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Distribution and chemotaxonomic significance of phenolic compounds in *Spermacoce verticillata* (L.) G. Mey

[Distribución e importancia quimiotaxonómica de los compuestos fenólicos en *Spermacoce verticillata* (L.) G. Mey]

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Abstract

Context: *Spermacoce verticillata*, known as "poaia and vassourinha de botão", is a species widely used in Brazilian traditional medicine as anti-inflammatory, antipyretic and analgesic. It is a native species, small, upright perennial, and broadly distributed throughout Brazil. Until now, few chemical studies have focused on the phenolic composition of this species.

Aims: Evaluate the phytochemical profile of phenolic compounds from *Spermacoce verticillata* and search new compounds that have chemotaxonomic significance.

Methods: Leaves of *S. verticillata* were extracted using distilled water. The extract (SVL) was purified by several chromatography processes. Extract and compounds were analyzed by HPLC-DAD and NMR.

Results: Phytochemical analysis led to identification, for the first time, of three compounds (**1-3**) for the specie. Chlorogenic acid (**1**) was identified by HPLC-DAD compared with reported in the literature. Quercetin-3-O-rutinoside (rutin) (**2**) and kaempferol-3-O-rutinoside (**3**) were isolated from butanolic fraction and identified by spectroscopic analysis comparison with data reported in the literature. The flavonoid rutin is the major compound in SVL followed by kaempferol-3-O-rutinoside and chlorogenic acid.

Conclusions: This is the first report for these compounds (**1-3**) in *S. verticillata*. The presence of these three new compounds indicates chemical markers of the species for this genus and family. This information is extremely important because increases the resources for chemotaxonomic classification of these species.

Keywords: *Borreria verticillata*; kaempferol-3-O-rutinoside; phenolic.

Resumen

Contexto: *Spermacoce verticillata*, conocida como "poaia y vassourinha de botão", es una especie ampliamente utilizada como antiinflamatoria, antipirética y analgésica en la medicina tradicional de Brasil. Es una especie nativa, pequeña, perenne, erguida y ampliamente distribuida en todo Brasil. Hasta el presente, pocos estudios químicos se han centrado en la composición fenólica de esta especie.

Objetivos: Evaluar el perfil fitoquímico de compuestos fenólicos de *Spermacoce verticillata*, en la búsqueda de nuevos compuestos que tienen importancia taxonómica.

Métodos: Hojas de *S. verticillata* fueron extraídas con agua destilada. El extracto (SVL) fue purificado por varios procedimientos cromatográficos y, éste y los compuestos aislados, se analizaron por HPLC-DAD y RMN.

Resultados: El análisis fitoquímico condujo a la identificación, por primera vez, de tres compuestos (**1-3**) para la especie. El ácido clorogénico (**1**) fue identificado por HPLC-DAD en comparación con lo reportado en la literatura. Quercetina-3-O-rutinósido (rutina) (**2**) y campferol-3-O-rutinósido (**3**) fueron aislados de la fracción butanólica e identificados por análisis espectroscópico en comparación con los datos reportados en la literatura. El flavonoide rutina es el compuesto principal en SVL, seguido de campferol-3-O-rutinósido y ácido clorogénico.

Conclusiones: Este es el primer informe para estos compuestos (**1-3**) en *S. verticillata*. La presencia de estos tres compuestos nuevos, para esta especie, están presentes como marcadores químicos de las especies de este género y familia. Esta información es muy importante para aumentar los recursos para la clasificación taxonómica de esta especie.

Palabras Clave: *Borreria verticillata*; campferol-3-O-rutinósido; fenólico.

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INTRODUCTION

The ethnopharmacological knowledge is useful to identify potential therapeutic targets from medicinal plants. Substances from vegetal kingdom, which have already contributed with several compounds in prophylaxis and treatment of a large variety of pathologies, have been investigated for their potential as anti-inflammatory and antithrombotic agents (Chaves et al., 2011).

The Rubiaceae family comprises about 650 genera and 13 000 species (Bremer et al., 2009). The genera *Spermacoce* (formerly called *Borreria*) is composed of 280 species distributed throughout the world (Dessein et al., 2006), including Brazil, where several species are native as *Spermacoce verticillata* (Chiqueiri et al., 2004).

Spermacoce verticillata (L.) G. Mey. (Rubiaceae), known as “vassourinha de botão”, is a species that occurs over the entire Brazilian territory and is commonly used in traditional folk medicine to treat gastrointestinal disorders, ulcers and as anti-inflammatory agent. The plant contains indole alkaloids, which borrevine and borreverine are the major compounds, as well as flavonoids, terpenes, anthraquinone, phytosteroids and iridoids (Conserva et al., 2012; Ferreira Júnior et al., 2012).

Recently, a review was published showing the main chemical components of genera *Spermacoce* (Conserva et al., 2012). For *Spermacoce verticillata* have been found mainly indole alkaloids (borrerine, verticillatines A e B), borreverine, isoborreverine and spermacoceine, and (-)-emetine (Moreira et al., 2010) have been found. Iridoids such as asperuloside, asperulosidic acid, borrieriagenin, daphylloside deacetyl asperuloside, deacetyl-asperulosidic acid (Moreira et al., 2010) and terpenoids such as cariophyllene, guaiane, campesterol, β -sitosterol, and stigmasterol (Moreira et al., 2010; Ferreira Júnior et al., 2012) are present. Other compounds as phytol, 1,8-cineol, α -pinene, and p-cymene were also identified (Ogunwande et al., 2010).

Recently, some new terpenes (ursolic, oleanolic and morolic acid), flavonoids (quercetin and quercetin-3-O- α -L-rhamnopyranosyl), anthraquinone (3,5-dioxo-friedelane), phytosteroids as 2-hydroxy-3-methylanthraquinone and sitostenone in *Spermacoce verticillata* were isolated and identified (Ushie et al., 2010; Ferreira-Júnior et al., 2012), pointing to

the wealth of secondary metabolites in this species and the need for further studies to elucidate these substances, which was the aim of this work.

MATERIAL AND METHODS

Chemical and instruments

Thin layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ (SilicCycle) eluted with n-butanol/acetic acid/water (BAW) 8:1:1, visualized under UV light (254 and 365 nm) and developed with ceric sulphate solution and aluminium chloride 2% ethanol solution. All 1D and 2D experiments were performed on a Bruker 400 MHz spectrometer. The NMR spectra were recorded in MeOD.

HPLC separation was performed using a Shimadzu liquid chromatograph Prominence LC-20AT coupled to a SPD-20A diode array detector (DAD) (column oven CTO-20A, communications bus module CBM-20A). The reversed-phase column used was Betasil Thermo C18 (250 mm x 4.6 mm, 5 μ m) with mobile phase consisted of water containing acetic acid 1% (A) and methanol (B) and the injection volume for all samples was 20 μ L. The samples were run for 18 minutes at a flow rate of 1 mL/min, with oven set at 30°C and absorbance monitored between 200 – 450 nm. The gradient used was 0 – 15 min (35 - 70% B), 15 – 17 min (70 - 80% B) and 17 – 18 min (80 - 35% B). The compounds were quantified from a calibration curve of rutin in triplicates of five concentrations (0.02 – 0.1 mg/mL). The phenolic compounds were analysed by matching the retention time and their spectral characteristics against those of standards. Standard of quercetin-3-O-rutinoside (rutin) was purchased from Sigma Chemical. Kaempferol-3-O-rutinoside isolated was used as standard in analysis of HPLC after their structural elucidation.

Plant material and extraction

Leaves of *Spermacoce verticillata* (L.) G. Mey. were collected at Volta Redonda - Rio de Janeiro, Brazil, in January 2012. A voucher specimen (RBR 26925) of this plant was identified by Dr. Pedro Germano Filho of the Institute of Botany, at Federal Rural University of Rio de Janeiro, where it was deposited.

Isolation and identification of flavonoids

The leaves (100 g) were triturated using a food processor and extracted with distilled water (10% w/v) by decoction (15 min). After the filtration, the extract (SVL) was frozen at -20°C and lyophilized (12.1 g). SVL was purified by ethanol precipitation and partitioned successively with ethyl acetate (3 x 400 mL), affording 199.0 mg of acetate fraction, and n-butanol (3 x 400 mL), affording 1.7 g of butanolic fraction, which was purified on an RP-2 column (30 x 1.2 cm; H₂O/MeOH), allowing for eight fractions. The sixth fraction showed a precipitate yellow crystalline (24.0 mg) that was separated by centrifugation and identified as quercetin-3-O-rutinoside known as rutin, by spectroscopic analysis comparison with data reported in the literature (Zuhal et al., 2006). The eighth fraction (83.0 mg) was purified on an RP-2 column (15 x 0.7 cm; H₂O/EtOH), giving five fractions. The third fraction showed a yellow compound (16.0 mg) identified as kaempferol-3-O-rutinoside by spectroscopic analysis comparison with data reported in the literature (Song et al., 2007).

RESULTS

HPLC analysis

This study led to the identification of chlorogenic acid, and flavonoids quercetin-3-O-rutinoside (rutin) and kaempferol-3-O-rutinoside in leaves of *Spermacoce verticillata* (SVL), according to reported HPLC-DAD data (Figs. 1-2) and NMR data (Zuhal et al., 2006; Song et al., 2007).

During the analysis of HPLC another compound (retention time at 7.934 min) was identified as a flavonoid derived, which has absorption in 254 and 365 nm. However, this compound wasn't isolated at this moment.

The HPLC conditions, described in the experimental section, allowed good separation for the phenolic acid and flavonoids (Fig. 1). The amount of chlorogenic acid, quercetin-3-O-rutinoside (rutin) and kaempferol-3-O-rutinoside, in leaves of *Spermacoce verticillata*, were 0.022, 0.248 and 0.003%, respectively (Table 1).

Table 1. Data obtained from HPLC analysis of flavonoids (quercetin-3-O-rutinoside and kaempferol-3-O-rhamnoside) and phenolic acid standards and of aqueous extracts (SVL).

Compounds	Retention time (min)	UV λ_{max} (nm)	Total area SVL (%)
Standard chlorogenic acid	4.190	263; 353	100
Rutin	9.235	255; 353	100
Kaempferol-3-O-rutinoside	11.066	265; 353	100
Compound 1	4.190	263; 327	0.022
Compound 2	9.236	255; 353	0.248
Compound 3	11.069	265; 347	0.030

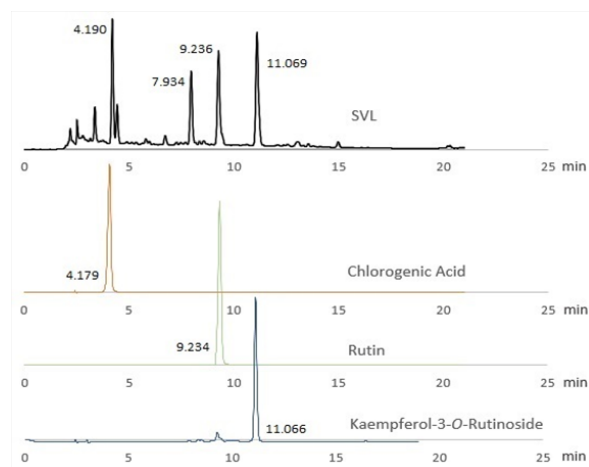


Figure 1. HPLC analysis of major compounds of aqueous extract of *Spermacoce verticillata* (SVL). Chlorogenic acid (Rt = 4.190 min), rutin (Rt = 9.236 min) and kaempferol-3-O-rutinoside (Rt = 11.069 min) were identified. The monitoring wavelength was 340 nm.

NMR data of quercetin-3-O-rutinoside (rutin) (2)

Yellow amorphous powder, ¹H NMR (MeOD, 400 MHz): δ_H 6.20 (1H, d, J = 1.8 Hz, H-6), 6.39 (1H, d, J = 2.2 Hz, H-8), 7.66 (1H, d, J = 1.8 Hz, H-2'), 6.86 (1H, d, J = 8.0 Hz, H-5'), 7.60 (1H, dd, J = 8.0/1.8 Hz, H-6'), 5.09 (1H, d, J = 7.8 Hz, H-1''), 3.25-3.47 (4H, m, H-2'', H-3'', H-4'', H-5''), 3.38 (1H, m, Ha-6''), 3.80 (1H, d, J = 10.5 Hz, Hb-6''), 4.51 (1H, d, J = 1.8 Hz, H-1'''), 3.63 (1H, dd, J = 3.5/1.5 Hz, H-2'''), 3.53 (1H, dd, J = 9.5/3.5 Hz, H-3'''), 3.28 (1H, m, H-4'''), 3.44 (1H, m, H-5'''), 1.11 (3H, d, J = 6.0 Hz, CH₃-6'''); ¹³C NMR (MeOD, 100 MHz): δ_C 158.0 (C-2), 135.0 (C-3), 178.0 (C-4), 161.6 (C-5), 98.6 (C-6),

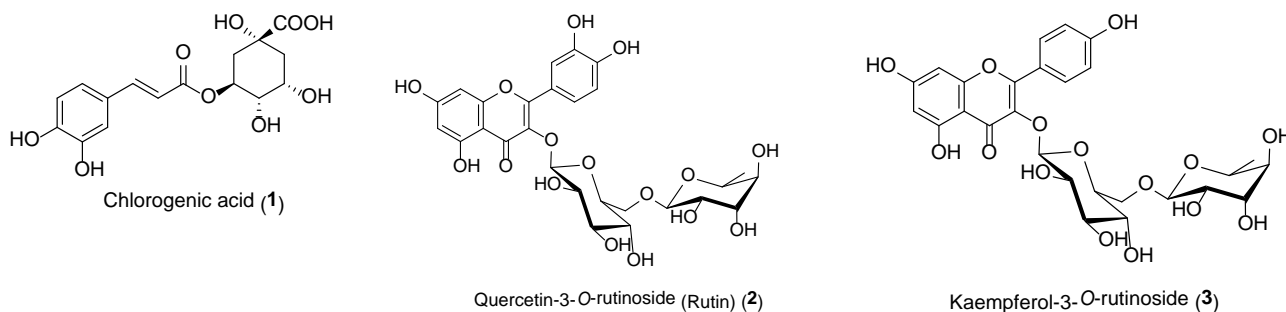


Figure 2. Structures of compounds **1-3** present in *Spermacoe verticillata*.

164.6 (C-7), 93.5 (C-8), 158.0 (C-9), 104.3 (C-10), 121.7 (C-1'), 116.3 (C-2'), 144.5 (C-3'), 148.5 (C-4'), 114.7 (C-5'), 122.2 (C-6'), 103.3 (C-1''), 74.3 (C-2''), 76.7 (C-3''), 70.8 (C-4''), 76.8 (C-5''), 67.2 (C-6''), 101.1 (C-1'''), 70.9 (C-2'''), 72.6 (C-3'''), 73.9 (C-4'''), 68.3 (C-5'''), 16.5 (C-6''').

NMR data of kaempferol-3-O-rutinoside (3)

Yellow amorphous powder, ^1H NMR δ (400 MHz, MeOD) 7.98 (2H, d, $J = 8.7$ Hz, H-2', 6'), 6.88 (2H, d, $J = 8.7$ Hz, H-3' 5'), 6.40 (1H, br.s, H-8), 6.20 (1H, br.s, H-6), 5.30 (1H, d, $J = 6.9$ Hz, H-'), 4.39 (1H, br.s, H-'''), 3.0-4.0 (16H, rut), 1.10 (3H, d, $J = 6.4$ Hz, $-\text{CH}_3$). ^{13}C NMR δ (100 MHz, MeOD) 157.0 (C-2), 134.1 (C-3), 177.9 (C-4), 161.4 (C-5), 98.6 (C-6), 164.5 (C-7), 93.6 (C-8), 158.1 (C-9), 104.2 (C-10), 121.3 (C-1'), 131.0 (C-2', C-6'), 114.7 (C-3', C-5'), 160.6 (C-4'), 101.1 (C-1''), 74.3 (C-2''), 76.7 (C-3''), 68.3 (C-4''), 75.7 (C-5''), 67.1 (C-6''), 100.9 (C-1'''), 70.0 (C-2'''), 70.6 (C-3'''), 72.5 (C-4'''), 68.3 (C-5'''), 16.6 (C-6''').

DISCUSSION

To the best of our knowledge, this is the first report of the compounds (**1-3**) in *S. verticillata*. Flavonoid quercetin-3-O-rutinoside (**2**) is the major compound in SVL followed by kaempferol-3-O-rutinoside (**3**) and chlorogenic acid (**1**), identified by retention time and UV absorption in comparison with the respective standards (Table 1). Through study were identified the presence of one phenolic acid (chlorogenic acid) and flavo-

noids (quercetin-3-O-rutinoside, kaempferol-3-O-rutinoside). Flavonoids are common in the family Rubiaceae and the genus *Spermacoe*. Currently there are ten molecules of this class described in four of ten species of *Spermacoe* (*S. stricta*, *S. laevis*, *S. articularis* and *S. verticillata*) (Noiarsa et al., 2007). This work reports for the first time the identification of chlorogenic acid, rutin and kaempferol-3-O-rutinoside for *Spermacoe verticillata*. The presence of these three new compounds indicates chemical markers of the species for this genus and family. This information is extremely important because it increases the resources for chemotaxonomic classification of these species (Somporn et al., 2012; Lallemand et al., 2012; Bolzani et al., 2001).

CONCLUSIONS

This is the first report for these compounds (**1-3**) in *S. verticillata*. The presence of these three new compounds indicates chemical markers of the species for this genus and family. This information is extremely important because increases the resources for chemotaxonomic classification of these species.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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