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Total Phenolic, Flavonoid Content and Antioxidant Activity of Vitex megapotamica (Spreng.) Moldenke

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ABSTRACT

In recent years, a substantial amount of evidence has pointed to the key role of free radicals and other oxidants as the main culprits for aging and degenerative diseases associated with aging, such as cancer, cardiovascular diseases, cataract, decline of the immune system and brain dysfunctions. The objective of this work was therefore to detect variations in total phenol and flavonoid content, and to define the antioxidant activity of samples of Vitex megapotamica. Dried samples were submitted to extraction to obtain the hexane, ethyl acetate and ethanol fractions. Phytochemical prospecting and an analysis of the phenolic content and antioxidant activity was carried out. The data was analyzed according to the mean±standard deviation and submitted to analysis of variance followed by Tukey's test with a significance of ($p < 0.05$). Flavonoids, tannins, coumarins, terpenoids, steroids, alkaloids and anthraquinones were detected in the samples. The content of total flavonoids varied between 3.18±0.58 and 7.22±0.17 g/100g, while the total phenols ranged from 1.13±0.16 to 18.44±0.21 g/100g. The extracts produced EC50 between 339.75±6.56 and 278.33±23.11 μg/mL. The results indicate that the use of solvents of different polarities in the extraction process is an important strategy to detect variations in the levels of total phenols and flavonoids, and of antioxidant activity, in samples of V. megapotamica.

Keywords: *Vitex montevidensis, phenol, flavonoids, antioxidant activity, phytochemical prospecting.*

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Introduction

The genus *Vitex* L. (Verbenaceae) has approximately 250 species with tropical and subtropical distribution, with many of them being considered medicinal. Several species occur in Brazil, from the Amazon (*V. triflora* Vahl.; *V. odorata* Hub.) and central Brazil (*V. cynomosa* Bertero), to the southern region (*V. megapotamica* (Spreng.) Moldenke) (Evans et al., 2009).

The species *Vitex megapotamica* (Spreng.) Moldenke also cited in literature as its synonym *Vitex montevidensis*, belongs to the family of Verbenaceae and is characterized as a tree of 3 to 10 meters in height, with a dark grayish bark that detaches with longitudinal blades. It has compound, digit-form leaves, generally with 5 elliptical-oval folioles and with an acute apex and long petiole. It has numerous, small, purplish-white, terminal or axillary flowers (Harley et al., 2010).

It's a species that partially sheds its leaves in the dry season, its popular names include "tarumã", "azeitona do mato", "azeitona brava", "sombra de touro", five leaves and "copiúba". It is found in the southern region of Brazil, Uruguay, Paraguay and Argentina. The fruits are edible, sweet, and very sought after by the fauna as food. It blooms from September to January and fructifies from December to February (Cardoso, 2004; ESALQ-USP, 2015).

In popular medicine, the infusion of the leaves of this plant is used in the treatment of hemorrhoids, as blood purifier (hypocholesterolemic agent), as diuretic agent, for cutaneous afflictions, as expectorant, for arterial hypertension, and as anti-inflammatory agent, among other therapeutic uses (Franco and Fontana, 1997). The isolation of steroids and iridoids (Rimpler, 1969; Rimpler, 1972), a hypoglycemic activity of the leaves of *V. megapotamica* (Zanatta et al., 2007), the hypolipidemic effect of the crude hydroalcoholic extract and the decoction of the bark, including their contribution in reducing serum levels of cholesterol and triglyceride (Brandt et al., 2009), have been reported for the species under study.

According to Ballvé et al., (1991) and Boligon (2011), the leaves of *V. megapotamica* (cited under *V. montevidensis*) have anthocyanin heterosides, phenols and tannins, catechins, flavonoids and xanthones, steroids and triterpenoids (free steroids), cardio-active heterosides, coumarins, organic acids and phenols. As such, it is crucial to study the levels of total phenols, total flavonoids and the antioxidant activity of *Vitex megapotamica* - Verbenaceae.

Materials and Methods

Collection and preparation of the raw material

The leaves of *Vitex megapotamica* - Verbenaceae were collected in the city of Nova Prata do Iguaçu, in the southwest region of the State of Parana, Brazil, between December 2014 and March 2015. The collection site can be found at an altitude of 867m, at latitude 25° 33' 46" S and longitude 53º 18' 45" W. Dried herbarium specimens were made containing the branches and leaves of the plant species (sterile plant). Three samples (A, B and C) were collected during the blooming period (spring). The samples were exsiccated in the Department of Bothany of the União de Ensino do Sudoeste do Paraná - Unisep - Francisco Beltrão Campus - Paraná - Brazil. One voucher specimen was deposited in the Herbarium of Unisep under the number 1,212. A sample of the same material was delivered to the Municipal Botanical Garden of Curitiba-PR.

The plants were then stored in a dehumidification chamber at a temperature of 24ºC during 45 days for drying. After this period, the leaves were separated and crushed, obtaining the plant biomass (powder) for the preparation of the extracts. The extracts of *V. megapotamica* were obtained in the Laboratory of Chemistry and Phytochemistry of the União de Ensino do Sudoeste do Paraná - Unisep - Francisco Beltrão - Paraná - Brazil.

Extraction Process

Samples of dried and crushed *V. megapotamica* were extracted by static maceration using solvents of increasing polarity: hexane, ethyl acetate and ethanol. The hexane (EH), ethyl acetate (EA) and ethanolic (EE) extracts were then filtered through filter paper and the solvents removed through evaporation, obtaining dry extracts.

Preparation of Stock Solutions

The stock solutions with a concentration of 5mg/mL were prepared based on 0.250 g of each dry extract solubilized in Dimethyl sulfoxide (DMSO). The dilution of each solution produced concentrations of 1mg/mL for the realization of the tests.

Phytochemical Prospecting

Chemical classes of secondary metabolism have been investigated in the extracts of *V. megapotamica* hrough identification reactions according to Matos (1997): flavonoids (reactions with $AlCl_3$, H_3BO_3, NaOH 1N, and Shinoda reactions), tannins (reactions with lead acetate, copper acetate, iron salts, alkaloids and gelatin), coumarins (reaction with KOH 5%), steroidal heterosides (Kedde, Libermann-Buchard and Baljet reactions), saponins (foam index), alkaloids (Bertrand, Bouchardat, Dragendorff and Mayer reactions) and anthraquinones (Borntraeger reaction).

Determination of Total Phenol Content

Total phenol contents were quantified by the spectrophotometric method using the Folin-Ciocalteu reagent (Sousa et al., 2007) and gallic acid as standard. A sequence of five test tubes, in triplicate, 0.01 mL (tube1), 0.02 mL (tube 2), 0.03 mL (tube 3), 0.04 mL (tube 4) and 0.05 mL (tube 5), was prepared from a stock solution of gallic acid 1 mg/mL. 5 mL of the diluted Folin-Ciocalteau reagent, 4 mL of sodium carbonate and water were then added to make up the volume to 10 mL. The tubes were placed in the dark for 1 hour at room temperature. The readings were performed with a spectrophotometer at 773 nm and the absorbances were used to obtain the calibration curve and coefficient of determination (R^2) by the least squares method. Solutions of the extracts were prepared for the acquisition of the absorbances that were substituted in the curve equation, determining the levels of total phenols.

Determination of Total Flavonoid Content

The quantification of total flavonoids was done with the spectrophotometric method according to Sobrinho et al., (2008). A 0.5 mg/mL solution of rutin was prepared. Aliquots of 0.02 mL, 0.05 mL, 0.1 mL, 0.2 mL and 0.3 mL of this solution were transferred to test tubes, in triplicate, and added to 0.12 mL of glacial acetic acid, 2 mL of pyridine:ethanol (2:8), 0.4 mL of ethanol, 0.5 mL of aluminum chloride 8%, and water to obtain a final volume of 5 mL. The readings were performed with a spectrophotometer at 418 nm and the absorbances were used to obtain the calibration curve and coefficient of determination (R^2) by the least squares method. Solutions of the extracts were prepared for the acquisition of the absorbances that were substituted in the curve equation, determining the levels of total flavonoids.

Antioxidant Activity

The antioxidant activity of the extracts was determined through the spectrophotometric method using the free radical DPPH (2.2-diphenyl-1-picrylhydrazyl) as described by Mensor et al., (2001). Stock solutions of dry extracts and rutin (positive control) at 1 mg/mL in ethanol 98% were prepared and diluted to various μg/mL concentrations for the spectrophotometric readings. A solution of 0.3 mM DPPH was also prepared to perform the test. After 60 minutes of reaction, the ability of the extracts and of the rutin to reduce 2.2-diphenyl-1-picrylhydrazyl to 2.2-diphenyl-1-picryl hydrazine was verified. The color change from purple to yellow was detected through the decrease in absorbance in a spectrophotometer at a wavelength of 520 nm. Based on the absorbance readings, the percentage of antioxidant activity (%AA) corresponding with the amount of DPPH reduced by the extracts was determined. The antioxidant activity was therefore expressed according to equation 1 from Mensor et al., (2001), described below:

$$\%AA = 100 - \{[(Abs_{amostra} - Abs_{branco}) \times 100] / Abs_{controle}\} \quad (1)$$

Where:

Aa = Sample absorbance of the sample; Ab = white -light absorbance; Ac = control absorbance.

After obtaining the antioxidant activity, the half maximal effective concentration (EC_{50}) of extracts was obtained by linear regression analysis using the least squares method, obtaining the equation of the curve and the coefficient of determination (R^2). The tests were performed in triplicate.

Statistical Analysis

The results presented in this study correspond to the average of three repetitions (n=3) ± standard deviation from the mean. If the null hypothesis had a probability of less than 5% ($p < 0.05$) of occurring after applying ANOVA, followed by multiple comparisons by Tukey's test, then the antioxidant activity results were considered statistically different. The correlation coefficients were determined between the total phenol content and the effective concentration of each extract, EC_{50}.

Results and Discussion

The identification reactions of chemical classes of secondary metabolites revealed the presence of flavonoids, tannins, coumarins, terpenoids and steroids, alkaloids and anthraquinones in samples of *V. megapotamica* (Table 1).

In the three samples (A, B and C), however, it was observed that the classes of constituents were identified according to the polarity of the solvent used in the extraction. Since flavonoids and tanninds are more polar, for example, their reactions were positive in the extracts in the ethyl acetate and ethanolic solution (Tiwari et al., 2011).

The negative reactions are indicative of the absence or low levels of constituents in the analyzed extracts. The absence of a constituent, such as of saponins, may be a result of it not being present, or of the decrease in gene expression of enzymes involved in the biosynthesis of secondary metabolites (Pichersky and Gang, 2000). Unlike the results shown in Table 1, saponins were found in a study of seasonal variation (Borella et al., 2006). The absence of saponins may therefore be associated with the time of collection.

The chemical prospecting also demonstrates, through the absence or presence of reactions, the difference between the analyzed extracts (Table 1). This means that the use of solvents of different polarities in the extraction process is an important strategy to detect variations in the chemical composition of the samples of *V. megapotamica*. In addition, it is possible that temporal and spatial variations arising from seasonality, circadian rhythm

Table 1- Phytochemical prospection of the samples A, B and C of *Vitex megapotamica* - Verbenaceae

Class	Reactions	EH	EA	EE	EH	EA	EE	EH	EA	EE
Flavonoids	AlCl3	-	-	-	-	-	-	-	-	+
	H3BO3	-	+	+	-	-	+	-	-	+
	NaOH	-	+	+	-	+	+	-	+	+
	Shinoda	-	-	-	-	-	-	-	-	-
Tannins	Lead acetate	-	+	-	-	-	+	-	+	-
	Copper acetate	-	+	-	-	-	+	-	+	-
	Iron Salts	-	-	-	-	-	-	-	-	-
	Alkaloids	-	-	-	-	-	-	-	-	-
	Gelatin	-	-	-	-	-	-	-	-	-
Coumarins	KOH	-	-	-	-	-	-	-	-	-
Terpenoids/Steroids	Baljet	+	+	+	+	+	+	+	+	+
	Kedde	-	-	+	-	-	+	-	+	+
	Lieberman-Buchard	+	-	+	+	-	+	+	+	+
Saponins	Foam index	-	-	-	-	-	-	-	-	-
Alkaloids	Dragendorff	+	-	+	+	-	+	+	+	+
	Mayer	-	-	-	-	-	-	-	-	-
	Bertrand	-	-	-	-	-	-	-	-	-
	Bouchardat	-	-	-	-	-	-	-	-	-
Anthraquinones	Borntraeger	-	-	-	-	-	-	-	+	+

Hexane extract (EH); Ethyl acetate extract(EA); Ethanolic extract (EE). (+) positive reaction; (-) negative reaction.

and development, temperature, the availability of water, ultraviolet radiation, nutrients, air pollution, induction by mechanical stimuli or attack by pathogens, among others, are related to the alteration in the synthesis of special metabolites (Gobbo-Neto and Lopes, 2007).

The results showed the presence of different chemical classes, which may be associated with the biological activities of *V. megapotamica*, especially those related to free radicals.

After defining the wavelength with a maximum absorption at 773 nm, the calibration curve of gallic acid (y = 0.115x + 0.005) was obtained to quantify total phenol contents. The absorbance values of extracts were replaced in this equation, determining the total phenol contents, which ranged from 1.13±0.16 to 18.44±0.21 g/100g equivalent to gallic acid (Table 2). In sample A, the highest total phenol content was obtained in the ethanolic extract, while samples B and C had more significant levels in the ethyl acetate extracts.

The means differ among themselves after ANOVA followed by Tukey's Test for $p < 0.05$.

The ethyl acetate extract revealed a higher total phenol content for samples B and C ($p < 0.05$), probably due to the affinity of these substances with the employed solvent. Phenolic substances have a higher affinity with polar solvents, such as ethanol and ethyl acetate (Tiwari et al., 2011). In this sense, the extraction process was fundamental to identify the difference in total phenol levels in the products analyzed. This difference may be related to intrinsic or extrinsic environmental factors that influence the biosynsthesis of special metabolites in plants (Gobbo-Neto and Lopes, 2007).

When the total phenol contents in the ethyl acetate extract are considered, it is possible that the main phenolic constituents found in this extract are tannins and flavonoids, as shown in table 1. The result for total phenols in the ethyl acetate extract therefore corroborates the findings of the phytochemical prospecting.

The absorbance values of the extracts of samples of *V. megapotamic*a were replaced in the equation of the calibration curve of rutin (y = 0.0104x + 0,0593) and the levels of total flavonoids equivalent to rutin were determined, producing a variation of 3.18±0.49 to 7.22±0.17 g/100g (Table 3). The ethyl acetate and ethanolic extracts of sample C had higher quantities of flavonoids. As expected, the hexane extracts did not reveal the presence of flavonoids.

The means differ among themselves after ANOVA followed by Tukey's test for $p < 0.05$.

Although the samples were taken from the same plant species, it is clear that they differ in the levels of flavonoids, which is an active substance group found in *V. megapotamica* (Ballvé et. al., 1991; Borella et al., 2006;

Table 2.-Mean total phenol content of the extracts of the samples of *Vitex megapotamica* - Verbenaceae

Extracts	Total Phenols g/ 100g		
	Sample A	Sample B	Sample C
Hexane	1.13 ± 0.16	1.80 ± 0.10	1.65 ± 0.15
Ethyl acetate	5.78 ± 0.34	9.65 ± 0.15	18.44 ± 0.21
Ethanolic	8.15 ± 0.20	7.60 ± 0.05	9.13 ± 0.12

Table 3- Mean total flavonoid content of the extracts of the samples of *Vitex megapotamica* - Verbenaceae

Extracts	Total Flavonoids g/100g		
	Sample A	Sample B	Sample C
Hexane	---	---	---
Ethyl acetate	3.18 ± 0.58	4.78 ± 0.23	7.22 ± 0.17
Ethanolic	3.66 ± 0.40	---	4.88 ± 0.54

Table 4- Antioxidant activity of the aerial extracts of *Vitex megapotamica* - Verbenaceae through the DPPH test

Extracts	Total Flavonoids g/100g			
	Sample A	Sample B	Sample C	Standard
Hexane	> 320	278.33±23.11	> 320	---
Ethyl acetate	163.11 ±20.12	126.38 ± 9.21	46.11 ± 3.66	---
Ethanolic	39.75 ± 6.56	67.57 ± 1.16	75.44 ± 8.84	---
Rutin	---	---	---	14.55±0.56

Boligon, 2011; Saldanha; Vilegas and Dokkedal, 2013).

This difference is related to the use of solvents of different polarities employed in the extraction process, which influenced the clarity with which the variation of flavonoids in the samples could be detected. In addition, factors associated with temporal and spatial variations can change the synthesis of special metabolites, including flavonoids (Gobbo-Neto and Lopes, 2007).

The results of this study corroborate those described by Borella et al., (2006) who showed variation in the flavonoid contents in different samples of *Baccharis trimera*. It is important to note that the changes in the levels of total flavonoids may cause a variation in biological activities.

This same behavior was observed by Onofre et al., (2015), who observed that the flavonoid levels varied in different samples and in different periods for *Stachytarpheta cayennensis.*

The levels of total constituents in plant derivatives depend on the extraction process and its variables, such as the solvent used. The polarity of the solvent interferes with the extraction, potentially extracting flavonoid glycosides in the ethanolic extract or free flavonoids in the ethyl acetate extract (Cechinel Filho and Yunes, 1998). The solvent can therefore guide the isolation of certain groups of flavonoids. Some free flavonoids, such as quercetin, luteolin and nepetin, have been isolated in the extract with ethyl acetate of *V. megapotamica* (Ballvé et. al., 1991; Borella et al., 2006; Boligon, 2011; Saldanha; Vilegas and Dokkedal, 2013; Onofre et al., 2015; Tan et al., 2016).

Table 4 presents the half maximal effective concentrations (EC_{50}) of the rutin standard and the hexane, ethyl acetate and ethanolic extracts of the three samples of *V. megapotamica.* The values shown represent the effective concentrations able of reducing 50% of the DPPH present in the solution. The EC_{50} of the extracts varied between 39.75 ± 6.56 to 278.33±23.11 μg/mL. Rutin produced an EC_{50} of 14.55±0.56 μg/mL. All samples showed less antioxidant activity than the rutin standard.

The means differ among themselves after ANOVA followed by Tukey's Test for $p < 0.05$.

The hexane extracts of samples A and C produced EC50 values above 320 μg/ mL, the highest concentration used in this assessment, showing less antioxidant activity due to the low extraction of phenolic substances. The EC_{50} of the hexane extract of sample B was equal to 278.33±23.11 μg/ mL, which can be explained by the higher content of total phenols. Polar solvents, such as ethanol and ethyl acetate, extract greater quantities of phenolic substances with antioxidant activity. These substances, such as flavonoids, can react with the free radicals, neutralizing their oxidant effect.

The results of this study corroborate the data obtained by other authors, who've correlated the content of phenolic substances and the antioxidant potential of plant extracts (Onofre et al., 2015; Tan et al., 2016). By observing the total phenolic compound content of the crude methanolic extract and fractions of the leaves of the species *Palicourea rigida*, Rosa et al., (2010), found that despite the low activity presented by the crude extract (500 ppm), the ethyl acetate fraction showed moderate activity (192 ppm) and the highest total phenolic content among the fractions tested. Similarly, by analyzing the content of total phenols and antioxidant activity of five medicinal plants, Souza et al., (2007) concluded that three species (*Terminalia brasiliensis, Cenostigma macrophyllu* and *Copernicia cerifera*) showed a positive relationship between phenol contents and antioxidant capacity, as analyzed by the DPPH method.

Conclusion

The use of solvents with different polarities in the extraction process was crucial to detect variations in the chemical composition and antioxidant activity in samples of *V. megapotamica*. In addition, the antioxidant potential of *V. megapotamica* involves mechanisms of sequestration of free radicals, particularly in the more polar extracts, which produced higher amounts of phenolic constituents.

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