.45 µm filter, prior to injection of 30.0 µl into the HPLC system. Spectral UV data from all peaks in the range 240-400 nm was collected and chromatograms were recorded at 360 and 270 nm for phenolic compounds. The HPLC-DAD-ESI-MS system consisted of a Shimadzu SPD-M10AVP DAD HPLC detector equipped with a photodiode array detector coupled to a Esquire 3000 Plus, Bruker Daltonics quadrupole, fitted with an electrospray ionization source running in negative mode. The mass detector was a quadrupole ion trap equipped with an atmospheric pressure ionization source through electrospray ionization interface. All the operations, acquisition and data analysis were controlled by SCL-10AVP software. The mobile phases consisted of eluent A (0.1% aq. formic acid) and eluent B (methanol), and the elution was programmed as follows: 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 finally returned to the initial conditions (20% B) to re-equilibrate the column prior to another run, using a reversed phase C18 column (Zorbax 5B RP-18) (4.6×250 mm, 5 µm), connected to a guard column. Formic acid is a common modifier for reversed-phase HPLC and its volatility also makes it highly suitable for mass spectrometry. This acid added in the mobile phase improved the ionization of the constituents and had shown not to alter the chromatographic retention time significantly. The flow rate was kept constant at 0.5 ml/min^{-1}, and the temperature of the column was maintained at 28°C. The ionization conditions were adjusted as follows: electrospray voltage of the ion source was -38 V, a capillary voltage of 4400 V and a capillary temperature of 325°C. Helium (He) was used as the collision gas and nitrogen (N_2) as the nebulizing gas. Nebulization was aided with a coaxial nitrogen sheath gas provided at a pressure of 26 psi. Desolvation was facilitated using a counter-current nitrogen flow set at a flux of 7.0 l/min. The data dependent MS/MS events were acquired on the most intense ions detected in full scan analysis. For deprotonated molecules, collision induced dissociation (CID) spectra were performed in the ion trap using helium as the collision gas. The collision energy was set with voltage ramping cycles from 0.5 up to 1.3 V. The MS conditions were mass range of 100-1000 with the resolution set at 30 000, using the normal scan rate tube lens-110.0 V. The constituents were detected through ion trap mass spectrometry in negative ion mode and on-line diode array ultraviolet visible spectroscopy. The structures of 16 compounds found in this extract were proposed mainly based on the MS data conjugated with the UV-DAD spectra, reference compounds and available mass spectra data in literature.

Results

The hydroethanolic extract of *P. dulcis* was obtained with a yield of 35.1 g using 100 g of dried roots. The data obtained in the analysis of the dried roots for water content (6.13%), total ash (3.78%) and acid-insoluble ash (2.1%), contribute to the quality control of the plant extract, since the levels are within the parameters found in most plant drugs in the pharmacopeia (Brandão et al., 2006; Farmacopéia Brasileira, 1977; 2010).

To characterize the qualitative chemical profile, this extract was initially analyzed via thin-layer chromatography (TLC) (Stahl, 1969; Wagner and Bladt, 1996). Dried TLC plates were sprayed with specific reagents and heated to observe the color reaction. The presence of phenolic hydroxyl groups was observed through positive reaction with ferric chloride and aluminum chloride. The presence of saponins was observed using TLC by positive Liebermann-Burchard test (Lu et al., 2007).

Table 1 lists the retention times (Rt), MS data spectra and maximal ultraviolet wavelength (λ_{max}) for the chemical constituents found in the extract. The total ion current chromatogram obtained in MS negative mode is shown in Fig. 1. The low resolution ESI-MS analysis did not provide the molecular mass nor, in consequence, the molecular formula of the compounds found in the extract (Abad-Garcia et al., 2009; Vukics and Guttman 2010; Kaume et al., 2012). For the MS/MS spectrum the ion abundance depends on the stabilities of the fragments. Generally, the number of phenolic hydroxyls determines the stability of the structure in negative ion mode (Vessecchi et al., 2011). The interpretation of the MS/MS and UV spectra compared to reported data and reference compounds were the main tool for the proposal of the structure of the compounds found in this extract. The ESI-MS and ESI-MS/MS spectra were acquired in negative ion mode. Through MS analyses no information can be obtained about the stereochemistry of the glycan part of the flavonoids glycosides. However, the sugar types (hexoses, deoxyhexoses and pentoses) can be indirectly deduced from the difference of the mass of pseudo-molecular ions and the masses of corresponding fragment (Ma et al., 1997; Vukics and Guttman 2010; Ferreres et al., 2011).

Table 1
The retention times (Rt), MS data spectra and maximal ultraviolet wavelength (λ_{max}) for the chemical constituents found in the hydroethanolic extract from roots of *Periandra dulcis* through HPLC-DAD-ESI-MS/MS.

	Rt	UV (λ_{max}) nm	[M-H]- *m/z*	MS/MS *m/z* and intensity of peak	Suggested structure	References
1	10.6	265-355	685	300	quercetin-*O*-malonyl galloyl rhamnoside	Rodriguez-Perez et al., 2013; Simirgiotis et al., 2013
2	13.0	280	935	917 (50), 783 (40), 633 (100)	di(hexahydroxydiphenoyl) galloyl glucoside	Bubba et al., 2012; Fernandes et al., 2011; Romani et al., 2012
3	14.6	280	951	907 (100), 783 (20)	trisgalloyl (hexahydroxydiphenoyl) glucose	Gordon et al., 2011; Bubba et al., 2012; Romani et al., 2012
4	21.4	-	445 (30)	313 (100), 343 (30)	3,5,4'-trihydroxy-6,7-methylenedioxyflavone-*O*-arabinoside	Ferreres et al., 2011
5	22.5	260, 290 sh, 355	631	479 (100), 316 (10)	myricetin-3-*O*-(2"-*O*-galloyl) glucoside	Rodriguez-Perez et al., 2013; Simirgiotis et al., 2013; Gouveia and Castilho, 2010
6	25.4	265-355	463	316 (100)	myricetin-3-*O*-rhamnoside	Fracasseti et al., 2013; Simirgiotis et al., 2013; Gouveia and Castilho, 2010
7	27.3	265-355	505	463 (20), 316 (100)	myricetin-3-*O*-acetyl rhamnoside	Faria et al., 2011; Gordon et al., 2011; Rodriguez-Perez et al., 2013; Fracasseti et al., 2013
8	29.6	265-355	519	315 (100)	rhamnetin-3-*O*-(6-*O*"-acetylglucoside)	Simirgiotis et al., 2013; Rodriguez-Perez et al., 2013; Gouveia and Castilho, 2010
9	33.2	-	955	937 (100), 893 (10), 747 (30), 611 (30), 539 (60)	hederagenin-3-*O*-ramnosyl glucosyl glucuronide	Pollier et al., 2011; Jin et al., 2006; 2007; Tava et al., 2009; 2011
10	33.9		925	907 (100), 863 (20); 717 (40); 629 (40); 539 (40)	hederagenin-3-*O*-rhamnosyl arabinosyl glucuronide	Pollier et al., 2011; Jin et al., 2006; 2007; Tava et al., 2009; 2011
11	35.4		823	805 (30), 761 (50), 647 (20), 687 (30), 351 (100)	periandrin isomer	Hashimoto et al., 1982; 1980
12	35.8		825.2	ND	bayogenin-3-*O*-glucosyl glucuronide	Pollier et al., 2011
13	37.5		925	907 (100), 811(30), 779 (20), 585 (40), 539 (50)	soyasapogenol D-3-*O*-rhamnosyl arabinosyl glucuronide	Jin et al., 2006; 2007; Tava et al., 2009; 2011; Pollier et al., 2011; Sagratini et al., 2009
14	40.9		823	ND	periandrin isomer	Hashimoto et al., 1982; 1980
15	43.2		909	891 (100), 847 (20), 763 (20); 701 (20), 595 (10), 523 (40)	soyasapogenol E-3-*O*-rhamnosyl arabinosyl glucuronide	Jin et al., 2006; 2007; Tava et al., 2009; 2011; Pollier et al., 2011; Sagratini et al., 2009
16	44.0		939	921 (100), 793 (30), 731 (30), 595 (40), 523 (70)	soyasapogenol E-3-*O*-rhamnosyl glucosyl glucuronide	Jin et al., 2006; 2007; Tava et al., 2009; 2011; Pollier et al., 2011; Sagratini et al., 2009

e.g. For compound **2**, the value 935 corresponds to *m/z* (mass/charge) of deprotonated molecule; the value 917 is the *m/z* of a fragment ion and (50) is its intensity.

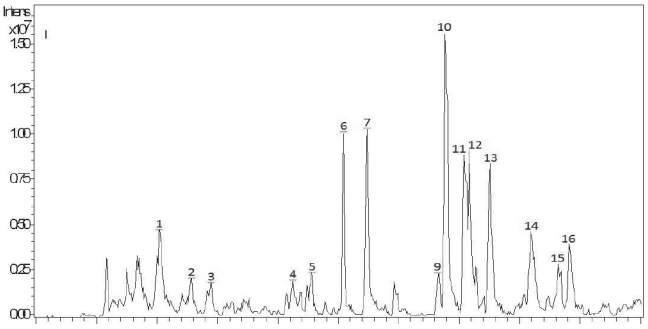


Figure 1 - Total ion current chromatogram obtained through the analysis of hydroethanolic extract from roots of *Periandra dulcis* in negative ion mode. The structure of compounds among 5 and 10 min could not be suggested due to the low content of these compounds in this extract.

Discussion

The different types of flavonoids have different UV spectra, which can be used to differentiate isomers possessing similar mass spectra (Abad-Garcia et al., 2009; Vukics and Guttman 2010). Flavonols had a characteristic UV spectrum with two bands at 260 and 370 nm, and shoulders occurring at 270 and 300 nm (Abad-Garcia et al., 2009). Compounds **1**, **4**-**8** showed UV spectra characteristic of flavonols. Compounds **2** and **3** showed UV spectra data that are in agreement with those of gallic acid and hexahydroxydiphenoyl (HHDP) derivatives, with a maximum absorption at 271-280 nm (Gordon et al., 2011). Saponins show maximum UV detection at wavelengths of 203 nm and were not detected through HPLC/DAD (Pollier et al., 2011). Little structural information regarding the aglycone fragment could be obtained from spectra data (Table 1). Acidic hydrolysis followed by HPLC-DAD-MS analyses was performed for the characterization of the aglycone of flavonols-*O*-glycosides. After hydrolyses, quercetin, rhamnetin and myricetin were identified by comparing the HPLC retention times, UV and mass spectra against those of a reference standard and published data (Ma et al., 1997). The MS/MS results are very similar to those from the standard solutions of quercetin, rhamnetin and myricetin fragmentation (data not shown).

Compound **1** at 10.6 min showed a deprotonated molecule at *m/z* 685 in the ESI-MS spectrum. The MS/MS fragment ion occurred at *m/z* 300. According to Rodriguez-Perez et al. (2013) and Simirgiotis et al. (2013), the fragment at *m/z* 300 could be formed through the homolytic cleavage of the *O*-glycosidic bond, resulting in the formation of a radical aglycone anion, which suggests quercetin as the aglycone. The loss of 384 u from deprotonated molecule could be attributed to the presence of malonyl (86 u), galloyl (152 u) and rhamnosyl (146 u) moieties as substituents (Table 1). According to Sanz et al. (2010), the 152 u correspond to the molecular weight of a galloyl moiety ($C_7H_4O_4$). For flavonols the most favoured glycosilation postions are aglycone positions 3 and 7 (Abad-Garcia et al., 2009; Vukics and Guttman 2010; Kaume et al., 2012; Gouveia and Castilho, 2010). Based only on MS/MS data, neither the nature of the hexoside residue nor the sugar linkage position to the aglycone could be determined (Giorgi and Ponticelli 2005). The hexoside was suggested as rhamnose. When the MS/MS spectrum base peak ion corresponds to the aglycone ion, the substituent groups could be attached to the same aglycone hydroxyl characteristic of flavonols-*O*-diglycosides (Abad-Garcia et al., 2009; Vukics and Guttman 2010; Gouveia and Castilho, 2010). Compound **1** was suggested as quercetin-*O*-malonyl galloyl rhamnoside.

The ESI-MS/MS spectra of hydrolysable tannins provide important information about gallic and ellagic tannins. Hydrolysable tannins possess a central core of polyhydric alcohols, such as, glucose and hydroxyl groups, which are esterified by gallic acid (gallotannins) or by hexahydroxy diphenic acid (HHDP) (ellagitannins) (Sanz et al., 2010; Bubba et al., 2012). The loss of 152 u (galloyl moiety fragmentation) and 170 u (gallic acid neutral loss) indicate the presence of galloyl groups, while the loss of 302 u from deprotonated molecules indicate the presence of hexahydroxydiphenoyl (HHDP) groups. The ESI-MS spectrum of compound **2** showed at 13.0 min a deprotonated molecule at *m/z* 935. The MS/MS spectrum of the deprotonated molecule produced a fragment at *m/z* 917 (50), which could be attributed to the loss of water (18 u); a base peak at *m/z* 633, which could be attributed to the loss of HHDP moiety; and a fragment ion at *m/z* 783 (30), that probably corresponds to the loss of the galloyl (152 u) moiety. Compound **2** was suggested as di (hexahydroxydiphenoyl) galloyl glucoside (Table 1), after data published by Fernandes et al. (2011). The ESI-MS spectrum of compound **3** at 14.6 min showed deprotonated molecule at *m/z* 951, which after fragmentation yielded a base peak at *m/z* 907 (100), that could be assigned to the loss of 44 u (carboxyl group). This result agrees with the presence of a unesterified carboxyl group (Bubba et al., 2012), while the fragment ion at *m/z* 783 (30) indicates the presence of an additional galloyl residue, suggested by the loss of 168 u (951-783) from deprotonated molecule; probably linked to one HHDP molecule by a C-C- bond, according to Gordon et al. (2011). Compound **3** was suggested to be trisgalloyl hexahydroxydiphenoyl glucoside by comparison with data published by Gordon et al. (2011) that found this tannin in *Psidium quineense* fruits.

For compound **4** at 21.4 min, the ESI-MS spectrum displayed deprotonated molecule at *m/z* 445 and the base peak at *m/z* 313, which could be taken as indication of the presence of a 3,5,4'-trihydroxy-6,7-methylenedioxyflavone as aglycone, also known as gomphrenol (Ferreres et al., 2011). The loss of 132 u suggests a pentoside sugar, probably arabinoside, as substituent. Gomphrenol is a flavonoid that has a methylene dioxy group, which is rare in nature. Gomphrenol glycosides are found in *Gomphrena globosa* (Ferreres et al., 2011). Compound **4** was suggested to be 3,5,4'-trihydroxy-6,7-methylenedioxyflavone-*O*-arabinoside.

For compound **5** at 22.5 min, the ESI-MS spectrum exhibited deprotonated molecule at *m/z* 631 and a base peak at *m/z* 479, which probably corresponds to the loss of galloyl moiety (152 u) that could also be accounted for the presence of

myricetin glucoside. Myricetin glycosides showed a similar UVspectra, with maximum absorption at 265-355 nm. The maximum absorption value at 349-355 nm, which is lower than that aglycone myricetin (369 nm) indicate the typical hypsochromic effect of flavonol glycosides in relation to its aglycone (Rodriguez Peres et al., 2013; Gouveia and Castilho, 2010). According to Fracassetti et al. (2013) myricetin-*O*-glycoside with a free hydroxyl at position 3 shows a UV band I maximum at 374 nm, while myricetin with a blocked hydroxyl at aglycone position 3 showed UV band I maximum at 356 nm. The presence of myricetin as aglycone was also suggested by a fragment ion at *m/z* 316 (10), which, according to Rodriguez-Perez et al. (2013), and Simirgiotis et al. (2013), is produced from the homolytic cleavage of the *O*-glycosidic bond, resulting in the formation of a radical aglycone anion. The linkage of gallic acid moiety in 3-*O*-acylated flavonol was suggested to be located in position 2" of the sugar hydroxyl of the glucose by characteristic MS fragmentation, showing a base peak at *m/z* 479. In this case it was suggested that the galloyl group was not linked at 6" position of glucose sugar, because in this position the fragmentation is difficult and the resultant from the loss of the galloyl group would not be observed. Compound **5** was suggested to be myricetin-3-*O*-(2"-*O*-galloyl) glucoside by comparison against published data (Fracassetti et al., 2013, Rodriguez Perez et al., 2013, Simirgiotis et al., 2013, Gouveia and Castilho, 2010).

The ESI-MS spectrum of compound **6** at 25.4 min displayed deprotonated molecule at *m/z* 463. The neutral loss of 146 u (rhamnose moiety) yielded the radical aglycone ion at *m/z* 316, which according to Fracassetti et al. (2013), Rodriguez Perez et al. (2013) and Simirgiotis et al. (2013) could be attributed to myricetin. Compound **6** was suggested as myricetin-3-*O*-rhamnoside compared against published data (Rodriguez Perez et al., 2013; Gouveia and Castilho, 2010).

For compound **7** at 27.3 min, the ESI-MS spectrum presented deprotonated molecule at *m/z* 505, 42 u (acetyl group) more than that of compound **6**, with which it shares a similar fragmentation pattern and UV spectrum. The fragment ion at *m/z* 463 (10) and base peak at *m/z* 316, could suggest that this compound is an acetyl derivative of compound **6**. Compound **7** was suggested as myricetin-3-*O*-acetyl rhamnoside by comparison with data published by Faria et al. (2011) and Gordon et al. (2011) that found compounds **6** and **7** in *Syzygium cumini*. For myricetin derivatives on reversed phase conditions, the acylated derivatives eluted after their corresponding non-acylated flavonol (Faria et al., 2011; Rodriguez-Perez et al., 2013).

Compound **8** at 29.6 min, was suggested as rhamnetin-3-*O*-(6"-*O*-acetylglucoside), based on UV and ESI-MS spectra that showed deprotonated molecule at *m/z* 519 and a base peak at *m/z* 315; this suggested the presence of rhamnetin or isorhamnetin as aglycone. (Abad-Garcia et al., 2009; Simirgiotis et al., 2013). The loss of 204 u (162 + 42) could be attributed to the presence of glucose and acetyl moieties as substituents (Gouveia and Castilho, 2010). The absence of the fragment ion (M-H-42)- in its MS/MS spectrum suggested the substitution of an acetyl group occur at the 6"-*O* position of the hexose, where fragmentation is difficult (Rodriguez Peres et al., 2013; Gouveia and Castilho, 2010).

Pentacyclic triterpenoids of the oleanane type are compounds commonly found in higher plants in the form of free acids or, more frequently, as aglycones of saponins (Jin et al., 2006, 2007; Pollier et al., 2011). Glycosides of oleanane triterpene aglycones, such as, soyasapogenols (Zhang and Popovich, 2009; Sagratini et al., 2009; Pollier et al., 2011), hederagenin derivatives (Pollier et al., 2011) and periandrin isomers (Hashimoto et al., 1984a,b; 1982; 1980) were the main constituents found in this extract (Table 1). The most common sugar residues in saponin compounds are hexoses (glucose, galactose), pentoses (arabinose, xylose), methylpentoses (furanose, quinovose, rhamnose) and uronic acids (glucuronic acid, galacturonic acid), which are linked to aglycone through the ether glycosidic bonds generally in 3-*O* position (Chapagain and Wiesman, 2008; Tava et al., 2009; 2011). Soyasaponins are glycosides of oleanane triterpene aglycones known as soyasapogenols, which are classified into four groups A, B, E and DDMP (Zhang and Popovich, 2009), and are common constituents of soy, *Glycine max*, Fabaceae (Kang et al., 2010). Identification of saponins using MS/MS technique was conducted by comparing results against MS data of the known saponin reported in literature (Jin et al., 2007; Pollier et al., 2011, Chapagain and Wiesman., 2008, Tava et al., 2009, 2011, Sagratini et al., 2009).

The ESI-MS spectra of saponins hederagenin derivatives, compounds **9** and **10** at 33.2 and 33.9 min, generated deprotonated molecules at *m/z* 955 and *m/z* 925, respectively. Compared to the results obtained by Pollier et al. (2011) and Jin et al. (2007), the fragmentation pattern of compound **9** indicated the presence of glucuronic acid, rhamnose and a hexose, probably linked to oxygen in position 3 of aglycone (Chapagain and Wiesman, 2008; Tava et al., 2009; 2011). The ESI-MS/MS spectra of compounds **9** and **10** showed base peak ions produced by loss of water at *m/z* 937 and *m/z* 907; fragment ions originated by the loss of carbon dioxide (44 u) at *m/z* 893 (10) and *m/z* 863 (10); and by the loss of rhamnose (146 u) at *m/z* 747 (20) and *m/z* 717 (35), respectively. Both compounds exhibited the fragment ion at *m/z* 539 (70) with 70% of intensity. Based on the results obtained by the same authors, principally Pollier et al. (2011), hederagenin (3,23-dihydroxyolean-12-en-28-oic acid) a triterpenic acid with molecular formula $C_{30}O_4H_{48}$ and molecular mass (MM) 472 u was suggested as the aglycone for both saponins. For compound **9**, according to the data published by Pollier et al. (2011) and Jin et al. (2007), the fragment at *m/z* 611 (40) which corresponded to the loss of 344 u from deprotonated molecule could be suggested by the loss of (rhamnose+glucose + 18 + 18) moieties, while that for compound **10**, the fragment ion at *m/z* 629 (40) could be suggested by the loss of 296 u (132 + 146 + 18) moieties from deprotonated molecule. For compound **9**, the fragmentation pattern corresponded to the previously reported MS data for hederagenin-3-*O*-glucosyl rhamnosyl glucuronide published by Pollier et al. (2011) who identified this compound in *Medicago truncatula* hairy roots, also a species of the Fabaceae family. On the basis of the excellent agreement of the mass spectrum of compound **9** with that present in literature, this compound was suggested to be hederagenin-3-*O*-glucosyl rhamnosyl glucuronide. Compound **10**, the main constituent found in

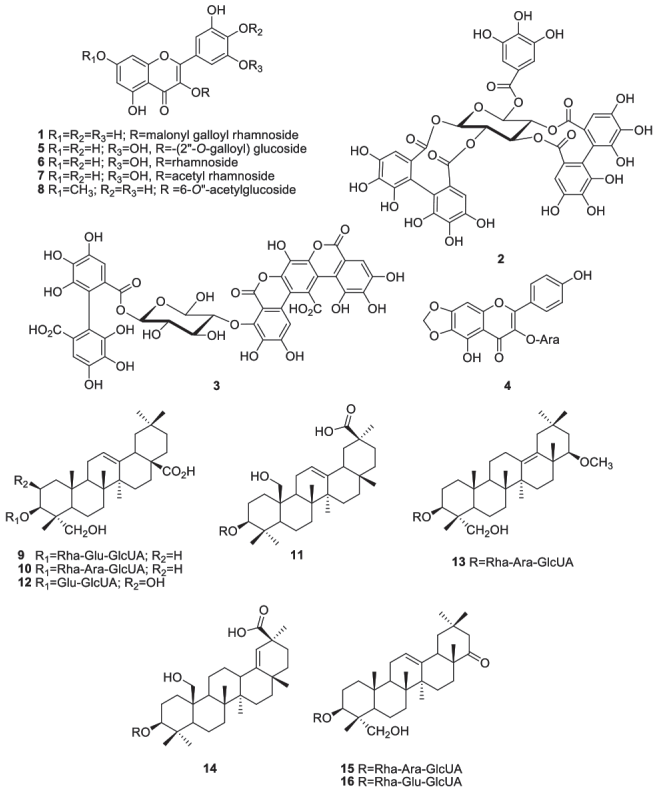
this extract, was tentatively assigned as hederagenin-3-*O*-rhamnosyl arabinosyl glucuronide.

The ESI-MS spectrum of compound **13** at 37.5 min, also showed deprotonated molecule at *m/z* 925, but this saponin differed from saponin **10** in retention time, structure and, as a consequence, in polarity. The ESI-MS/MS spectrum exhibited different fragmentation pattern, except for the fact that some fragments, such as a base peak at *m/z* 907 and fragment ion at *m/z* 539 were similar to those found in compound **10**. Different product ion sss were observed at *m/z* 779 associating the sequential loss of 146 u, probably of rhamnose from the parent ion. For compound **13**, soyasapogenol D [21α-methoxyolean-13(18)-ene-3β,24-diol], that possesses molecular formula $C_{31}O_3H_{52}$ and also MM 472 u was suggested as aglycone, according to the data published by Pollier et al. (2011) and Jin et al. (2007). Soyasapogenol D is an isomer of hederagenin and the difference in retention times on reversed phase can be explained by the structure of these triterpenoids, where the carboxylic group (COOH) present in hederagenin was substituted for a methoxy group in soyasapogenol D, a substituent with lesser polarity. Thus, compound **13** was suggested as soyasapogenol D-3-*O*-rhamnosyl arabinosyl glucuronide.

Two periandrin isomers, compounds **11** and **14** were found at 35.4 and 40.9 min showing deprotonated molecule at *m/z* 823 in ESI-MS spectra. Periandrin III and IV were found in the roots of *P. dulcis* by Hashimoto et al. (1980; 1982). Periandrin III 3-β-*O*-[β-D-glucuronopyranosyl-β-D-glucuronopyranosyl]-25-hydroxyolean-18(19)-en-30-oic acid), possesses [25-hydroxyolean-18(19)-en-30-oic acid] as aglycone, and a sugar chain consisting of two glucuronic acids. Periandrin IV (3-β-*O*-[β-D-glucuronopyranosyl-(1-2)-β-D-glucuronopyranosyl]-25-hydroxyolean-12(13)-en-30-oic acid) possesses [25-hydroxyolean-12(13)-en-30-oic acid] as aglycone and also has a sugar chain consisting of two glucuronic acids (Hashimoto et al., 1980, 1982). Only compound **11** fragmented when submitted to collision-induced dissociation MS/MS conditions used in this analyzes (see Material and Methods). For compound **11**, the MS/MS product ion obtained from deprotonated molecule showed a base peak at *m/z* 351 and fragment ions at *m/z* 805 $(M-H-18)^-$, at *m/z* 761 $(M-H-18-44)^-$ and at *m/z* 647 suggesting the loss of 176 u (glucuronic acid) (Table 1). Compounds **11** and **14** were suggested as periandrin isomers, periandrin III and IV.

Compound **12** at 35.8 min generated deprotonated ion at *m/z* 825 in ESI-MS spectrum, and on the base of literature data published by Pollier et al. (2011) it was suggested as bayogenin-3-*O*-glucosyl glucuronide. According to Pollier et al. (2011), bayogenin [2,3,23-trihydroxyolean-12(13)-en-28-oic acid] possesses the molecular formula $C_{30}O_5H_{48}$ and MM 488 u. Bayogenin-3-*O*-glucosyl glucuronide was found by Pollier et al. (2011) in *Medicago truncutula*, Fabaceae.

Compounds **15** and **16** were suggested to be soyasapogenol E glycosides derivatives. According to Pollier et al. (2011) and Jin et al. (2006; 2007), soyasapogenol E is a pentacyclic triterpenoid oleanane derivative, containing a double bond among positions



12 and **13** and is substituted by hydroxyl groups at the 3β and 24 positions, possessing an oxo group at position 22 having molecular formula $C_{30}O_3H_{48}$ and MM 456 u (Zhang and Popovic, 2009). Compound **15** at 43.2 min and compound **16** at 44.0 min exhibited deprotonated molecule at *m/z* 909 and *m/z* 939, respectively. The ESI-MS/MS spectra of compounds **15** and **16** showed base peak ions produced by loss of water at *m/z* 891 and *m/z* 921, and fragments produced by the loss of rhamnose at *m/z* 763 (30) and *m/z* 793 (30) from deprotonated molecule, respectively. Both compounds exhibited the fragment ion at *m/z* 523 (70). According to the data published by Pollier et al. (2011) and Jin et al. (2007), for compound **15**, the fragment ion at *m/z* 701 (40) and for compound **16** the fragment ion at *m/z* 731 (40), these fragments were suggested by the loss of 208 u (146 + 44 + 18) from deprotonated molecule and the fragment ion at *m/z* 595 (40). It was suggested by the loss of 314 u (146 + 132 + 18 + 18) in compound **15**, and by the loss of 344 u (146 + 162 + 18 + 18) in compound **16**. Compound **15** was suggested as soyasapogenol E-3-*O*-rhamnosyl arabinosyl glucuronide. Compound **16** was plausibly characterized as soyasapogenol E-3-*O*-rhamnosyl glucosyl glucuronide, on the basis of the excellent agreement with data obtained by Pollier et al. (2011). In this extract saponins, such as hederagenin-3-*O*-rhamnosyl arabinosyl glucuronide, periandrin isomers, bayogenin-3-*O*-glucosyl glucuronide and soyasapogenol D-3-*O*-rhamnosyl arabinosyl glucuronide were found as main constituents; although the flavonols, myricetin-3-*O*-rhamnoside and myricetin-3-*O*-acetyl rhamnoside were also obtained in high content.

Conclusion

The analysis of the hydroethanolic extract of *P. dulcis*, a species belonging to the Fabaceae family, showed predominance of saponins among their main constituents although phenolic compounds (principally myricetin derivatives) also were detected in significant amounts. Based on literature, it can be suggested that all saponins present in this extract are 3-*O*-glucuronide of hederagenin and soyasapogenols derivatives, and the flavonols are myricetin-3-*O*-glycosides derivatives. Except for saponins, such as periandrin and periandradulcis derivatives, there is no information about the chemical composition of this species.

Authors' contributions

GN carried out the laboratory work, analysis of the data and drafted the paper. RT designed the study and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved for submission.

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