Flavonol glycosides found in hydroethanolic extracts from *Tilia cordata*, a species utilized as anxiolytics

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ABSTRACT: *Tilia* species, among which is *Tilia cordata* Mill. (Tiliaceae), have been used in folk medicine as anxiolytic. The hydroethanolic extract was analyzed by using liquid chromatography with mass spectrometry HPLC-DAD-ESI-MS/MS in negative ion mode, and its chemical composition was compared to flavonoids reported as anxiolytics. The major flavonoids found were: quercetin-3,7-di-*O*-rhamnoside, kaempferol-3,7-di-*O*-rhamnoside and kaempferol 3-*O*-(6"-*p*-coumaroyl glucoside) or tiliroside. The anxiolytic activity of the genus *Tilia* has been attributed to the presence of quercetin and kaempferol derivatives, while the anxiolytic activity of *T. americana* var. *Mexicana* was attributed to tiliroside, which was also found among the major constituents of this species.

Key words: *Tilia cordata*, HPLC-DAD-ESI-MS/MS, quercetin and kaempferol glycosides, tiliroside.

RESUMO: Flavonóides glicosídeos encontrados no extrato hidroalcoólico de *Tilia cordata*, espécie usada como ansiolítico. As espécies de *Tilia*, entre elas, a *Tilia cordata* Mill. (Tiliaceae) são utilizadas como ansilíticas na medicina popular. O extrato hidroalcoólico foi analisado usando cromatografia líquida acoplada à espectrometria de massas HPLC-DAD-ESI/MS/MS no modo negativo e a sua composição química foi comparada com os flavonóides já reportados como ansiolíticos. Os principais flavonóides encontrados foram: quercetina-3,7-di-*O*-rhamnosideo, canferol-3,7-di-*O*-rhamnosideo, e canferol 3-*O*-(6"-*p*-cumaroil glucosideo) ou tirilosideo. A atividade ansiolítica do gênero *Tília* tem sido atribuída à presença de derivados de canferol e quercetina, enquanto que a atividade ansiolítica da *T. americana* var. *Mexicana* foi atribuída ao tilirosideo, o qual também foi encontrado entre os principais constituintes desta espécie.

Palavras-chave: *Tilia cordata*, HPLC-DAD-ESI-MS/MS, glicosídeos de quercetina e canferol, tilirosideo

INTRODUCTION

Tilia cordata Mill. (Tiliaceae) has been used in folk medicine, primarily as a non-narcotic sedative for sleep disorders or anxiety. The anxiolytic effect of *Tilia* species, such as *T. americana* var. *Mexicana*, has been attributed to the presence of tiliroside (Anesini et al., 1999; Perez-Ortega et al., 2008). Phytochemical studies have demonstrated that *Tilia* species possess hydrocarbons, esters, aliphatic acids (Fitsiou et al., 2007), terpenoids, quercetin and kaempferol derivatives (Pietta et al., 1994), phenolic compounds, condensed tannins (Behrens et al., 2003) and a coumarin scopoletin (Arcos et al., 2006). *Tilia americana* var. *Mexicana* has several flavonoids such as rutin, hyperoside, quercitrin and tiliroside (Aguirre-Hernandez et al., 2010).

The combination of liquid chromatography with electrospray ionization mass spectrometry (LC-ESI-MS) provides the molecular weight and a partial characterization of flavonoid glycosides present in complex mixtures. The structural characterization of flavonoid glycosides has been carried out with mass spectral methods based on collision-induced dissociation (CID) of molecular species, such as protonated molecules $[M + H]^+$, deprotonated molecules $[M – H]^-$ and sodiated molecules $[M + Na]^+$, generated by a ionization technique like ESI (Kachlicki et al., 2008; Shahat et al., 2005). Thus, MS/MS spectra of protonated, deprotonated

and sodiated molecules can be used to obtain information about several structural features, such as the aglycone and the types of carbohydrates that are present (mono-, di-, tri- or tetrasaccharides and hexoses, deoxyhexoses or pentoses) (Kachlicki et al., 2008; Shahat et al., 2005).

Type-A α-aminobutyric acid (GABAA) receptors are the major inhibitory neurotransmitter receptors in the human central nervous system, which are involved in epilepsy, sedation and anxiolysis, producing these effects by binding to GABAA receptors. The GABAergic system has á-aminobutyric acid as neurotransmitter. Anxiolytics facilitate the coupling of GABAergic receptors to GABAA (Mewett et al. 2009) and produce their pharmacological effect by binding to a benzodiazepine recognition site on the GABAA receptor complex. GABAA receptors are heteromeric GABA-gated chloride channels. GABA released from GABAergic interneurons exerts inhibition by acting on GABA receptors at pre-synaptic terminals and post-synaptic neurons to reduce pre-synaptic glutamate release and produce inhibitory postsynaptic currents, or membrane hyperpolarization, in post-synaptic neurons (Mewett et al. 2009). Benzodiazepines (BDZ) are the most common anxiolytic drugs used today. Benzodiazepines bind to the so-called benzodiazepine site, where they modulate the receptor to be more sensitive to GABA, thereby yielding an anticonvulsant, sedative or anxiolytic effect (Huen et al., 2003).

Flavonoids have recently increased in importance because they have been identified as a new type of ligand with *in vivo* anxiolytic properties (Jager & Saaby, 2011). Flavonoids are present in food and medicinal plants and are thus consumed by humans. The flavones apigenin and luteolin derivatives have shown an anxiolytic effect in rodents exposed to behavioral tests (Coleta et al., 2008). In the Central Nervous System (CNS) several flavonoids bind to the benzodiazepine site on the GABAA receptor and modulate the á-aminobutyric acid (GABA)ergic system to produce the biological effect of sedation, anxiolytic or anti-convulsive (Aguirre-Hernandez et al., 2010). Flavonoids of several classes are also inhibitors of monoamine oxidase A or B (Zhu et al., 2006). Various *in vivo* studies have shown that flavonoids can be absorbed after oral administration, pass the blood-brain barrier and produce various effects on the CNS (Jager & Saaby, 2011).

In this study, the major flavonoids present in the hydroethanolic extract of *Tilia cordata* were characterized by using HPLC–DAD–ESI-MS/MS in negative ion mode and a correlation was established between them and the flavonoids reported as anxiolytics. Quercetin and kaempferol

derivatives were found in *Tilia* species. But there is no literature report regarding the determination of phenolic compounds from hydroethanolic extract of *T. cordata*. *Tilia* species have been used in folk medicine due to their anxiolytic and sedative activities, which have been attributed to flavonoids, such as tiliroside. Therefore, the aim of this study was to compare the flavonoids identified in this species with the flavonoids reported as anxiolytics. Tiliroside is one of the main flavonoids found in this species.

MATERIAL AND METHOD

Plant Material

The leaves of *T. cordata* were purchased from "Quimer Ervas & Especiarias S. A." and came with their respective certificates. Quercetin (Q4951, > 95%), apigenin (A3145, > 95%), kaempferol (K0133, > 96%) and quercetrin (Q3001, > 90%) 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 standards (100µg / mL) were prepared in methanol. 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).

Sample preparation

The leaves 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 powder were extracted with 1 L of hydroethanolic solution at 50% (V/V) by maceration (Mendes et al., 2002). 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° C) for 48 hours. The lyophilized extracts were stored in amber flasks at 5° C (freezer).

Phytochemical Assays

The lyophilized hydroethanolic extract 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). For alkaloid analyses, lyophilized samples (50 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) to remove neutral compounds, 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 Assays

The free flavonoid aglycones of flavonoid-*O*-glycosides were released by acidic hydrolysis, as follows: 60mg of samples from *T. cordata* were dissolved in 4mL of 10% (V/V) H_2SO_4 solution, 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 obtained after the hydrolysis reactions was concentrated to approximately 1mL and analyzed by HPLC/DAD using the same chromatographic conditions that were utilized for the analyzes of standards of quercetin and kaempferol.

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

For reversed phase high performance liquid chromatography (RPHPLC) analysis, lyophilized extract was dissolved in water : methanol (80:20) v/v (10mg / 3mL) and filtered with a 0.45 µm filter, prior to injection of 30.0µL into the HPLC system. Spectral UV data from all peaks were collected in the range 240 – 400nm, and chromatograms were recorded at 370 and 260nm 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 the system controller Shimadzu VP series HPLC system (SCL-10A VP). 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 (4.6×250mm, 5µm), connected to a guard column and a gradient of 20–90% B (V/V) over 50 min were utilized for separations, as follows: 0min –20% B in A; 10min – 30% B in A; 20min – 50% B in A; 30 min – 70% B in A; 40min– 90% B in A; 45min – 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 0.5mL min⁻¹, and the temperature of the column was maintained at 30°C. The ionization conditions were adjusted as follows: electrospray ionization was performed using an ion source voltage of - 39 V and a capillary offset voltage of 4400V. 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 1.3V. 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 26 psi. Desolvation was facilitated by using a counter current nitrogen flow set at a flux of 7.0 liter / minute and a capillary temperature of 325°C. The full scan mass acquisition both in negative and positive ion mode were performed by scanning from *m/z* 100 up to 900. 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 (ESI/MS and ESI/MS/MS) obtained, including their respective aglycone, which are determined after hydrolysis reaction using standards of quercetin, kaempferol and also taking into account the ESI-MS and ESI-MS/MS data provided by the literature.

RESULTS AND DISCUSSION

RPHPLC-DAD-ESI-MS/MS analyses

The hydroethanolic extract of *T. cordata* has an acidic pH (5.5). Flavonoids are acidic due to phenolic hydroxyl groups that interface with the neighboring benzene ring, causing a conjugation effect. The yield of the hydroethanolic extract of *T. cordata* was 9.2 g per 100 g of crude plant material. To characterize the qualitative chemical profile, the extract was initially analyzed via TLC (Stahl, 1969; Wagner & Bladt, 1996; Jayaprakasha et al., 2006). Dried TLC plates were sprayed with specific reagent (methanolic hydrochloric acid 2 M; ferric chloride; dragendorff reagent; Liebermann-Burchard; Carr-Price reagent and antimony pentachloride) and heated to observe the color reaction characteristic for each chemical class. The spots of procyanidins 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. This extract showed the presence of flavonoids and procyanidins (condensed tannins). Alkaloids were not detected.

Formic acid is a common modifier for RPHPLC, and its volatility also makes it highly suitable for mass spectrometry. Table 1 summarizes the following information on peaks observed during RPHPLC-DAD-ESI-MS/MS analyses: (1) peak labels, (2) retention times (RT) (min), (3) proposed structure, (4) wavelengths of absorbance maxima (ëmax), (5) *m/z* ratios for the protonated [M + H]⁺ molecules, (6) *m/z* ratios for the deprotonated [M – H]⁻ molecules and (7) the major MS/MS fragments obtained in negative mode. The retention time (RT) on the column is governed not only by the polarity of the molecules but also by their size. Besides, RT of

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the separated substances depend on their solubility in water and/ or their hydrophobicity, and RT having been demonstrated to increase as the hydrophobicity of the compound on the reverse phase columns increase. The sugar position is more important for the retention time than the nature of the sugar (Abad-Garcia et al., 2009).

The susceptibility of the sugar aglycone in acidic hydrolysis depends on the attachment position of the sugar. The loss of the glycan substituent at the 3' or 5' positions occur more readily in comparison with 7' and 3' positions (Abad-Garcia et al., 2009). Compounds **2 - 8** (Figure 1) were easily hydrolyzed in an acidic medium. Identification of the kaempferol and quercetin aglycones after hydrolysis reactions were confirmed by comparing retention time, UV, MS and co-injection with standards. In addition, inspection of the UV spectra of compounds **2 - 7** showed absorptions typical of flavonol derivatives, with maximum absorption at band I (347–370nm) and band II (250–267nm) (Abad-Garcia et al., 2009).

Most of the authentic compounds exhibited [M – H]⁻ ions of sufficient abundance that could be subjected to MS/MS analysis. The first-order mass spectra obtained during analyses after the LC separation provided information about molecular weight of flavonoid glycosides. The number and size of sugars (hexose, deoxyhexose or pentose) substituted to the aglycone and also the aglycone were established from the second-order product ion mass spectra, which were performed in the Collision-Induced Dissociation (CID) MS/MS negative mode. Ions of deprotonated molecules [M - H]⁻ are usually more stable than their protonated [M + H]⁺ counterparts; thus, higher collision energy is necessary for the fragmentation of the precursor ions (Kachlicki et al., 2008; Shahat et al., 2005;

Abad-Garcia et al., 2009). Protonated flavonol di-O-glycosides containing a substituent at both the 3-O and 7-O positions more easily lose the 3-O than the 7-O glycoside (Kachlicki et al., 2008; Shahat et al., 2005). Thus, while the loss of the 3-O glycoside is more pronounced than that of the 7-O glycoside in the MS/MS spectra of protonated molecules, the opposite behavior is noted for deprotonated and sodiated molecules (Kachlicki et al., 2008; Shahat et al., 2005).

The peak at 25.2 min for compound **2** (Table 1, Figure 1 and 2) exhibited deprotonated and protonated molecules at *m/z* 609.2 and 611.3, and a sodiated molecule [M + Na]⁺ at *m/z* 633.1, respectively, in the ESI/MS spectra. The MS/MS spectrum of deprotonated molecule gave rise to the *m/z* 447.0 [M – H - 162]⁻ as the base peak, formed after the loss of the glucose sugar attached to the C-7 carbon atom of flavonol. The fragment at *m/z* 462.9 probably correspond to the loss of rhamnose sugar at C-3 carbon atom of flavonol, and at *m/z* 301.0 (aglycone quercetin), being typical of di-O-glycosylflavonol. This type of fragmentation occurring in negative ion mode, in which the loss of a sugar unit gives the most abundant base peak (*m/z* 447.0) different from the peak of the aglycone (*m/z* 301.0), indicated that residues of glycosilation are present in more than one phenolic hydroxyl group of the aglycone (Kachlicki et al., 2008; Shahat et al., 2005). Glucose is more common than galactose. Compound **2** was tentatively characterized as quercetin-3-O-rhamnoside-7-O-glucoside or quercetrin-7-O-glucoside.

Compound **3** (Table 1, Figure 1 and 2), peak at 27.4 min, exhibited deprotonated and protonated molecules at *m/z* 593.1 and *m/z* 595.2, and a sodiated molecule [M + Na]⁺ at *m/z* 617.2, respectively, in the

TABLE 1. HPLC/MS data, protonated and deprotonated molecules (*m/z*) for peaks, including the retention times (RT), MS/MS experiments and maximal absorption wavelength (λ_max) of the constituents found in *T. cordata*.

	R_t (min)	Proposed structure	UV λ_max (nm)	(M + H)⁺ (m/z)	(M - H)⁻ (m/z)	MS/MS (m/z) (ESI⁻) (%) (m/z)
1	16.2	procyanidin dimer B2	275	579.2	577.1	407.1 (100), 425.0 (90), 451.0 (30), 559.0 (50), 289.0 (20)
2	25.2	quercetin-3-O- rhamnoside -7-O- glucoside	260, 355	611.3	609.2	447.0 (100), 462.9 (70), 301.0 (60)
3	27.4	quercetin-3,7-di-O-rhamnoside	263, 355	595.2	593.1	447.0 (100), 301.0 (30)
4	28.6	quercetin-3-O-glucoside	260, 355	465.1	463.1	301.0 (100)
5	29.5	kaempferol-3,7-di-O-rhamnoside	260, 350	579.2	577.1	431.0 (100), 285.0 (80)
6	30.6	quercitrin	260, 350	449.1	447.1	301.0 (100)
7	32.7	kaempferol-3-O-rhamnoside	ND	433.1	431.1	285.0 (100)
8	34.2	tiliroside	260, 315	595.2	593.1	285.0 (100) 447.0 (10)

ND – not determined
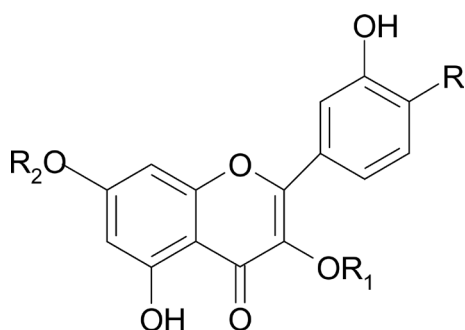
Compound	R	R$_1$	R$_2$
2	OH	Rha	Glc
3	OH	Rha	Rha
4	OH	Glc	H
5	H	Rha	Rha
6	OH	Rha	H
7	H	Rha	H
8	H	(6"-p-coum-glc)	H

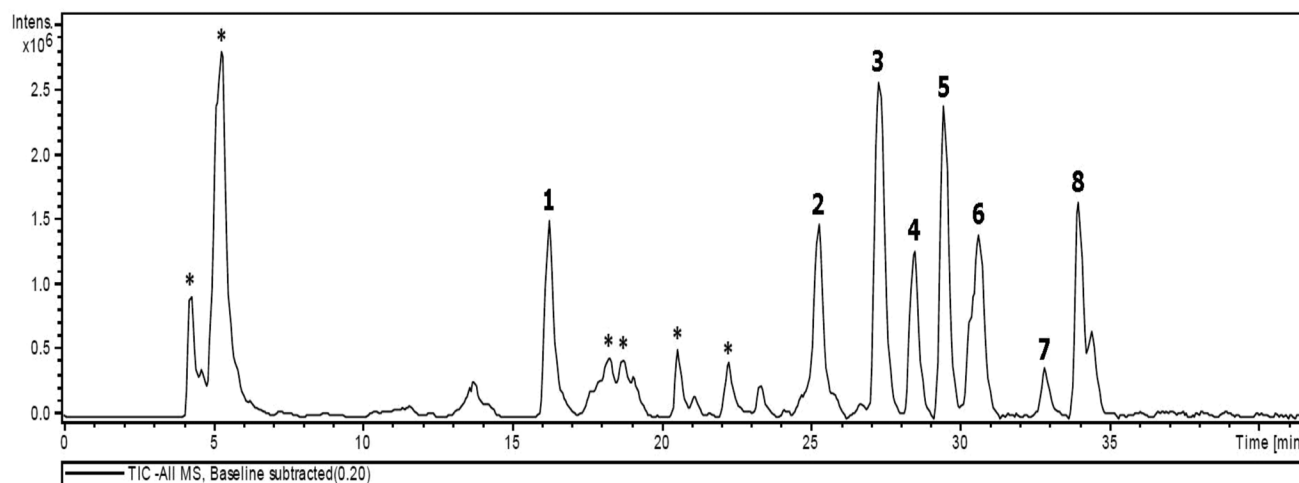**FIGURE 1**. Proposed structure of flavonols glycosides found in *T. cordata*.

FIGURE 2. HPLC-ESI/MS chromatogram of hydroethanolic extract of *T. cordata*.

ESI/MS spectra. The MS/MS spectrum in negative ion mode showed a base peak [(M – H) - 146]⁻ at *m/z* 447.0 and a fragment at *m/z* 301.0 (quercetin), typical of di-*O*-glycosylflavonol. The most abundant product ion obtained from deprotonated molecule was formed at *m/z* 447.0, after loss of the rhamnose residue probably attached to the C-7 carbon atom of flavonol (Kachlicki et al., 2008; Shahat et al., 2005). Compound **3** was tentatively characterized as quercetin-3,7-di-*O*-rhamnoside.

At the peak at 29.5 min, **c**ompound **5** showed deprotonated and protonated molecules at *m/z* 577.1 and 579.2 and a sodiated molecule [M + Na]⁺ at *m/z* 601.3, respectively, and the MS/MS spectrum in negative ion mode showed a base peak [(M – H) - 146]⁻ at *m/z* 431.0 and a fragment at *m/z* 285.0 (aglycone kaempferol), exhibiting the same pattern of fragmentation of compound **3**, leading to the characterization of this compound as kaempferol-3,7-di-*O*-rhamnoside.

In this species, flavonoids monoglycosides, such as quercetin-3-*O*-glucoside (peak at 28.6 min, compound **4**), quercitrin (quercetin-3-*O*-rhamnoside) (peak at 30.6 min, compound **6**) and kaempferol-

3-*O*-rhamnoside (peak at 32.7 min, compound **7**) were also found (Table 1, Figure 2), which were characterized by comparing with the previously reported MS/MS fragmentation data and UV spectra. Compound **4** exhibited deprotonated and protonated molecules at *m/z* 463.1 and *m/z* 465.1, respectively, and the MS/MS spectrum of deprotonated molecule showed a base peak at *m/z* 301.0 that indicated the presence of quercetin as aglycone and hexose (162 u) as sugar. The other quercetin derivative, compound **6** exhibited protonated and deprotonated molecules at *m/z* 449.1 and *m/z* 447.1, respectively. In this case, the base peak obtained at *m/z* 301.0 was formed by the loss of rhamnose (146 u). Compound **7** exhibited deprotonated and protonated molecules at *m*/z 431.1 and *m/z* 433.1, respectively, and the MS/MS spectrum of deprotonated molecule exhibited a base peak at *m/z* 285.0, indicating kaempferol as aglycone and rhamnose as glycoside. Identification of compounds **6** was also confirmed by comparing the retention times, UV and mass data with authentic standards.

Compound **8**, peak at 34.2 min (Table 1, Figure 1 and 2), exhibited deprotonated and

protonated molecules at *m/z* 593.1 and 595.2, and a sodiated molecule [M + Na]⁺ at *m/z* 617.2, respectively. This compound presented a UV spectrum with ëmax at 260.0 (band II) and 315.0 nm (band I), suggesting that this flavonol is acylated because it showed an absorption maximum for band I at 315nm. The main characteristics of *p*-coumaroylglycosylated flavonols are the shift of absorption for 315nm (Shahat et al., 2005; Abad-Garcia et al., 2009). The MS/MS spectrum in negative ion mode produced a base peak [(M – H) – 146 - 162]⁻ at *m/z* 285.0, resulting from the loss of the coumaroyl glucoside moiety and revealing the presence of kaempferol and another fragment [(M – H) - 146]⁻ at *m/z* 447 (10% of the base peak), showing the presence of coumaroyl group. Acyl groups are predominantly located at the 6-position of hexose moiety (Abad-Garcia et al., 2009; Kite et al., 2011). Generally, flavonoid glycosides esterified with aromatic acids have higher retention times on RP-HPLC columns than diglycosides and monoglycosides (Abad-Garcia et al., 2009). The MS/MS spectrum in positive ion mode exhibited fragments at *m/z* 577.1 (10%) [(M + H) – 18]⁺, at *m/z* 309.0 (100%), resulting from glucose more *p*-coumaroyl moiety and at m/z 287.1 (10%) (kaempferol). This compound was characterized as kaempferol 3-*O*-(6"-*p*-coumaroyl glucoside), known as tiliroside (Abad-Garcia et al., 2009). Kaempferol and quercetin derivatives were previously identified in *T. cordata* (Pietta et al., 1994; Loscalzo et al., 2009). Quercetin-3,7-di-*O*-rhamnoside and kaempferol-3,7-di-*O*-rhamnoside were also found in *Tilia argentea* and were shown to possess potent antinociceptive (reduced the sensitivity to painful stimuli) and anti-inflammatory activity (Toker et al., 2004).

Type B procyanidin dimers

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). The peak at 16.2min is probably procyanidin dimer B2 (dimer of flavonol catechin) (compound **1,** Table 1, Figure 2) based on UV absorption maximum at 275 nm, deprotonated molecule [M - H]⁻ at *m/z* 577.1, protonated molecule [M +H]⁺ at *m/z* 579.2, and sodiated molecule [M + Na]⁺ at *m/z* 599.2, respectively. The MS/MS spectrum of the deprotonated molecule 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* 289.0 (catechin). The MS/MS spectrum in positive ion mode 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. Retro-Diels-Alder fission of the heterocyclic ring system of the flavan-3-ol (Hellstrom et al., 2007) subunits gave rise to a fragment of *m/z* 425.0 from anion *m/z* 577.1. The ion at *m/z* 425.0 eliminates water, probably from ring C at position C3/C4, resulting in a fragment ion of *m/z* 407.1.

Relationship among flavonoids found in this species and flavonoids known as anxiolytics.

Tilia sp. (Tiliaceae) have been used around the world due to its anxiolytic and sedative activity, and quercetin and kaempferol flavonoids have been shown to be responsible for its sedative effect (Aguirre-Hernandez et al., 2010; Viola et al., 1994; Martinez et al., 2009). *Tilia* extracts acted as an agonist of the peripheral benzodiazepine receptor, suggesting the presence of flavonoids capable of binding to the peripheral type of benzodiazepine receptor binding sites (Aguirre-Hernandez et al., 2010; Viola et al., 1994). The pharmacological assay that guided a purification of an ethanol extract of *Tilia petiolaris* DC. inflorescences resulted in the isolation and identification of neuroactive flavonoid glycosides, among which areisoquercitrin, quercetin-3-O-glucoside-7-O-rhamnoside and kaempferol-3-O-glucoside-7-O-rhamnoside (Toker et al., 2004). *T. americana var. mexicana* aqueous extract produced an antinociceptive effect in models like formalin and arthritic pain, reinforcing its use for treating this type of affection in folk medicine, in which an anxiolytic compound is probably involved. The chromatographic analyses of aqueous extracts indicate the presence of glycosides from quercetin such as kaempferitrin, isoquercitrin, astragalin and tiliroside as flavonoids responsible for the antinociceptive activity (Loscalzo et al., 2009; Martinez et al., 2009). Kaempferitrin, isoquercitrin, astragalin and tiliroside have been reported as the major compounds in polar extracts of various *Tilia* species such as: *Tilia cordata*, *Tilia rubra*, *Tilia argentea*, and *Tilia platyphyllos* (Loscalzo et al., 2009; Martinez et al., 2009). Phytochemical analyses evidenced that flavonoids comprise the principal group of compounds present in the anxiolytic extract of *T. Americana* (Herrera-Ruiz et al., 2008) and the anxiolytic activity of *T. americana* var. *Mexicana*, widely used in Mexican traditional medicine to relieve sleeplessness, headache and nervous excitement, has been attributed to tiliroside (3-O-(6"-O-(E)-p-coumaroyl)-β-glucosylkaempferol) (Herrera-Ruiz et al., 2008), which was also found in the current study for *T. cordata* hydroethanolic extract. The anxiolytic activity of *Passiflora* species has been attributed to flavones *C*-glycosides (Li et al., 2011). Flavonols that act as monoamineoxidase A and B (MAO A and B) inhibitors can modulate monoamine levels in the brain (serotonine, dopamine,

and norepinephrine),which causes behavioral modifications in rodents, indicating an anxiolytic effect (Chimenti et al., 2006). Beyond its antioxidant effect, quercetin, like other flavonoids, exhibits a wide range of neuropharmacological actions including analgesia, sleep, anticonvulsant, sedative and anxiolytic effects (Dai et al., 2006). Acylated flavonol monorhaminoside have been identified as promising phytochemical class (Pfisterer et al., 2011).

In conclusion, since *T. cordata* exhibited a high content of flavonol *O*-glycosides (mono- and di-) quercetin and kaempferol derivatives and tiliroside, its medicinal use as anxiolytic could be attributed to the presence of these flavonoids, in special tiliroside, which has been reported as the anxiolytic constituent of *T. americana* var. *Mexicana*.

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