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Chemical composition of hydroethanolic extracts from *Siparuna guianensis*, medicinal plant used as anxiolytics in Amazon region

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Abstract: *Siparuna guianensis* Aubl., Siparunaceae, is used as anxiolytic plants in folk medicine by South-American indians, "caboclos" and river-dwellers. This work focused the evaluation of phenolic composition of hydroethanolic extract of *S. guianensis* through HPLC-DAD-ESI/MS/MS. The constituents exhibited protonated, deprotonated and sodiated molecules and the MS/MS fragmentation of protonated, deprotonated and sodiated molecules provided product ions with rich structural information. Vicenin-2 (apigenin-6,8-di-*C*-glucoside) was the main constituent found in *S. guianensis* together quercetin-3,7-di-*O*-rhamnoside and kaempferol-3,7-di-*O*-rhamnoside. A commercial extract of *Passiflora incarnata* (Phytomedicine) was used as surrogate standard and also was analyzed through HPLC-DAD-ESI/MS/MS, showing flavones *C*-glycosides as constituents, among them, vicenin-2 and vitexin. The main constituent was vitexin. Flavonols triglycosides were also found in low content in *S. guianensis* and were tentatively characterized as quercetin-3-*O*-rutinoside-7-*O*-rhamnoside, quercetin-3-*O*-pentosyl-pentoside-7-*O*-rhamnoside and kaempferol-3-*O*-pentosyl-pentoside-7-*O*-rhamnoside. Apigenin and kaempferol derivatives had been reported as anxiolytic agents. Flavonoids present in this extract were correlated with flavonoids reported as anxiolytics.

Introduction

Siparunaceae comprise *Glossocalyx* with one species in West Africa and Siparuna with 65 species in the neotropics. The monoecy species is confined in the Amazon basin and Southern Central America (Renner & Won, 2011). *Siparuna guianensis* Aubl., common name "Capitiú" had been utilized as an anxiolytic by South-American indians, "caboclos" and river-dwellers (Rodrigues et al., 2008). Other classes of constituents found in this genus were alkaloids, steroids (Braz Filho et al., 1976), essential oils (Viana et al., 2002; Valentini et al., 2010) and a mixture of diglycosyl and monoglycosyl flavonoids derivatives of quercetin and kaempferol (Leitão et al., 2005). Little is known on the chemistry of Siparuna species. However, a comprehensive profiling of *S. guianensis* flavonoids has not yet been reported.

Passiflora incarnata was used as surrogate standard. *Passiflora* species, Passifloraceae, are native to tropical and subtropical areas of the Americas. Many of these species are present as official drugs in the pharmacopeias of several countries (Dhawan et al., 2004). The anxyolitic activity of *Passiflora* genus had been attributed to flavones *C*-glycosides derivatives of apigenin and luteolin (Lolli et al., 2007; Sena et al., 2009), such as isoorientin, vicenin-2, spinosin, and 6,8-di-*C*-glycosylchrysin (Sena et al., 2009). Other classes of constituents found in this genus were cyanogenic glycosides, benzopyrones (Dhawan et al., 2004), volatile constituents, saponins (Birk et al., 2005) and simple indole alkaloids (Abourashed et al., 2003).

Research on flavonoids has increased because they have been identified as a new type of neuromimetic ligand with *in vivo* anxiolytic properties (de Castro et al., 2007). Flavonoid glycosides, showed to exert central nervous system mediated activities, particularly as sedative-hypnotics, analgesics and anxiolytic (de Castro et al., 2007; Fernandez et al., 2009; Elsas et al., 2010). Myricitrin and naringin exhibited anxiolytic effects with no signs of sedation (Fernandez et al., 2009).

Type A α-aminobutyric acid (GABAA) receptors are the major inhibitory neurotransmitter receptors in the Central Nervous System, which is involved in epilepsy, sedation and anxiolysis, producing these effects through binding to GABAA receptors (Melo et al., 2010). Anxiolytics facilitate the coupling of GABAergic receptors to GABAA and produce their pharmacological effect by binding to a benzodiazepine recognition site on the GABAA receptor complex (Harris et al., 2008; Ennaceur et al., 2008). The first drugs used to treat anxiety

were barbiturates, toxic compounds that produce a variety of adverse effects. These compounds have mainly been replaced by benzodiazepines (BDZ), the most common anxiolytic drugs used today. However, long-term BDZ use induces tolerance and dependence (Ennaceur et al., 2008). Phytomedicines are an interesting alternative to synthetic drugs for therapy. Beside this, they can offer the potential for the development of new drugs (Carlini, 2003). Flavonoids showed anxiolytic activity on rodent behavior with efficiency comparable to that of typical BDZ agents. Beside this, unlike BDZ, the flavonoid anxiolytics did not induce sedation and dependence as side effects (Sena et al., 2009; Birk et al., 2005; de Castro et al., 2007).

HPLC–DAD–ESI-MSn represents a powerful tool for the analysis of natural products. Structural characterization of flavonoid glycosides through spectrometric methods were based on collision-induced dissociation (CID) of molecular species, such as, protonated molecules $[M+H]^+$, deprotonated molecules $[M-H]^-$ and sodiated molecules $[M+Na]^+$. The CID experiment can be used with soft, such as ESI-MS/MS or hard ionization, because is dependent only of the analyser (Q-TOF, ion-trap and others) and collision gas (Shahat et al., 2005; Steinmann & Ganzera, 2011).

In this study, the main flavonoids presents in hydroethanolic extracts of *S. guianensis* were characterized using HPLC–DAD–ESI-MS/MS and their respective structure were correlated with flavonoids reported as anxiolytics, being also compared with an extract of *P. incarnata,* a phytomedicine (surrogate standard), used with anxiolytic purposes.

Materials and Methods

Chemical and reagents

The leaves of *Siparuna guianensis* Aubl., Siparunaceae, were harvested in the wild by the curator of the INPA (Instituto Nacional de Pesquisas da Amazônia) Manaus-AM, Brazil, and identified by taxonomist José Lima dos Santos and a voucher was deposited in Botanical Institute of São Paulo (E. Rodrigues 531). *P. incarnata* standardized extract used as phytomedicine, was acquired from Centroflora®. Quercetin, apigenin, kaempferol, vitexin and orientin standards were purchased from Sigma-Aldrich Chemical CO. (St. Louis, MO, USA); their purities were above 97% as determined by HPLC-DAD analysis. Stock solutions of these compounds (100 µg/mL) were prepared in methanol and further analyzed by HPLC-DAD. 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).

Preparation of extract

Fresh aerial parts were air-dried in the shade at room temperature to a constant weight, ground to pass through a 30 mesh screen, and stored in sealed glass vials. For preparation of lyophilized extracts, 100 g of the powders were extracted with 1 L of hydroethanolic solution 50% (v/v) by maceration. The crude preparation was filtered through Whatman paper nº 1 and concentrated under reduced pressure in a rotaevaporator to produce a crude extract, which was placed in a lyophilizer (4 atm of pressure and temperature of -40 ºC) for 48 h. The lyophilized extracts were stored in amber flasks at 5 ºC (freezer).

Phytochemical screening

The lyophilized hydroethanolic extracts of *S. guianensis* was screened via thin layer chromatography (TLC) for alkaloids, phenolic acids, steroids, terpenoids, cardioactive glycosides, flavonoids, coumarins, saponins, lignans, tannins and iridoids (Stahl, 1969; Wagner & Bladt, 1996). The extract was dissolved in methanol PA (10 mg/mL) and applied to silica-gel 60 F254 plates (Merck). Solution standards of pure compounds were prepared at concentration of 1 mg/mL. For alkaloid analyses, lyophilized samples (60 mg) were dissolved in 2 mL of water to form a suspension that was acidified with a solution of 20% of sulfuric acid (H_2SO_4) to pH 4. The acidic suspension was first partitioned with ethyl acetate (EtOAc) and chloroform to remove neutral components, and the aqueous phase was then basified with sodium carbonate (Na_2CO_3) to pH 10 followed by extraction with chloroform (Xu et al., 2006).

Hydrolysis experiments

The free flavonoid aglycones of flavonoid-*O*-glycosides were released by acid hydrolysis as follows: 50 mg of lyophilized extract of *S. guianensis* were dissolved in 4 mL of solution 10% (v/v) H_2SO_4, and heated in boiling water for 1 h (Chirinos et al., 2009). After cooling, the reaction mixture was neutralized with saturated aqueous sodium carbonate and filtered under reduced pressure. The filtrate was concentrated to approximately 1 mL.

Reversed Phase HPLC-DAD-ESI-MS/MS analysis

For reversed phase high performance liquid chromatography (RPHPLC) analysis, lyophilized and hydrolyzed extracts were dissolved in water:methanol (80:20) v/v (10 mg/3 mL) and filtered with a 0.45 µm filter, prior to injection of 31.2 µL (concentration of 104 µg/mL) into the HPLC system. Spectral UV data from all peaks were collected in the range 240-400 nm, and

chromatograms were recorded at 360 and 270 nm for phenolic compounds. A DADSPD-M10AVP Shimadzu equipped with a photodiode array detector was coupled to Esquire 3000 Plus, Bruker Daltonics mass spectrometer with electrospray ionization (ESI) source and ion-trap analyser. All the operations, acquisition and data analysis were controlled by SCL-10A VP software. The mobile phases consisted of eluent A (0.1% aq. formic acid) and eluent B (methanol). A reverse phase, C18, Zorbax-5B-RP-18 (Hewlett Packard) column (4.6×250 mm, 5 µm), connected to a guard column and a gradient of 20-90% B (V/V) over 50 min were utilized for separations, 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. 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 40 V, a capillary voltage 4500 V and a capillary temperature of 325 °C. Ultrahigh pure Helium (He) was used as the collision gas and high-purity nitrogen (N_2) as the nebulizing gas. Nebulization was aided with a coaxial nitrogen sheath gas provided at a pressure of 27 psi. Desolvation was facilitated using a counter current nitrogen flow set at a flux of 7.0 L/min. The full scan mass acquisition both in negative and positive ion mode were performed by scanning from 100 up to 1000 *m/z* range. Collision induced dissociation (CID) spectra were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.5 up to 1.3 V. Due to the unavailability of commercial standards of flavonoids glycosides, these compounds were characterized by the interpretation of their UV absorbance band, the mass spectra obtained through MS/MS fragmentation of protonated, deprotonated and sodiated molecules, including of their respective aglycone (standards of quercetin, kaempferol, apigenin and luteolin are used) and also taking into account the data provided by the literature (MS database) and the vicenin-2 present in commercial extract of *P. incarnata.*

Results and Discussions

Several flavonoid glycosides occurring in plant tissue have the same molar mass but due to isomeric substitutions of their aglycones have different chemical and biological properties. Thus, it is very important to obtain information that permit to differentiate glycosylation positions, localize interglycosidic linkages in glycan moieties and evaluate structures of aglycone and sugar rings (Abad-Garcia et al., 2009). The hydroethanolic extract of *Siparuna guianensis* Aubl., Siparunaceae, have an acid pH (5.0). The yield of this hydroethanolic extract was 12.0 g per 100 g of crude plant material. To characterize the qualitative chemical profile, this extract was initially analyzed via TLC (Stahl, 1969; Wagner & Bladt, 1996; Chirinos et al., 2009). Dried TLC plates were sprayed with specific reagent and heated to observe the color reaction. The spots of procyanidins (condensed tannins) exhibited a pink color upon heating with methanolic hydrochloric acid 2 M. The hydroethanolic extracts reacted positively with ferric chloride, indicating the presence of phenolic hydroxyl groups (Jayaprakasha et al., 2006), while alkaloids were not detected. Oxoaporphine alkaloids had been found in *S. guianensis* (Braz Filho et al., 1976), but it is likely that the relatively nonpolar alkaloids are not efficiently extracted by polar solvents (ethanol and water).

The results suggested that the mobile phase gave a good resolution (Rs of approximately 1.3). The formic acid concentration (0.1%) resulted in a good chromatographic peak shapes with a signal-to-noise (S/N) ratios of approximately 9. Source voltage proved to be an important factor in the quality of MS spectra (Abad-Garcia et al., 2009). This chemical analysis of phenolic compounds was qualitative. The quantification of constituents was not carried out, because no commercial standards were available. The intra- and inter-day

Table 1. LC/MS data, deprotonated and protonated molecules (*m/z*) for peaks, including the retention times (Rt), MS/MS experiments and maximal absorption wavelength (λ_{max}) of the constituents found in *Passiflora incarnata* (surrogate standard).

	Rt (min)	Proposed structure	UV λ_{max} (nm)	(M+H)+/ (M+Na)+ (*m/z*)	(M-H)- (*m/z*)	MS/MS (*m/z*) (ESI-) (%)
1	19.8	isoorientin-2”-*O*-glucoside	270, 349	611.3/633.2	609.2	429.0 (100), 309.1 (50), 489.0 (20)
2	22.2	vicenin-2	271, 335	595.3/617.2	593.1	473.1 (100), 503.0 (40), 353.4 (40), 575.1 (20), 383.2 (10)
3	23.2	isoschaftoside	270, 338	565.1/587.1	563.0	473.1 (100), 503.0 (60), 545.0 (60), 383.2 (50), 443.1 (40), 353.5 (40)
4	23.7	schaftoside	270, 340	565.1/587.1	563.0	443.1 (100), 473.0 (80), 545.0 (20), 503.0 (20), 383.1 (25), 353.4 (20)
5	24.5	orientin	269, 348	449.1	447.1	327.0 (100), 357.0 (30)
6	26.0	isovitexin-2”-*O*-glucoside	270, 340	595.3	593.1	413.0 (100), 293.1 (60), 473.0 (20).
7	26.9	vitexin	268, 338	433.2/455.1	431.1	311.0 (100), 341.0 (35)

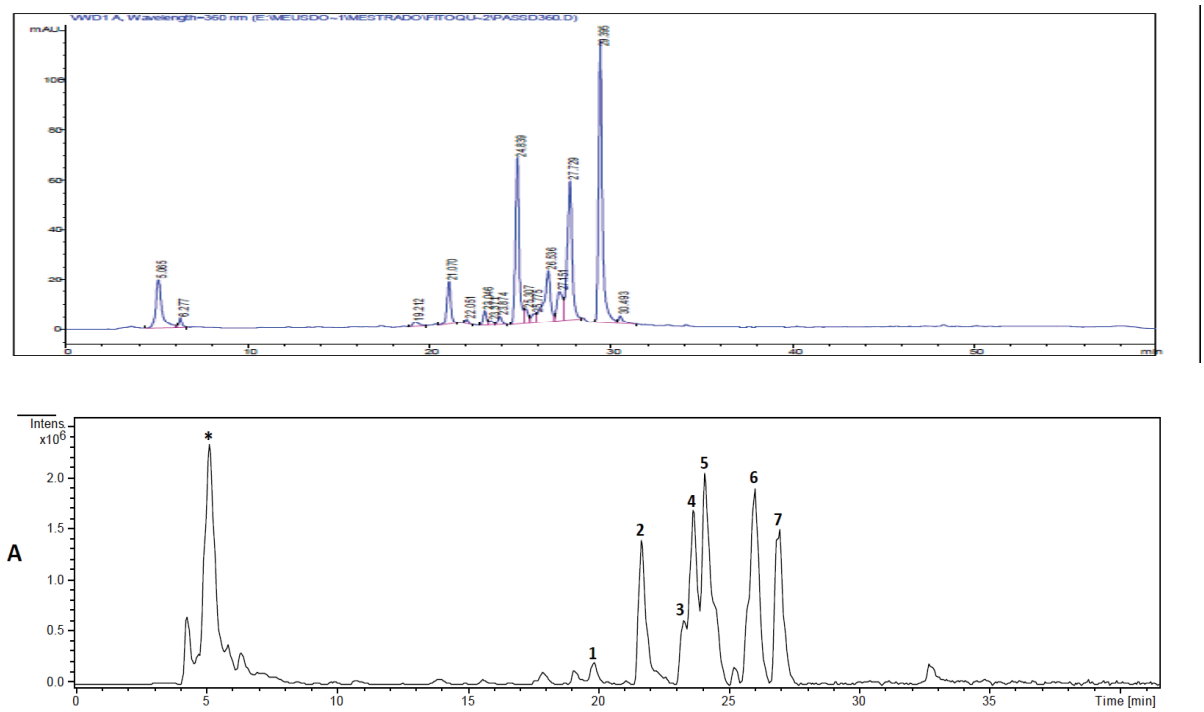


Figure 1. LC/DAD chromatogram (above) and LC-ESI/MS chromatogram (bellow) from extract of *P. incarnata.* The * mean a non-identified compound.

precisions were estimated by the relative deviations of the peak areas through replicate injections of solutions during one day and one month, respectively.

Different fragment ions were obtained when was applied collision induced dissociation (CID) at molecular species $[M+H]^+$, $[M+Na]^+$ and $[M-H]^-$ for the characterization of flavonoids. Tables 1 (*P. incarnata*, surrogate standard, Figure 1), and 2 (*S. guianensis*, Figure 2) summarizes the following information on peaks observed during RPHPLC-DAD-ESI-MS/MS analyses: peak labels, retention times (Rt) (min), proposed structure, wavelengths of absorbance maxima (λmax), ESI/MS and ESI/MS/MS spectra data. The retention time on the column is governed not only by the polarity of the molecules but also by their size. The sugar position is more important for the retention time than the nature of sugar (Abad-Garcia et al., 2009).

C-glycosylflavonoids are common constituents in flowering plants, being the major class of flavonoids present in *Passifloraceae* (Muller et al., 2005). The main compounds described in *P. incarnat*a include apigenin, luteolin and their *C*-glycosyl derivatives, such as vitexin, isovitexin, orientin, isoorientin, schaftoside and vicenin-2, among others (Muller et al., 2005; Wohlmuth et al., 2010). Analyses of flavones presents in commercial extract of *P. incarnata,* used as surrogate standard, was carried out by HPLC-DAD-MS/MS using the same conditions utilized for *S. guianensis*. The bioactive marker present in *P. incarnata* phytomedicine was vitexin. Compounds **1-7** and **9** were found to be resistant to acid hydrolysis. The on line UV-visible spectra of all flavonoids from *P. incarnata* and peaks at 20.1 (compound **9**) and 22.2 min (compound **2**) from *S. guianensis* exhibited band I (330-350 nm) and band II (254-272 nm) with similar intensities, typical of flavones (Abad-Garcia et al., 2009).

The peak at 19.8 min for compound **1** (Table 1, Figure 1) showed deprotonated and protonated molecules at *m/z* 609.2 and 611.3, and $[M+Na]^+$ at *m/z* 633.2, respectively. The MS/MS spectrum on precursor ion at *m/z* 609.2 showed fragments at *m/z* 489.0 [(M-H)-120]-, *m/z* 429.0 [(M-H)-180]- (base peak) and *m/z* 309.1, indicating luteolin as aglycone and exhibiting a fragmentation pattern of flavone *O*-glucosyl-*C*-glucoside. The base peak at *m/z* 429.0 indicated the fragmentation of the sugar moiety (glucose) from *O*-glycosylation. The characteristic (aglycon+41-18) ion for this type of flavonoid was detected at *m/z* 309.1, which corroborated with the *O*-glycosilation at the 2”-O position and luteolin as aglycone. The losses of 180 and 120 u are significant for diglucosides like sophoroside (1-2 linkages of two glucose molecules). Compound **1** was characterized as luteolin-6-*C*-glucosyl-2”-*O*-glucoside, also known as isoorientin-2”-*O*-glucoside.

The ESI-MS spectra for peak at 22.2 min (compound **2**) (Table 1, Figure 1) exhibited protonated

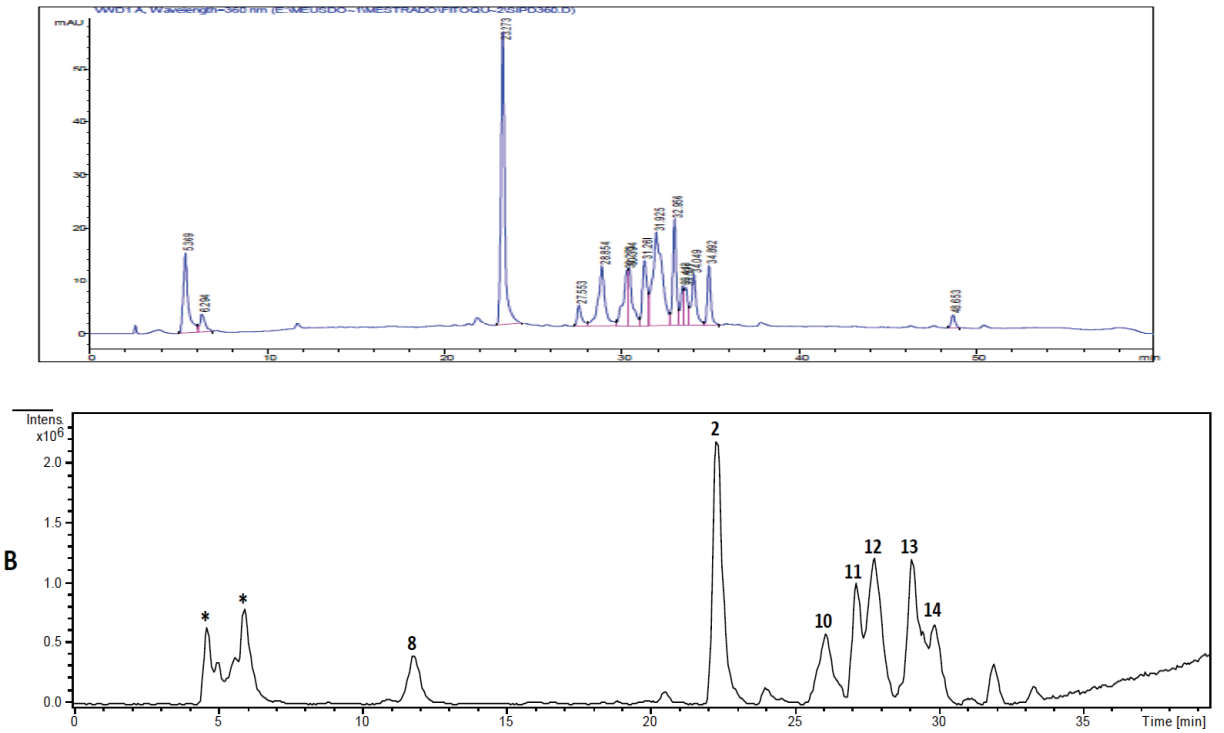


Figure 2. LC/DAD chromatogram (above) and LC-ESI/MS chromatogram (bellow) from extract of *S. guianensis*. The * mean a non-identified compound.

and deprotonated molecules at *m/z* 595.3 and 593.1 and $[M+Na]^+$ at *m/z* 617.2, respectively. The MS/MS spectrum on precursor ion at *m/z* 593.1 produced ions at *m/z* 575.1 $[(M-H)-18]^-$, *m/z* 503.0 $[(M-H)-90]^-$, and a base peak at 473.1 $[(M-H)-120]^-$, exhibiting a fragmentation pattern of flavones di-*C*-glycoside. The ions at *m/z* 353.4 $[(M-H)-(120+120)]^-$ and 383.2 $[(M-H)-(90+120)]^-$ indicated the presence of apigenin (MM 270) (Vessecchi et al., 2011) as aglycone and two hexose moieties (glucoses). Comparing with MS literature data (Piccinelli et al., 2008; Figueirinha et al., 2008) this compound was characterized as 6,8-di-*C*-glucosylapigenin, also known as vicenin-2.

The ESI-MS spectra of peaks observed at 23.2 (compound **3**) and 23.7 min (compound **4**) (Table 1, Figure 1) showed the same (+) and (-)-ESI-MS spectra at *m/z* 565.1 and at *m/z* 563.0, and $[M+Na]^+$ at *m/z* 587.1, respectively, suggesting that these compounds were isomers. Both compounds yielded the ion at *m/z* 503.0 showing a loss of 132 u, characteristic of a pentose sugar, a fragmentation pattern typical of the asymmetric di-*C*-glycosides. In general, a 6-*C*-pentosyl-8-*C*-hexosyl substitution lead to a higher abundance of the ion [(M-H)-90.0]- relatively to $[(M-H)-120.0]^-$ (Figueirinha et al., 2008). The $[(M-H)-60.0]^-$ ion comes from the cleavage of *C*-pentosyl, while the $[(M-H)-120.0]^-$ ions comes from the cleavage of *C*-hexosyl, and furthermore, glycosyl substitution at the C6 position of flavones produce the base peak. Compound **3** showed a base peak at *m/z* 473.1 $[(M- H)-90]^-$ what indicated the presence of a 6-*C*-pentosyl unit, characteristic for 6-*C*-arabinosyl-8-*C*-glucosyl apigenin (isoschaftoside), while that MS/MS spectrum of compound **4**, showed a base peak at *m/z* 443.1 $[(M-H)-120]^-$, indicating the presence of 8-*C*-pentosyl unit, being characterized as 6-*C*-glucosyl-8-*C*-arabinosyl apigenin (schaftoside).

The MS/MS spectrum of peak at 24.5 min, compound **5** (Table 1) obtained from $[M-H]^-$ at *m/z* 447.1, exhibited fragments at *m/z* 357.0 $[(M-H)-90]^-$ and *m/z* 327.0 $[(M-H)-120]^-$ as base peak, suggesting that the mono-*C*-glycosylation is in position 8. In general, the fragmentation of the 6-*C*-isomers is more extensive, giving a ion corresponding to $[(M-H)-18]^-$, probably due to the formation of an additional hydrogen bond between the 2"-hydroxyl group of the sugar and the 5- or 7- hydroxyl group of the aglycone, which confers additional rigidity (Abad-Garcia et al., 2009; Figueirinha et al., 2008). Thus, compound **5** was characterized as 8-*C*-glucosyl luteolin, also known as orientin.

For compound **6**, peak at 26.0 min (Table 1, Figure 1), the MS/MS spectrum obtained from $[M-H]^-$ at m/z 593.1, exhibited pattern of fragmentation [(M-H)-

120]$^-$ at *m/z* 473.0, base peak [(M-H)-180]$^-$ at *m/z* 413.0 and [(aglycon+41)-18)]$^-$ ion at *m/z* 293.1, characteristic of flavone *O*-glucosyl-*C*-glucoside, indicating the presence of sophoroside, and apigenin as aglycone. This compound was characterized as apigenin-6-*C*-glucosyl-2"-*O*-glucoside, also known as isovitexin-2"-*O*-glucoside.

The peak at 26.9 min (compound **7**) (Table 1, Figure 1), main constituent found in *P. incarnata*, used as biomarker for this commercial product, gave the [M-H]$^-$ ion at *m/z* 431.1, [M+H]$^+$ ion at *m/z* 433.2 and [M+Na]$^+$ ion at *m/z* 455.1, respectively. The MS/MS spectrum on precursor ion at *m/z* 431.1 yielded the ions at *m/z* 341.0 and *m/z* 311.0 (base peak), indicating the presence of hexose as monosaccaride and apigenin as aglycone. Compound **7** was characterized as 8-*C*-glucosyl apigenin, also known as vitexin. The proposed structures of compounds **5** and **7** were also confirmed via a comparison of retention time, UV spectra, and MS data with standards.

S. guianensis accumulates flavonoids that occur as *C*- and *O*-glycosides (Table 2). Based on acid hydrolysis, UV spectra and MS/MS fragmentation behavior, the distinction of flavones *C*-glycosides and flavonols *O*-glycosides is easily observed. The carbon-carbon bond is resistant to cleavage, thus in flavones *C*-glycosides the main cleavage are at the bonds of the sugar. For flavonols *O*-glycosides, the sugar moieties are easily lost by neutral losses (Abad-Garcia et al., 2009).

According to the chromatographic profile of *S. guianensis* (Figure 2), the predominant flavonoid, peak at 22.2 min exhibited retention time (Rt), UV and MS spectra similar to compound **2**, characterized as vicenin-2 in *P. incarnata*. No commercial standard of vicenin-2 are available, therefore, this peak was compared with vicenin-2 present in *P. incarnata* extract, used as a surrogate standard. A mixture (1:1, v/v) of *P. incarnata* and *S. guianensis w*as analyzed and vicenin-2 appeared as a single peak at 22.0 min in the chromatogram of the combined extracts. UV, MS and MS/MS spectra of vicenin-2 in the chromatogram of the mixture of *P. incarnata* and *S. guianensis* were consistent with those obtained in each extract. This compound also was analyzed through MS/MS in positive ion mode and MS/MS spectra of protonated and sodiated molecules exhibited fragments that corresponded to the loss of water molecules, which are characteristic of flavones-di-*C*-glycosides (Abranko et al., 2011). The MS/MS spectrum obtained from protonated molecule of vicenin-2 at *m/z* 595.3 showed fragments at *m/z* 577.1, 559.1, 529.0 and 511.0 that corresponded to the loss of water molecules and also the MS/MS spectrum obtained from sodiated molecule at *m/z* 617.3 showed fragments that corresponded to the loss of water molecules at *m/z* 599.1 and 581.1.

The peak at 20.1 min, compound **9** (Table 2, Figure 2) exhibited deprotonated and protonated molecules at *m/z* 609.2 and *m/z* 611.1, respectively.

Table 2. LC/MS data, deprotonated, protonated molecules (*m/z*) for peaks, including the retention times (Rt), MS/MS experiments and maximal absorption wavelength (λ_{max}) of the constituents found in hydroethanolic extract of *Siparuna guianensis.*

	Rt (min)	Proposed structure	UV λ_{max} (nm)	(M+H)+/ (M+Na)$^+$(*m/z*)	(M-H)$^-$ (*m/z*)	MS/MS (*m/z*) from [M-H]$^-$, [M+H]$^+$, [M+Na]$^+$ (%)
8	11.8	procyanidin dimer B1	275	579.1/599.2	577.1	[M-H]$^-$ 559.0 (50), 451.0 (30), 425.0 (90), 407.1 (100), 289.3 (20). [M+H]$^+$ 561.9 (40), 427.0 (100), 409.0 (80)
9	20.1	lucenin-2	271, 348	611.1	609.2	[M-H]$^-$ 591.0 (20), 519.0 (40), 489.1 (100), 399.0 (20), 369.0 (20)
2	22.2	vicenin-2	271, 335	595.3/617.3	593.1	[M-H]$^-$ 575.0 (20), 503.0 (40); 473.0 (100), 383.1 (10), 353.2 (40). [M+H]$^+$ 577.1 (100), 559.0 (40), 529.0 (60), 511.0 (50), 457.1 (50) [M+Na]$^+$ 599.1 (100), 581.1 (60)
10	26.0	quercetin-3-*O*-rutinoside-7-*O*-rhamnoside	263, 355	757.1/779.1	755.0	[M-H]$^-$ 609.0 (100) [M+H]$^+$ 611.1 (100), 594.7 (10), 449.0 (80), 302.9 (40) [M+Na]$^+$ 633.1 (100)
11	27.1	quercetin-3-*O*-pentosyl-pentoside-7-*O*-rhamnoside	263, 356	713.1/735.2	711.0	[M–H]$^-$ 565.0 (100) [M+H]$^+$ 567.1 (10), 449.0 (100), 302.9 (40) [M+Na]$^+$ 589.0 (100)
12	27.4	quercetin-3,7-di-*O*-rhamnoside	263, 354	595.3/617.2	593.1	[M-H]$^-$ 447.0 (100), 301.0 (60), [M+Na]$^+$ 599.0 (10), 471.0 (100), 325.0 (10)
13	29.0	kaempferol-3-*O*-pentosyl-pentoside-7-*O*-rhamnoside	263, 354	697.1/719.1	695.0	[M-H]$^-$ 549.0 (100) [M+H]$^+$ 565.2 (20), 433.0 (100), 286.9 (30) [M+Na]$^+$ 573.1 (100), 286.9 (10)
14	29.5	kaempferol-3,7-di-*O*-rhamnoside	263, 354	579.2/601.3	577.1	[M-H]$^-$ 431.0 (100), 285.0 (70)
15	28.4	quercetin-3-*O*-pentosyl rhamnoside-7-*O*-rhamnoside	ND	726.8/749.3	725.1	[M+Na]$^+$ 603.1 (100), 324.9 (40), 300.9 (20)

ND: The data were not determined.

The MS/MS spectrum on precursor ion at *m/z* 609.2 exhibited the fragmentation pattern $[(M-H)-18]^-$ at *m/z* 591.0, $[(M-H)-90]^-$ at *m/z* 519.0, base peak $[(M-H)-120]^-$ at *m/z* 489.1, $[(M-H)-(90+120)]^-$ at *m/z* 399.0, and $[(M-H)-(120+120)]^-$ at *m/z* 369.0, characteristic of di-*C*-glucosylflavones, indicating luteolin (5,7,3',4'-tetrahydroxyflavone, MM-286) as aglycone. This compound was characterized as 6,8-di-*C*-glucosyl luteolin, which is also known as lucenin-2. Kaempferol and quercetin derivatives were previously identified in *S. guianensis* (Leitão et al., 2005), while the occurrence of flavones di-*C*-glycosides is not known in this specie.

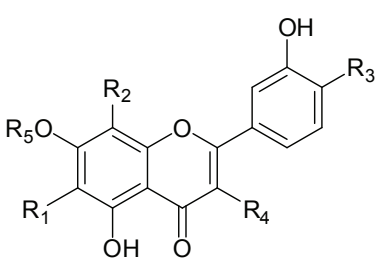
The different types of CID spectra were evaluated with respect to their structural information content, such as, their utility to locate the *O*-linked saccharide residues and to determine the sequence in the disaccharidic part. The glycan cleavage sequence in $[M+H]^+$ ions of flavonol *O*-glycosides started with the elimination of glycoside residue from C-3 carbon atom of flavonol (Shahat et al., 2005; Kachlicki et al., 2008). Ions of deprotonated molecules $[M-H]^-$ are usually more stable than their protonated counterparts, so higher collision energy is necessary for the fragmentation of the precursor ions. Generally, the most abundant product ions obtained from deprotonated molecules were formed after loss of glycoside residue attached to the C-7 carbon atom of flavonol (Shahat et al., 2005; Kachlicki et al., 2008). Thus, generally, the 3-*O* and 7-*O* glycosides in flavonol 3,7-di-*O*-glycosides can readily be located on the basis of ESI-MS/MS, while the loss of 3-*O* glycoside is more abundant than that the 7-*O* glycoside for protonated molecules, the opposite behavior is observed for deprotonated and sodiated molecules (Shahat et al., 2005; Kachlicki et al., 2008).

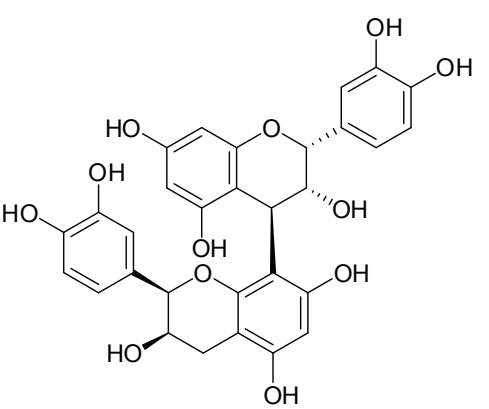
Compounds **10-15** (Table 2, Figure 2) were easily hydrolyzed in an acidic medium. The aglycones obtained through hydrolysis experiments were quercetin and kaempferol. Identification of the aglycones, kaempferol and quercetin, obtained after hydrolysis process, were confirmed by comparison of retention time, UV, MS and co-injection with standards. Inspection of the UV spectra of compounds **10-15** showed absorptions typical of flavonol derivatives, with maximum absorption at band I (347-365 nm) and band II (250-267 nm) (Abad-Garcia et al., 2009; Figueirinha et al., 2008).

Compound **12**, peak at 27.4 min, exhibited deprotonated and protonated molecules at *m/z* 593.1 and *m/z* 595.3, and $[M+Na]^+$ at *m/*z 617.2, respectively. The MS/MS spectrum on precursor ion at *m/z* 593.1 showed a base peak $[(M-H)-146]^-$ at *m/z* 447.0 and a fragment at *m/z* 301.0 (quercetin), typical of a di-*O*-glycosylflavonol. The MS/MS spectrum of sodiated molecule $[M+Na]^+$ at *m/z* 617.2 showed a base peak at *m/z* 471.0 corresponding to the loss of rhamnose, and fragment ions at *m/z* 599.0 corresponding to the loss of 18 u and at *m/z* 325.0, that contained $[agly+Na]^+$ ion product, that also show quercetin as aglycone. These data was also compared with literature data (Kachlicki et al., 2008) and compound **12** was characterized as quercetin-3,7-di-*O*-rhamnoside.

Compound **14**, peak at 29.5 min, exhibited deprotonated and protonated molecules at *m/z* 577.1 and 579.2 and $[M+Na]^+$ at *m/z* 601.3, respectively. The MS/MS spectrum on precursor ion at *m/z* 577.1 exhibited a base peak $[(M-H)-146]^-$ at *m/z* 431.0 and a fragment at *m/z* 285.0 (kaempferol), leading to the characterization of this compound as kaempferol-3,7-di-*O*-rhamnoside.

The most abundant product ion obtained from the MS/MS analysis of deprotonated molecules showed





8

1 R_1=sophoroside; R_3=OH; R_2=R_4=R_5=H
2 R_1=R_2=glucosyl; R_3=R_4=R_5=H
3 R_1=arabinosyl; R_2=glucosyl; R_3=R_4=R_5=H
4 R_1=glucosyl; R_2=arabinosyl; R_3=R_4=R_5=H
5 R_1=R_4=R_5=H; R_2=glucosyl; R_3=OH
6 R_1=sophoroside; R_2=R_3=R_4=R_5=H
7 R_1=R_3=R_4=R_5=H; R_2=glucosyl
9 R_1=R_2=glucosyl; R_3=OH; R_4=R_5=H
10 R_1=R_2=H; R_3=OH; R_4=rutinoside; R_5=rhamnosyl
11 R_1=R_2=H; R_3=OH; R_4=pentosyl-pentoside; R_5=rhamnosyl
12 R_1=R_2=H; R_3=OH; R_4=R_5=rhamnosyl
13 R_1=R_2=R_3=H; R_4=pentosyl-pentoside; R_5=rhamnosyl
14 R_1=R_2=R_3=H; R_4=R_5=rhamnosyl
15 R_1=R_2=H; R_3=OH; R_4=pentosyl-rhamnoside; R_5=rhamnosyl

that compounds **10, 11** and **13** (Table 2) first lost a rhamnose ($C_6O_4H_{10}$) moiety [M-H-146]-, given the fragments at *m/z* 609.0 (compound **10**), 565.0 (compound **11**) and 549.0 (compound **13**) (see Table 2) indicating the presence of 7-*O*-rhamnosyl group linked to hydroxyl group of aglycone (Shahat et al., 2005; Kachlicki et al., 2008). However, MS/MS spectra in negative ion mode did not provide any evidence regarding the nature of the aglycones. Positive mode is more useful for structure elucidation, because can be used to characterize the aglycone type, while the negative mode is more sensitive (Abad-Garcia et al., 2009).

Compound **10**, peak at 26.0 min (Table 2) exhibited protonated and deprotonated molecules at *m/z* 757.1 and 755.0, and $[M+Na]^+$ at *m/z* 779.1, respectively. The following MS/MS event in negative ion mode showed the loss of deoxyhexosyl moiety (146 u) at *m/z* 609.2 and the occurrence of this fragment was coincident with quercetin-3-*O*-rutinoside (Abad-Garcia et al., 2009, Kachlicki et al., 2008). The MS/MS spectrum on precursor ion at *m/z* 757.1 exhibited a base peak at *m/z* 611.1 $[M+H-146]^+$, also coincident with quercetin-3-*O*-rutinoside (Kachlicki et al., 2008), and fragments at *m/z* 449.0 $[M+H-146-162]^+$ and the aglycone quercetin at *m/z* 302.9. In this case, the fragment at *m/z* 611.1 was obtained by the loss of rhamnosyl (146 u) at 3-*O*-hydroxyl group of quercetin. In the MS/MS spectrum of sodiated molecule was observed a base peak at *m/z* 633.1, corresponding to the loss of rhamnosyl moiety (146 u) also indicative the presence of of 7-*O*-rhamnosyl group in aglycone quercetin. The irregular ion at *m/z* 594.7 (Table 2) can be rationalized by loss of an internal glucose residue (Shahat et al., 2005; Kachlicki et al., 2008). Compound **10** was tentatively characterized as quercetin-3-*O*-rutinoside-7-*O*-rhamnoside.

Compound **13**, peak at 29.0 min exhibited protonated and deprotonated molecules at *m/z* 697.1 and 695.0, and $[M+Na]^+$ at *m/z* 719.1, respectively. The MS/MS spectrum on precursor ion at *m/z* 697.1 showed fragments at *m/z* 565.2, corresponding to the loss of a pentosyl moiety (132 u), a base peak at *m/z* 433.0, corresponding to the loss of other pentosyl moiety (arabinose or xylose) and a fragment at *m/z* 286.9 showing kaempferol as aglycone. The base peak at *m/z* 433.0 suggested the presence of dipentosyl moiety located at 3-*O* position of aglycone. In the MS/MS spectrum of sodiated molecule was observed a base peak at *m/z* 573.1, corresponding to the loss of rhamnosyl moiety (146 u) in the 7-*O*-position of aglycone and a fragment at *m/z* 286.9 indicating kaempferol as aglycone. The irregular ion at *m/z* 565.2 can be rationalized by loss of an internal pentoside residue. This compound was tentatively characterized as kaempferol-3-*O*-pentosyl-pentoside-7-*O*-rhamnoside.

Compound **11**, peak at 27.1 min, exhibited protonated and deprotonated molecules at *m/z* 713.1 and 711.0, and $[M+Na]^+$ at *m/z* 735.2, respectively; with 16 mass units more than compound **13**, what suggested quercetin as aglycone. Compounds **11** and **13** showed the same pattern of fragmentation in both positive and negative MS/MS spectra. The retention time of quercetin derivative (27.1 min) was lesser than for kaempferol derivative (29.0 min), which confirmed to the general rule that an increase in the number of hydroxyl groups results in a shorter HPLC retention time in reversed phase (Abad-Garcia et al., 2009; Figueirinha et al., 2008). The MS/MS spectrum on precursor ion at *m/z* 713.1 gave a base peak at *m/z* 449.0, indicating the loss of 264 u (two pentosyl moieties) and a fragment at *m/z* 302.9, that confirmed quercetin as aglycone. The ion at *m/z* 567.1 can be rationalized by loss of rhamnosyl moiety (146 u). Since the loss of rhamnosyl group resulted in a product ion at *m/z* 567.1 with a much smaller relative abundance than the product ion at *m/z* 449.0 formed by loss of the dipentosyl residue, the latter at *m/*z 449.0 indicated the presence of dipentosyl at the 3-*O* and the former at *m/z* 567.1 indicated the presence of rhamnose at 7-*O* position of the aglycone. The MS/MS spectrum of sodiated molecule at *m/z* 735.2 exhibited a fragment at *m/z* 589.0 (100%), corresponding to the loss of rhamnosyl moiety (146 u), indicative the presence of 7-*O*-rhamnosyl group in quercetin. Compound **11** was tentatively characterized as quercetin-3-*O*-pentosyl-pentoside-7-*O*-rhamnoside.

In this extract was also found a trace content of compound **15**, peak at 28.4 min that showed protonated and deprotonated molecule at *m/z* 726.8 and 725.1 and a sodiated adduct at *m/z* 749.3, respectively. The MS/MS spectrum of sodiated molecule at *m/z* 749.3 exhibited a base peak at *m/z* 603.1, that probably correspond to the loss of rhamnosyl group at 7-*O* position of aglycone and a fragment ion at *m/z* 324.9, that contained $[agly+Na]^+$ ion product and a fragment corresponding to quercetin at *m/z* 300.9. Compound **15** was tentatively characterized as quercetin-3-*O*-pentosyl rhamnoside-7-*O*-rhamnoside.

Condensed tannins consist of polyhydroxyflavan subunits with interflavonoid C-C-linkages. The fragmentation reflects the oligomeric composition and the major fragment ions are due to the cleavage of the interflavonoid C-C linkages with losses of catechin units (288 mass units). Procyanidin sequence ions $[(M-H)-288]^-$ were mainly observed, especially in the MS/MS spectra in negative mode of higher oligomers. The peak at 11.8 min (Table 2) found in *S. guianensis* is probably procyanidin B1 (compound **8**), which was detected based on UV absorption maximum at 275 nm and the ions $[M-H]^-$ at *m/z* 577.1, $[M+H]^+$ at *m/z* 579.1, and $[M+Na]^+$ at *m/z* 599.2, respectively. The MS/MS spectrum on precursor ion at *m/z* 577.1 gave several product ions characteristic of procyanidins $[(M-H)-18]^-$ at *m/z* 559.0, $[(M-H)-126]^-$ at *m/z* 451.0, $[(M-H)-152]^-$ at *m/z* 425.0, a base peak $[(M-H)-170]^-$ at *m/z* 407.1, and $[(M-H)-288]^-$

at *m/z* 289.3 (catechin). For the molecular anions (*m/z* 577.1), the sequences end at the monomer catechin. The MS/MS spectrum on precursor ion at *m/z* 579.1 gave a fragment at *m/z* 561.9 $[(M+H)-18]^+$, a base peak at *m/z* 427.0 and a fragment at *m/z* 409.0. There are two possible mechanisms for production of ions *m/z* 425.0 and *m/z* 407.1: first, direct cleavage of the interflavanoid bonds, and second, quinone methide cleavage of the interflavanoid bonds. Retro-Diels-Alder reaction of the heterocyclic ring system of the flavan-3-ol (Hellstrom et al., 2007) subunits gave rise to a fragment of *m/z* 425.1 from anion *m/z* 577.1. The ion at *m/z* 425.1 eliminates water, probably from ring C at position C3/C4, resulting in a fragment ion of *m/z* 407.0.

Biological activity-relationship among flavonoids found in S. guianensis *and anxiolytic activity*

In this study HPLC-ESI-MS/MS technique was applied to the characterization of flavonoids presents in *S. guianensis*, which are compared with the flavonoids found in extract of *Passiflora incarnata* and others reported as anxiolytics. In the Central Nervous System (CNS) several flavonoids bind to the benzodiazepine site on the GABAA receptor producing sedation, anxiolytic or anti-convulsive effects (Jager & Saaby, 2011). Flavonoids of several classes are inhibitors of monoamine oxidase A or B, causing anti-depressants effects and also improving the conditions of Parkinson's patients (Ji & Zhang, 2006). Kaempferol showed an anxiolytic-like activity (Grundmann et al., 2009) and the anxiolytic activity of *Tilia* species had been attributed to the kaempferol 3-*O*-(6"-*p*-coumaroyl glucoside), also known as tiriloside (Aguirre-Hernandez et al., 2010).

Flavones are important active constituents present in *Passiflora* species and appeared to have positive effects on anxiety through their capability to interact with GABAA receptors (Barbosa et al., 2008; Grundmann et al., 2008; Deng et al., 2010; Li et al., 2011). Flavone derivatives of apigenin were reported as anxiolytic agents in different studies using animal models of anxiety (Kumar & Sharma, 2006). Besides this, flavonoids with benzodiazepine receptors specificity and/or anxiolytic activity were obtained from medicinal plants that are traditionally used in folk medicine for their anxiolytic/sedative properties, such as *Turnera aphrodisiaca* and *Passiflora* species (Grundmann et al., 2008; Deng et al., 2010; Li et al., 2011; Kumar & Sharma, 2006).

Aqueous extracts of *P. edulis* and *P. alata* induced anxiolytic activity in rats without disrupting memory processes, and the differences in flavonoid contents were used to explain the differences observed in anxiolytic effects of these plants (Barbosa et al., 2008). Extracts of *P. incarnata*, containing flavones glycosydes as major constituents, exhibited GABA-mediated anxiolytic activity, in vivo (Grundmann et al., 2008). *Passiflora edulis* fo. *flavicarpa* exhibited anxiolytic and sedative activity and the six major flavonoid compounds isolated from the leaves of *Passiflora edulis* fo. *flavicarpa*, lucenin-2, vicenin-2, isoorientin, isovitexin, luteolin-6-*C*-chinovoside, and luteolin-6-*C*-fucoside, had not been detected in Passiflora edulis fo. edulis, which did not exhibited this activity (Kumar & Sharma, 2006).

The anxiolytic activity of *Passiflora* species had been attributed to flavone derivatives of apigenin and luteolin (Grundmann et al., 2008; Deng et al., 2010; Li et al., 2011; Kumar & Sharma, 2006). *S. guianensis* showed a high content of flavonoids, such as, vicenin-2, quercetin-3,7-di-*O*-rhamnoside and kaempferol-3,7-di-*O*-rhamnoside, thus probably its medicinal use as anxiolytic could be attributed to these compounds.

Conclusion

The data obtained through MS/MS spectra in negative and positive ion mode and metal-adducted molecules gave complementary information allowing making of some considerations about structural features of the flavonoids glycosides. The medicinal use of this plant due to anxiolytic activity could be attributed to a high content of flavonoids, which had been reported as anxiolytics. The principal constituent found in *S. guianensis* was vicenin-2. Considering that some flavonoids exhibit antioxidant, anxiolytic and sedative properties, it is reasonable to propose that flavonoids should be considered as a possible tool to complement pharmacological therapies for anxiety disorders and delay the aging process.

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