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## HPLC/DAD Profile and Determination of Total Phenolics, Flavonoids, Tannins and Alkaloids Contents of *Scutia buxifolia* Reissek Stem Bark

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### ABSTRACT

The species *Scutia buxifolia* Reissek, belongs to Rhamnaceae family and is popularly known in Brazil as coronilha. In this study, total phenolics, flavonoid, tannin and alkaloids contents from the stem bark of the plant were investigated. Additionally, HPLC/DAD analysis of ethyl acetate and butanolic fractions revealed six active phenolic compounds (gallic, chlorogenic and caffeic acids; and quercetin, rutin and kaempferol). Total phenolics were measured using folin ciocalteau and total flavonoid, using aluminium chloride. HPLC/DAD profiles were performed using gradient system. The stem bark of *S. buxifolia* exhibited as a large content of phenolic compounds and flavonoids. Total phenolic varied from  $323.47 \pm 2.62$  to  $141.09 \pm 0.71$  mg g<sup>-1</sup>, the contents of flavonoids ranged from  $145.72 \pm 0.27$  to  $100.37 \pm 0.56$  mg g<sup>-1</sup>, tannin and alkaloids varied from  $176.70 \pm 0.24$  to  $66.67 \pm 0.17$  mg g<sup>-1</sup> and  $3.07 \pm 1.13$  to  $0.28 \pm 0.19$  mg g<sup>-1</sup>, respectively. The species *S. buxifolia* exhibited high phenolics contents, being ethyl acetate and butanolic the most active fractions. Several bioactive phenolics were identified and quantified for the first time in these fractions.

**Key words:** *Scutia buxifolia*, free radicals, HPLC-DAD

### INTRODUCTION

*Scutia buxifolia* Reissek belongs to the Rhamnaceae family and is popularly known as coronilha. It is native tree from South America, with a dispersion area that comprises Rio Grande do Sul State in Brazil and the countries Argentina and Uruguay. The root bark infusion is popularly used as cardiogenic, antihypertensive and diuretic (Wasicky *et al.*, 1964). Antimicrobial activities of some cyclopeptide alkaloids isolated from the root bark of this specie were reported by Morel *et al.* (2005) using the bioautography method. Cytotoxicity of extracts from leaves, twigs and stem bark of the plant was evaluated by the *Artemia salina* assay, as well as the antimicrobial activity against a panel of microorganism strains (Boligon *et al.*, 2011). Previously studies indicated that the leaves and stem bark of *S. buxifolia* contains flavonoids and steroids compounds, the extracts also were effective inhibitors of tbars production and presented DPPH scavenger activity (Boligon *et al.*, 2009, 2010).

Polyphenols are a group of secondary metabolites involved in the H<sub>2</sub>O<sub>2</sub> scavenging in plant cells. Interest in plant materials rich in polyphenolic compounds are on the increase due to their high antioxidant potency which may offer protection against chronic diseases, such as cardiovascular disease, neuronal disease, cataracts and several forms of cancer (Halliwell, 1997; Aliyu *et al.*, 2011; Prasong, 2011). The antioxidative property of polyphenols is a predominant feature of their radical-scavenging capacity (Cotelle, 2001; Lau *et al.*, 2005; Pandey *et al.*, 2011; Anokwuru *et al.*,

2011). They possess ideal structural chemistry for radical scavenging activity and are more effective than tocopherol (vitamin E) and ascorbate (Pandhair and Sekhon, 2006). The polyphenolic compounds in plant extracts most commonly found are phenolic acids, flavonoids and tannins (Naik *et al.*, 2006; Sati *et al.*, 2010). Flavonoids are 15-carbon compounds generally distributed throughout the plant kingdom (Harborne, 1988), this compounds together with other phenolics structures of plant origin have been reported as scavengers of Reactive Oxygen Species (ROS) and are seen as promising therapeutic drugs for free radical pathologies (Chang *et al.*, 2007; Khasawneh *et al.*, 2011).

Tannins are naturally occurring, high molecular weight polyphenols which can be divided into hydrolysable tannins and condensed tannins. Tannins are the most abundant antioxidants in the human diet and they exhibit many biologically important functions which include protection against oxidative stress and degenerative diseases, this oxidation inhibiting activities of tannins have been known for a long time (Atanassova and Christova-Bagdassarian, 2009).

Considering the importance of identifying and quantifying compounds with capacity antioxidant, mainly in the crude extract because it is the common from of usage in popular medicine (Kintzios *et al.*, 2010; Muchuweti *et al.*, 2007), this study were determined the levels of phenolics, flavonoids, tannins and total alkaloids in crude extract and dichloromethane, ethyl acetate and n-butanol fractions from the stem bark of *Scutia buxifolia*. Simultaneously, HPLC/DAD was performed to identify which were the mainly flavonoids and phenolics compounds and to quantify them.

## MATERIALS AND METHODS

**Chemicals, apparatus and general procedures:** All chemicals were of analytical grade. Solvents for the extractions and analytical procedures were purchased from Merck (Darmstadt, Germany). Acetonitrile was of HPLC grade. Folin-Ciocalteau phenol reagent 2 N, bismuth nitrate pentahydrate, thiourea, sodium sulfide, nitric acid, quercetin, rutin, kaempferol, gallic, caffeic and chlorogenic acids were procured from Sigma Chemical Co. (St. Louis, MO, USA). Deionized water was prepared by a Milli-Q water purification system. High performance liquid chromatography of the samples was performed with the HPLC system (Shimadzu, Kyoto, Japan), prominence auto sampler (SIL-20A), equipped with Shimadzu LC-20 AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV-VIS-DAD (diode-detector) SPD-M20A and Software LC solution 1.22 SP1. The absorbance for phenolics, flavonoids, tannin and alkaloids assay were performed in a Shimadzu-UV-1201 (Shimadzu, Kyoto, Japan) spectrophotometer.

**Plant collection and extractions:** Stem bark of *S. buxifolia* were collected in Dom Pedrito (Rio Grande do Sul, Brazil) in October of 2007 (coordinates 30°59'09"S and 54°27'44" W). Exsiccate was archived as voucher specimen in the herbarium of Department of Biology at Federal University of Santa Maria by register number SMBD 10919.

The stem bark were dried at room temperature and powdered in a knife mill (0.86 µm), resulting in a mass of 651.52 g of plant material which was submitted to maceration at room temperature with ethanol 70% for a week with daily shake. After filtration, the extract was evaporated under reduced pressure to remove the ethanol and after this step the aqueous extract was partitioned successively with dichloromethane, ethyl acetate and n-butanol. The yield of the extract and fractions was calculated by the formula:

$$\text{Yeild}(\%) = \frac{(\text{Mass of the extract})}{(\text{Mass of material})} \times 100$$

**Determination of total phenolics:** The determination of total phenolic contents was performed by the Folin-Ciocalteu method with slightly modifications (Boligon *et al.*, 2009). The samples were read at 730 nm in spectrophotometer. The total phenolics content was expressed in milligrams equivalents of gallic acid (GAE) per gram of each fraction. The equation obtained for the calibration curve of gallic acid in the range of 0.005-0.030 mg mL<sup>-1</sup> was  $Y = 11.969x - 0.0454$  ( $r = 0.9984$ ). The experiments were conducted in triplicate.

**Determination of total flavonoids:** The determination of flavonoids was performed as described by Woisky and Salatino (1998). The absorbance was determined by spectrophotometer at 420 nm. Ethanol was used as a blank. The equation obtained for the calibration curve of quercetin in the range of 0.012-0.200 mg mL<sup>-1</sup> was  $Y = 0.0045x - 0.014$  ( $r = 0.9952$ ). The content of flavonoids was established as quercetin mg g<sup>-1</sup> dry extract. The experiments were conducted in triplicate.

**Determination of total tannins:** The tannins content was performed using the method described by Morrison *et al.* (1995). Samples in concentrations of 0.25 mg mL<sup>-1</sup>, 5 mL of solution A (1 g vanillin in 100 mL of methanol) and solution B (8 mL HCl in 100 mL of methanol) were used to experiment. The samples were read at 500 nm in spectrophotometer. The total tannins content was expressed in milligrams equivalents of catechin per gram of each fraction. The equation obtained for the calibration curve of catechin in the range of 0.001-0.025 mg mL<sup>-1</sup> was  $Y = 0.00015x + 0.005$  ( $r = 0.9979$ ). The experiments were conducted in triplicate.

**Determination of total alkaloids:** The alkaloids content was performed using the method described by Sreevidja and Mehrotra (2003), where Dragendorff's reagent precipitates alkaloids in plants materials. It is based on the formation of yellow bismuth complex in nitric acid medium with thiourea. Crude extract and fractions of *S. busifolia* in concentrations of 20 mg mL<sup>-1</sup> were used in experiment. Mixture of thiourea and nitric acid were used as a blank. The samples were read at 435 nm in spectrophotometer. The equation obtained for the calibration curve of bismuth nitrate pentahydrate solution in the range of 0.01-0.09 mg mL<sup>-1</sup> was  $Y = 2.2783x + 0.0361$  ( $r = 0.9997$ ). The experiments were conducted in triplicate.

**HPLC/DAD qualitative and quantitative analysis:** Reverse phase chromatography analyses were carried out under gradient conditions using a Phenomenex C-18 column (4.6×250 mm) packed with 5 µm diameter particles. The mobile phase was solvent A = water/acetic acid (99:1 v/v) and solvent B = acetonitrile. The gradient program was started with 13% of B until 10 min and changed to obtain 20, 30, 50, 60, 70, 2 and 10% B at 20, 30, 40, 50, 60, 70 and 80 min, respectively. The flow rate was 0.5 mL min<sup>-1</sup> and the injection volume was 50 µL (Ozturk *et al.*, 2009). Detection was performed with three wavelengths, 271 nm for gallic acid, 327 nm for caffeic and chlorogenic acids and 365 for quercetin, rutin and kaempferol. The mobile phase was filtered through a membrane filter 0.45 µm and then degassed by an ultrasonic sound before use. The fractions and standards solutions (quercetin, rutin, kaempferol, gallic, caffeic and chlorogenic acids) were prepared in the same mobile phase of HPLC. Standard calibration curves were constructed in the concentration range of 0.010 to 0.200 mg mL<sup>-1</sup>. The chromatographic peaks were confirmed by comparing its retention time with those of

reference standards and by DAD spectra (200 to 500 nm), the quantification was performed by peak integration using the external standard method.

**Statistical analysis:** One-way ANOVA followed by Tukey test were performed in the total phenolics, flavonoids, tannins and alkaloids assays. Results were expressed as the Mean±Standard error deviation (SE) and differences were considered statistically significant when  $p < 0.05$  and  $p < 0.001$ .

## RESULTS AND DISCUSSION

Compositions for different extracts of stem bark of *Scutia buxifolia* are reported in Table 1. The solvent extraction systems used showed a variation in the concentration of the compounds, ethyl acetate and butanolic fractions exhibited a high content of phenolics ( $322.69 \pm 1.20$  and  $323.47 \pm 2.62$  mg g<sup>-1</sup> GAE, respectively) when compared to the dichloromethane fraction ( $166.88 \pm 0.66$  mg g<sup>-1</sup> GAE) and crude extract ( $141.09 \pm 0.71$  mg g<sup>-1</sup> GAE). The phenolic substances such as tocopherols, flavonoids and phenolic acids are specially mentioned as antioxidants due to their efficient scavenging capacity of reactive oxygen species which may cause a large number of disorders by reacting with cellular lipids, proteins, carbohydrates and nucleic acids (Chanwitheesuk *et al.*, 2005; Anokwuru *et al.*, 2011). Due to their natural origin, the antioxidants obtained from plants are of greater benefit in comparison to synthetic ones (Stankovic *et al.*, 2010).

For the determination of flavonoids contents, the ethyl acetate fraction exhibited the highest value ( $145.72 \pm 0.27$  mg g<sup>-1</sup> quercetin), butanolic fraction expressed the second highest value ( $138.92 \pm 0.83$  mg g<sup>-1</sup> quercetin), on the other hand, crude extract showed the lowest flavonoids content ( $100.37 \pm 0.56$  mg g<sup>-1</sup> quercetin). Flavonoids exhibit inhibition of mutagenicity induced by chemical mutagens and have anticarcinogenic, antioxidant and anti-inflammatory activities (Miyazawa *et al.*, 2000; Gill *et al.*, 2011).

The quantification of tannin and alkaloids followed the order: ethyl acetate fraction > butanolic fraction > crude extract > dichloromethane fraction (Table 1). Tannins have shown potential antiviral, antibacterial and antiparasitic effects. In the past few years tannins have also been studied for their potential effects against cancer through different mechanisms (Atanassova and Christova-Bagdassarian, 2009; Lu *et al.*, 2004). When comparing the results obtained with phenolics, flavonoids, tannins and alkaloids contents assays, we may observe a relation between four dosages for all fractions. Regarding the presence of alkaloids found in this study using

Table 1: Yield of crude extract and fractions in percentage (%), contents of total phenols, flavonoids, tannins and alkaloids of *S. buxifolia* stem bark

Crude extract fractions	Stem bark				
	Yield (%)	Phenols <sup>1</sup> ±SE	Flavonoids <sup>2</sup> ±SE	Tannin <sup>3</sup> ±SE	Alkaloids±SE
		----- (mg g <sup>-1</sup> ) -----			
Butanolic	8.32	323.47±2.62 <sup>a</sup>	138.92±0.83 <sup>a</sup>	174.83±0.65 <sup>a</sup>	1.86±0.09 <sup>a</sup>
Ethyl acetate	6.03	322.69±1.20 <sup>a</sup>	145.72±0.27 <sup>b</sup>	176.70±0.24 <sup>a</sup>	3.07±1.13 <sup>b</sup>
Dichloromethane	1.53	166.88±0.66 <sup>b</sup>	137.23±0.68 <sup>a</sup>	79.61±0.51 <sup>b</sup>	0.28±0.19 <sup>c</sup>
Crude extract	10.97	141.09±0.71 <sup>c</sup>	100.37±0.56 <sup>c</sup>	66.67±0.17 <sup>c</sup>	1.59±0.08 <sup>d1</sup>

<sup>1</sup>Phenols: Expressed as gallic acid equivalents (GAE); <sup>2</sup>Flavonoids: Expressed as quercetin (mg g<sup>-1</sup> fraction); <sup>3</sup>Tannin: Expressed as catechin (mg g<sup>-1</sup> fraction); SE: Standard error. Values followed by different letters in each column differ by Tukey's test at  $p < 0.05$

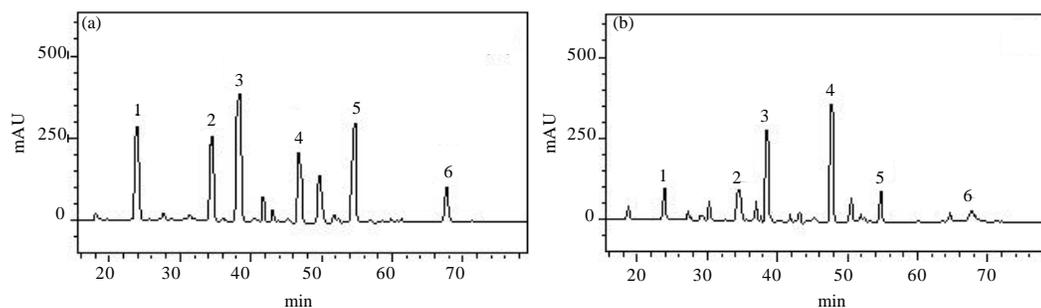


Fig. 1(a-b): High performance liquid chromatography phenolics profile of ethyl acetate (a) and butanolic fractions (b) of *Scutia buxifolia* stem bark. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercetin (peak 5) and kaempferol (peak 6)

spectrophotometric assay, our results support published works previously which describe the isolation of cyclopeptide alkaloids in this species. However, the merit of our analysis is to quantify totality these compounds in each extract.

Schubert *et al.* (2007) working with *Ilex paraguariensis* found phenolics contents ranging from 86.82 to 199.91 mg g<sup>-1</sup>, in our study we found higher values. The *Cariniana domestica* showed a variation of the 510.00 to 214.32 mg g<sup>-1</sup> and 39.92 to 15.26 mg g<sup>-1</sup> for phenolics and flavonoids, respectively (Janovik *et al.*, 2011). Previous work has found similar good results for ethyl acetate and butanolic fractions and have documented that these fractions are source of antioxidant substances (Kang *et al.*, 2010; Aliyu *et al.*, 2011), some investigations of plants composition have demonstrated that polar fractions usually concentrates high amounts of phenolics compounds (Canadanovic-Brunet *et al.*, 2008).

Besides the aforementioned high content of flavonoids, phenolics and tannins in the *S. buxifolia*, a previous work developed by Boligon *et al.* (2010) obtained from the stem bark and leaves of the plant led to the isolation and identification of several triterpenes that may also contribute, in part, by antioxidant properties previously described for this species (Sunitha *et al.*, 2001; Boligon *et al.*, 2009).

HPLC/DAD fingerprint should be considered to evaluate the quality of herbal medicines all over the worlds considering multiple constituents present in the herbal medicines and its products (Giri *et al.*, 2010). Ethyl acetate and butanolic fractions furnished high concentrations of polyphenols, flavonoids and tannins. Therefore, we have performed an HPLC/DAD analysis with the purpose of identify and quantify its mainly phenolic compounds. These fractions were investigated for the presence of the following compounds: gallic, chlorogenic and caffeic acids, quercetin, rutin and kaempferol. Identification was performed by comparison of their retention's time and UV absorption spectrum with those of the standards.

The results and chromatograms are shown in Table 2 and Fig. 1. All tested substances are well-known antioxidants. The ethyl acetate fraction presented as major component caffeic acid (9.25%), followed by quercetin and gallic acid (7.00 and 6.72%, respectively); whereas the butanolic fraction was mainly characterized by the presence of rutin and caffeic acid (6.94 and 9.10%, respectively). Gallic acid is a derivative from benzoic acid widely distributed in various plants and foods and its various biological effects have been reported, such as anti-proliferative, pro-apoptotic and

Table 2: HPLC/DAD of identified and quantified phenolic compounds in ethyl acetate and butanolic fractions obtained from the stem bark of *Scutia buxifolia*

<i>S. buxifolia</i>	Ethyl acetate fraction		Butanolic fraction	
	Dried fraction (mg g <sup>-1</sup> )	%	Dried fraction (mg g <sup>-1</sup> )	%
Gallic acid	67.19±0.03 <sup>a</sup>	6.72	10.11±0.07 <sup>a</sup>	1.01
Chlorogenic acid	59.40±0.01 <sup>b</sup>	5.94	18.59±0.09 <sup>b</sup>	1.86
Caffeic acid	92.53±0.06 <sup>c</sup>	9.25	61.04±0.02 <sup>c</sup>	6.10
Rutin	38.87±0.09 <sup>d</sup>	3.88	69.43±0.03 <sup>c</sup>	6.94
Quercetin	70.05±0.13 <sup>a</sup>	7.00	9.85±0.10 <sup>d</sup>	0.98
Kaempferol	15.26±0.08 <sup>e</sup>	1.53	6.32±0.05 <sup>d</sup>	0.63

Results are expressed as mean±S.E. of three determinations. Averages followed by different letters in each column differ by Tukey test at  $p < 0.001$

anti-tumorigenic effects against prostate (Kaur *et al.*, 2009). Rutin (quercetin 3-O-rhamnoglucoside) which is commonly found in plants, upon the hydrolysis of the glycoside bond produces quercetin, a highly antioxidative aglycon. This flavonoid is an active and natural antioxidant, involved in protection and prevention of pathologies like diabetes mellitus, as an example (Fernandes *et al.*, 2010).

## CONCLUSION

*S. buxifolia* is used in medicine popularly of Brazil, our results suggest that the species has strong free radical scavengers and can be considered as good sources of natural antioxidants. Ethyl acetate and butanolic fractions presented the highest flavonoid, phenolic, tannin and alkaloids contents indicates that these compounds contribute to the antioxidant capacity.

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