HPLC Analysis and Phytoconstituents Isolated from Ethyl Acetate Fraction of *Scutia buxifolia* Reiss. Leaves

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SUMMARY. Fractionation of the ethyl acetate soluble fraction from the ethanol extract of the leaves of *Scutia buxifolia* (Rhamnaceae) led to the isolation of quercetin, quercetin 3-O-rhamnoside (quercitrin), quercetin 3-O-glucoside (isoquercitrin) and rutin. The structures of the isolates were elucidated by spectroscopic analysis and comparison with literature data and analyzed for high performance liquid chromatography (HPLC). The isolated compounds are reported for the first time to the species *S. buxifolia*.

INTRODUCTION

The Rhamnaceae family includes 58 genera and approximately 900 species occurring in tropical and subtropical areas around the world 1,2. *Scutia buxifolia* Reissek, popularly known as “coronilha”, is a local plant from South America, with a dispersion that comprise Rio Grande do Sul state in Brazil, Argentina and Uruguay. The plant is popularly used as cardiotonic, antihypertensive and diuretic 3. Notwithstanding its popular use, there are little bibliographic sources concerning its secondary metabolites, with the exception of the papers from Morel’s research group that describes the isolation of ciclopeptides alkaloids from the root bark extract of the plant and antimicrobial activities observed in some isolated ciclopeptides alkaloids 4-7.

Phytochemical investigations of our group in the leaves of the plant indicate a large number of phenolic compounds, including flavonoids (Thin Layer Chromatography (TLC), data not shown). Flavonoids are of particular importance in the human diet as there is evidence that they act as antioxidants, antimicrobial and antiviral agents and epidemiological studies have indicated that their consumption is associated with a reduced risk of cancer and cardiovascular disease 8-11. Therefore we decided to isolate and quantify the major flavonoids compounds from the leaves of the plant and consequently, this work describes the structural elucidation of four flavonol-derived compounds isolated from the ethyl acetate fraction as well their HPLC (combined with diode array detection) quantification.

MATERIALS AND METHODS

Reagents, standards and apparatus

All chemicals were of analytical grade. Silica Gel 60 for column chromatography, Silica Gel 60 F254 coated plates, solvents for the extractions and analytical procedures, dichloromethane, ethyl acetate, ethanol, acetic acid and n-butanol, were purchased from Merck (Darmstadt, Germany). Quercetin and rutin reference standards were obtained from Sigma Chemical. Methanol and acetonitrile were of HPLC grade. Deionized water was prepared by a Milli-Q water purification system. High performance liquid chromatography (HPLC) 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 detector DAD (diode) SPD-M20A and Software LC solution 1.22 SP1. NMR spectra were carried out on a Bruker AMX 400 spectrometer equipped with a broadband 5-mm probe, using a spectral width of 10 ppm (parts per million). 1HNMR recorded 400 MHz and 13C NMR at 100 MHz. Chemical shifts were expressed as ppm relative to the TMS. Deuterated methanol (methanol-d4, 99.8 atom % of deuterium, solvent peaks dH 3.34 and dC 49.0 ppm) was used as solvent for the samples.

Plant collection and extraction

Leaves of *Scutia buxifolia* were collected in...
Dom Pedrito (Rio Grande do Sul State) 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 leaves were dried at room temperature and powdered in a knife mill, resulting in a mass of 1.5 Kg 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 (3 x 200 mL for each solvent).

**Isolation and purification**

The ethyl acetate fraction (1.0 g) was submitted to column chromatography on silica gel 60 using initially CH2Cl2 (700 mL) as mobile phase. Afterward the column was eluted with a binary mixture of increase polarity, starting with CH2Cl2:EtOH (9:1 v/v, 700 mL) followed by CH2Cl2:EtOH (8:2 v/v, 700 mL), CH2Cl2:EtOH (7:3 v/v, 700 mL), CH2Cl2:EtOH (6:4 v/v, 700 mL), and CH2Cl2:EtOH (5:5 v/v, 600 mL). The procedure describe above furnished forty-one (41) fractions of ± 100 mL each, which were analyzed by TLC and pooled together on the basis of similarities in their chromatographic profile (solvent system: chloroform:ethanol:water, 60:40:5, v/v). The separated fractions were observed under UV light (254 and 366 nm) and detection was performed with anisaldehyde-H2SO4/100 °C for ten minutes. Authentic samples of quercetin and rutin were used as reference standards in order to guide the fractions pool process. Fractions 8 to 28 furnished a sub-fraction (0.292 g), which was further chromatographed under silica gel 60 and eluted with CH2Cl2:EtOH (4:6 v/v) to give isolated compounds 1 (0.034 g), 2 (0.017 g), 3 (0.023 g) and 4 (0.029 mg). Compounds 3 (0.034 g) and 4 (0.023 g) were obtained from the sub-fraction 32-41 (0.325 g) after this step, the aqueous extract was partitioned successively with dichloromethane, ethyl acetate and n-butanol (3 x 200 mL for each solvent).

**Preparation of standard and sample solutions for HPLC quantification**

Standard stock solutions of quercetin and rutin were prepared in mobile phase, at a concentration range of 0.018 to 0.280 mg/mL for quercetin and 0.0125 to 0.200 mg/mL for rutin. The ethyl acetate fraction was dissolved in the mobile phase. All solution were filtered through a filter paper and a 0.45 µm membrane filter (Millipore). Triplicate injections were made for each level, and a linear regression was generated.

**Chromatographic conditions**

Chromatographic analyses were carried out in isocratic conditions using RP-C18 column (4.6 mm x 250 mm) packed with 5µm diameter particles. The mobile phase was methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0% acetic acid. The mobile phase was filtered through a 0.45 µm membrane filter and degassed in ultrasonic bath previous to use. Flow rate and injection volume were 1.0 ml/min and 10 µl, respectively. The chromatographic peaks were confirmed by comparing their retention time and UV spectra with those of the reference standards. Quantification was carried out by the integration of each peak using the external standard method. All chromatographic operations were carried out at ambient temperature. Quercetin and rutin reference standards, ethyl acetate fraction from the leaves of S. buxifolia and isolated compounds (1-4) were quantified at 257 nm (band II, characteristic of flavonol nucleus).

**RESULTS AND DISCUSSION**

Successive column chromatographic procedures with ethyl acetate fraction led to the isolation of four flavonol compounds (Fig. 1), whose structures were identified based on 1H NMR and 13C NMR spectra and by comparison with literature 12-15.

The 1H NMR spectrum of compound 1 showed two peaks at 6.17 (1H, d, J = 2.0 Hz) and 6.37 ppm (1H, d, J = 2.0 Hz ) consistent with the meta protons H-6 and H-8 on A-ring and an ABX system at δ 7.72 (1H, d, J = 2.1 Hz, H-2'), 7.62 (1H, dd, J = 8.4, 2.1 Hz, H-6') and 6.87 (1H, d, J = 8.4 Hz, H-5') corresponding to the catechol protons on B-ring. The 13C NMR indicated the presence of 15 carbon atoms, the signal at: δ 177.3 was attributed to a carbonyl carbon placed at C-4, the other signals were: 165.6 (C-7), 162.6 (C-8), 158.4 (C-9), 148.8 (C-4'), 148.1 (C-2), 146.3 (C-3'), 137.3 (C-3), 124.2 (C-1'), 121.8 (C-6'), 116.2 (C-5'), 116.0 (C-2'), 104.6 (C-10), 99.2 (C-6'), 94.4 (C-8). The spectral data were compatible with those of quercetin 12-15.

<table>
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<th>Compound</th>
<th>1H NMR data (ppm)</th>
<th>13C NMR data (ppm)</th>
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<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
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<tr>
<td>2</td>
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<td>3</td>
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was indicative of a rhamnopyranose moiety 16,19. The principal signals of quercetin aglycone were: δ 7.32 (1H, d, J = 2.0 Hz, H-2'), 7.29 (1H, dd, J = 2.0 and 8.4 Hz, H-6'), 6.90 (1H, d, J = 8.0 Hz, H-5'), 6.32 (1H, d, J = 2.0 Hz, H-8), 6.16 (1H, d, J = 2.0 Hz, H-6). The 13C NMR spectrum indicated the presence of 21 carbon atoms: δ 179.5 (C-4), 165.6 (C-7), 163.3 (C-5), 159.2 (C-9), 149.6 (C-4'), 158.4 (C-2), 146.2 (C-3'), 136.2 (C-3), 122.9 (C-1), 122.9 (C-6), 116.3 (C-5), 116.3 (C-2'), 110.3 (C-10), 99.8 (C-6), 94.7 (C-8), 105.8 (C-1'), 71.8 (C-2'), 71.2 (C-3'), 71.9 (C-4'), 71.5 (C-5') and 17.6 (C-4'). The data were in agreement with literature data reported to quercetin 3-glucoside (3), indicating a 1-6 linkage between the C3-glucose and the rhamnose 18. The identity of this compound was further confirmed by cochromatography with authentic rutin standard and literature data comparisons 16-18.

The ethyl acetate fraction from the leaves of S. buxifolia was analyzed by Liquid Chromatography. A simple and rapid reversed-phase HPLC method was utilized for the determination of quercetin, quercitrin, isoquercitrin and rutin 20. Figure 2 shows a representative chromatogram obtained for ethyl acetate fraction and the isolated flavonol compounds. The ethyl acetate contains other minor compounds in addition to quercetin (retention time-tR 12.4 min, peak 1), quercetin-3-glucoside (3), indicating a 1-6 linkage between the C3-glucose and the rhamnose 18. The identity of this compound was further confirmed by cochromatography with authentic rutin standard and literature data comparisons 16-18.

Since extracts of natural origin usually contain a range of chemically diverse constituents occurring in varying concentrations, it is important to use chromatographic methods to analyze these inherently complex mixtures. The HPLC profile of ethyl acetate fraction was acquired, as well the quantification of rutin and quercetin by HPLC-DAD based in the reference rutin and quercetin standards calibration curves. Calibration curve for quercetin: Y = 30153x – 235135, r = 0.9983, calibration curve for rutin: Y = 19217x - 16949, r = 1.000. Quercitin and isoquercitrin were also quantified but they were expressed separately as quercitin contents (Table 1). The major component was quercitrin, followed by rutin, quercitin and isoquercitrin.

CONCLUSION

Quercetin, quercitrin, isoquercitrin and rutin were isolated from the plant for the first time.
Quercitrin was the major flavonol present in the plant. These results indicate that the plant *Scutia buxifolia* has many chemicals compounds able to catch free radicals. Therefore, it is assumed that the plant has, besides its popular uses, promising compounds in search of antioxidants and drugs for diseases resulting from oxidative stress. Given that the popular use of the plant point toward the daily intake of aqueous infusions for antihypertensive purposes, the beneficial effects of drinking large quantities of antioxidant substances should be thinking as another advantageous benefits of this plant because several studies and epidemiological data suggesting an association between diets rich in fruits, vegetables, red wine and the decline of degenerative diseases. Knowing that the activity of extracts of plants can not be judged by only few methods, it is necessary more studies to determine whether this medicinal plant could be used industrially.

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**REFERENCES**


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<th>Flavonol compound</th>
<th>Quantities 1</th>
<th>Percentual (%)</th>
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<tr>
<td>Quercitin</td>
<td>27.1 ± 0.03</td>
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<tr>
<td>Quercitrin 2</td>
<td>183.2 ± 0.24</td>
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<tr>
<td>Isoquercitrin 2</td>
<td>6.6 ± 0.04</td>
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<tr>
<td>Rutin</td>
<td>48.1 ± 0.18</td>
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Table 1. Flavonols composition of *S. buxifolia* leaf ethyl acetate fraction. 1 Results are expressed as mean ± S.E. of three determinations. 2 Quantified as quercetin.

![Figure 2. Chromatograms of ethyl acetate sample (a), and isolated quercetin (b), quercitrin (c), isoquercitrin (d) and rutin (e).](image-url)