

LC/ESI-MS METHOD APPLIED TO CHARACTERIZATION OF FLAVONOIDS GLYCOSIDES IN *B. forficata* subsp. *pruinosa***Lidiane da Silveira Farias[#] and Andreas S. L. Mendez^{*,#}**

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Recebido em 08/08/2013; aceito em 28/10/2013; publicado na web em 26/02/2014

Bauhinia forficata is used in folk medicine for its hypoglycemic effect. In the south of Brazil, the subspecies *pruinosa* is found in a region with the characteristic flora, pampa biome. This species has been consumed by the local population as a tea for diabetes treatment. We studied the chemical composition of hydroethanolic extracts using LC/ESI-MS. The leaf extracts were prepared by percolation with 50% (v/v) ethanol. The chromatographic analyses were performed using a reverse-phase system, gradient elution with acetonitrile:phosphoric acid 0.05%, and ESI-MS in the positive ion mode. The chemical profile of the flavonoids was suggested to involve four quercetin and kaempferol glycosides.

Keywords: *Bauhinia forficata* subsp. *pruinosa*; flavonoids; LC-ESI-MS.**INTRODUCTION**

Bauhinia forficata is a medicinal plant largely consumed by the Brazilian population. It is popularly known as “pata de vaca” (cow’s hoof) because of the large and bilobed aspect of the leaves, a characteristic distinctive to this genus. Traditionally, the aerial parts are used in folk medicine for diabetes treatment, and it is available as a tea in local herbal stores.¹⁻⁴ Two subspecies are also of interest, subsp. *forficata* and subsp. *pruinosa*. *B. forficata* subsp. *forficata* is well characterized with respect to its pharmacological aspects and phytochemical analysis.⁵⁻⁹ Their chemical composition is based on kaempferol and quercetin O-glycoside derivatives such as kaempferol 3,7-dirhamnoside (kaempferitrin) and quercetin 3,7-di-O- α -L-rhamnopyranoside.^{4,9,10} Several reports have discussed the hypoglycemic potential.^{2,7,11} They mention different experimental models, i.e. using alloxan-induced diabetic rats and the streptozotocin-induced diabetes model.^{2,7} Based on tests involving the crude extracts and their respective fractions, the observed effects demonstrated the biological potential for glucose and triglycerides level reduction, which are associated with improvement in the carbohydrate metabolism.²

B. forficata subsp. *pruinosa* is found in a characteristic region of South America (pampa biome) comprised of Argentina, Brazil, and Uruguay. In the south of Brazil, this plant is consumed by the local population as a tea for its known hypoglycemic and diuretic effects. In recent work by Arigony,¹² the hydroethanolic extracts obtained from this species were studied by LC-UV. Flavonoids were detected, and the compound, kaempferol-3-robinoside-7-rhamnoside, was preliminarily suggested as likely comprising the majority of the extract.

In the present work, an analytical investigation of hydroethanolic extracts obtained from *B. forficata* subsp. *pruinosa* was performed using LC/ESI-MS. The hyphenated system was applied to the characterization of the chemical composition in flavonoids, observing the presence in a majority of compounds.

EXPERIMENTAL**Chemicals**

Acetonitrile was purchased from Tedia (Fairfield, OH, USA). Phosphoric acid, ethanol, and methanol were purchased from Merck (Darmstadt, Germany). Purified water was obtained using the Milli-Q Plus[®] system from Millipore (Milford, MA, USA). All other reagents used in this study were of analytical or HPLC grade.

Plant material

Leaves of *Bauhinia forficata* subsp. *pruinosa* (Vogel) Fortunato & Wunderlin were collected at Uruguaiana city (Rio Grande do Sul, Brazil) in October of 2011 (spring). This region is located near Argentina along the western border and is predominantly pampa biome flora. The plant was identified and voucher specimens (ICN 167491) were deposited at the ICN Herbarium (Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Brazil).

Extracts

Air dried (at 35 °C) leaves of *B. forficata* subsp. *pruinosa* were reduced to a powder and submitted to extraction by percolation (1:1, v/v) using an ethanol-water mixture (1:2).^{8,13} The hydroethanolic extracts were then filtered and stored in a refrigerator until analysis. For chromatographic assays, the extracts were diluted in a 50% methanol solution at a ratio of 1:10 (v/v). All solutions were filtered through a 0.45 μ m membrane filter from Millipore (Milford, MA, USA) before injection.

LC/ESI-MS

The LC analyses were conducted using a Prominence Liquid Chromatograph (Shimadzu, Kyoto, Japan) equipped with an SLC-10A controller, LC-20AD pump, SIL-10AF auto sampler, and SPD-M10A PDA detector. A Phenomenex Luna C-18(2) column (250 x 4.6 mm, 5 μ m) was used. The mobile phase consisted of water containing 0.05% phosphoric acid (A) and acetonitrile (B) at a flow rate of 0.8 mL min⁻¹ using the following gradients: 0.1–23 min, 10–40% of solvent B in A; 23.01–40 min, 10% solvent B and 90% solvent A.

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The detection was done on a DAD detector set at 340 nm. The mobile phase was prepared daily, filtered through a 0.45 μm membrane filter (Millipore), and sonicated before use.

The MS analyses were performed on an Esquire plus 3000 (Bruker Daltonics, Billerica, MA, USA) ion trap mass spectrometer with an electrospray interface (ESI). The data acquisition software employed was Esquire CONTROL 5.2. The LC/ESI-MS was conducted in positive-ion mode and operated according to defined conditions: nitrogen gas temperature - 320 $^{\circ}\text{C}$; drying gas flow rate - 7 L min^{-1} ; capillary voltage - 4000 V; nebulizing pressure - 27 psi. Mass spectra were recorded using the full scan mode in the range of 200–800 Daltons.

RESULTS AND DISCUSSION

The phytoconstituents described for the *Bauhinia* species predominantly include flavonoid O-glycosides, the majority of which are derivatives of kaempferol and quercetin.^{3,4,10} *B. forficata* Link is known because of the presence of kaempferitrin, a flavonoid cited as a chemical marker for phytopreparations containing this species, and even mentioned because of its involvement with the observed

hypoglycemic effect.^{2,9,11} The rich flavonoid content has also been reported and is associated with biological effects such as glycemic response, cholesterol metabolism, and antioxidant activity.^{14,15} Recently, our group studied the aqueous and hydroethanolic extracts from *B. forficata* subsp. *pruinosa*, evaluating the phenolic content and antioxidant potential using an *in vitro* experimental model based on a DPPH (2,2-diphenyl-1-picrylhydrazyl) assay and lipid peroxidation prevention by measuring TBARS (thiobarbituric acid reactive substances).¹³ The potential of this species to reduce lipid peroxidation in an experimental model of hyperglycemia in human erythrocytes *in vitro* was verified.¹⁶

Considering the reports on the effectiveness of this plant for the treatment of diabetes, we proposed an investigation on the chemical composition of flavonoids. The previous chromatographic analysis by LC-UV was performed using defined conditions that provided good separation and rapid elution.¹³ The experimental conditions were applied to LC-MS analysis, which provided a good separation profile and detected intense peaks at retention times of 12.6 (compound 1), 13.9 (compound 2), 15.0 (compound 3), and 16.1 min (compound 4) (Figure 1). Performing a detailed evaluation of the mass spectra (Figure 2), led to the suggestion of four phenolic compounds, all

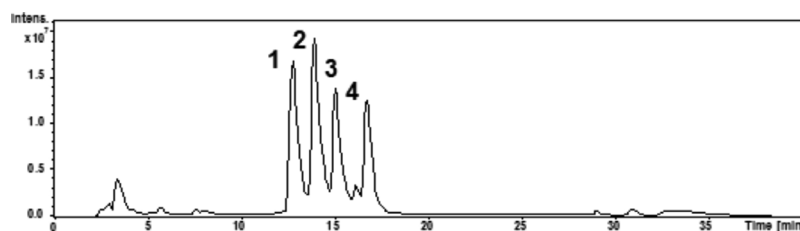


Figure 1. Representative LC/ESI-MS chromatograms obtained from the analysis of *Bauhinia forficata* subsp. *pruinosa*. Peak numbers correspond to the suggested chemical compounds. (1) quercetin-3-O-(2-rhamnosyl)rutinoside; (2) kaempferol-3-O-(2-rhamnosyl)rutinoside; (3) quercetin-3-O-rutinoside; (4) kaempferol-3-O-rutinoside

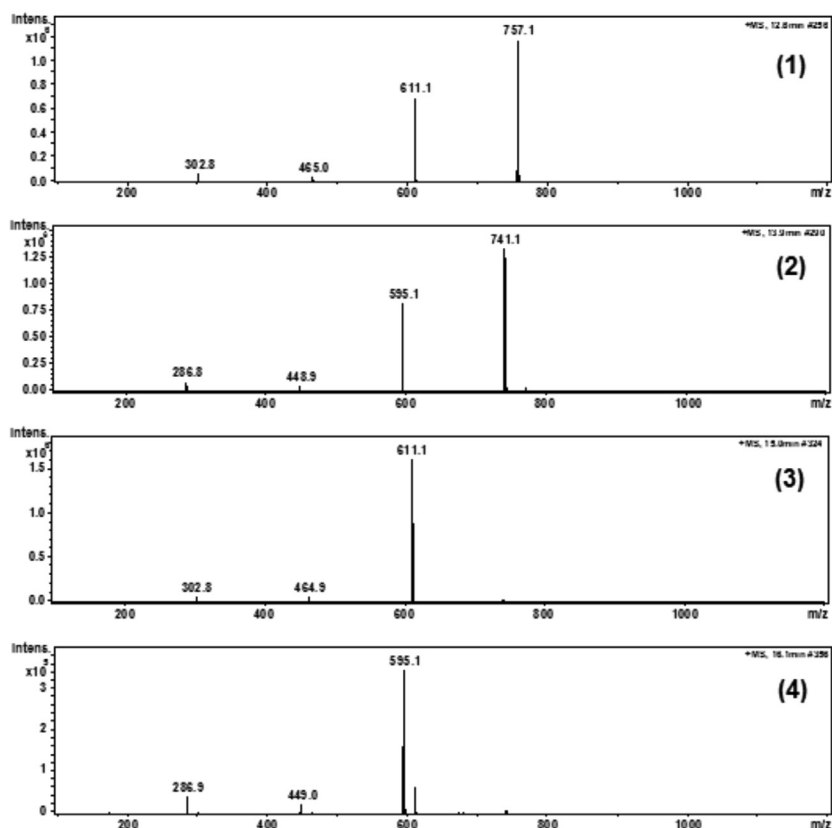


Figure 2. Mass spectra obtained using LC/ESI(+)-MS for analysis of *Bauhinia forficata* subsp. *pruinosa*. (1) quercetin-3-O-(2-rhamnosyl)rutinoside; (2) kaempferol-3-O-(2-rhamnosyl)rutinoside; (3) quercetin-3-O-rutinoside; (4) kaempferol-3-O-rutinoside

Table 1. Chemical constituents identified in *Bauhinia forficata* subsp. *pruinosa* with corresponding retention times, molecular ions in positive mode, and key fragments by LC/ESI-MS

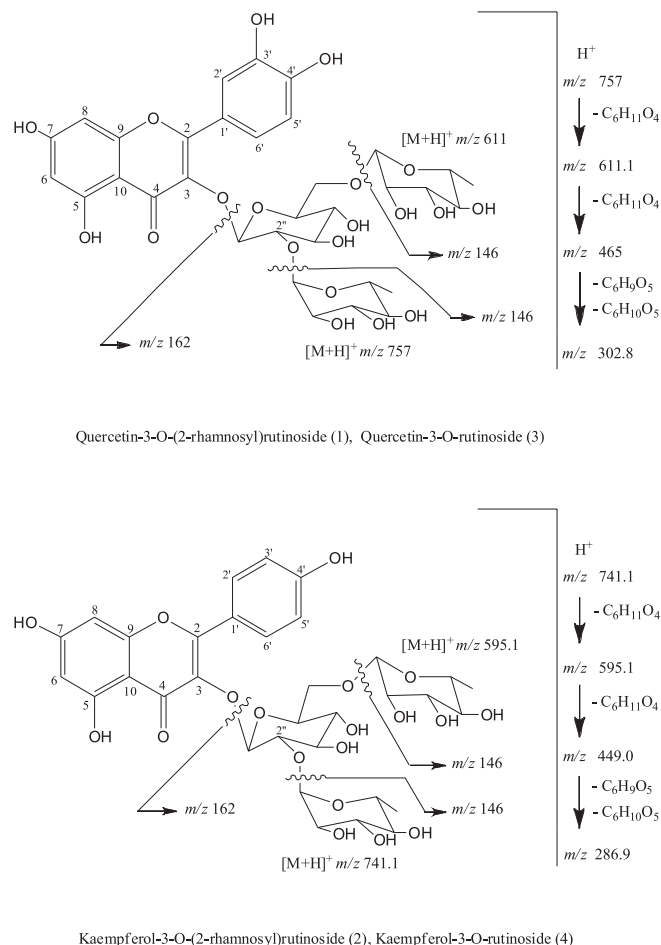
Compound	t ^R (min)	UV _{max} (nm)	[M+H] ⁺ (m/z)	MS fragmentation [M+H] ⁺ (m/z)	Suggested compound
1	12.6	255, 352	757.1	611.1; 465.0; 302.8	Quercetin-3- <i>O</i> -(2-rhamnosyl)rutinoside
2	13.9	262, 350	741.1	595.1; 448.9; 286.8	Kaempferol-3- <i>O</i> -(2-rhamnosyl)rutinoside
3	15.0	255, 352	611.1	464.9; 302.8	Quercetin-3- <i>O</i> -rutinoside
4	16.1	262, 350	595.1	449.0; 286.9	Kaempferol-3- <i>O</i> -rutinoside

containing a basic nucleus of quercetin or kaempferol. The identities, UV_{max}, and *m/z* of the ions are described in Table 1. Initially, in previous experiments using chemical standards, the flavonoid, kaempferitrin, had a retention time similar to the compound retained at 16.1 min. This first analysis indicated their presence, which had not yet been confirmed by MS analysis.

The proposed MS fragmentation and chemical structures of flavonoids 1–4 are illustrated in Figure 3. The chemical fragmentation involves cleavage of interglycosidic bonds, resulting in an aglycone ion at a characteristic *m/z*. In our case, O-glycosides are suggested because of the rearrangement and concomitant cleavage that occur at the interglycosidic bond during fragmentation, leading to the elimination of monosaccharide residues and generation of an OH terminal group.¹⁷ Compounds 1 and 3 are identified as quercetin-3-*O*-(2-rhamnosyl)rutinoside and quercetin-3-*O*-rutinoside. Compound 1 displayed a pseudo-molecular ion [M+H]⁺ at *m/z* 757.1 and fragments at *m/z* 611.1 and 302.8, indicating the loss of monosaccharide and disaccharide residues, rhamnosyl (146 u) and rutinoside (308.2 u). In reference to compound 3, the direct loss of the rutinoside residue results in the aglycone ion [M+H]⁺ at *m/z* 302.8, suggesting a flavonoid nucleus of quercetin. The residual disaccharide rutinoside was suggested based on the mass spectra and a recent literature report that mentions the compound in *B. forficata* subsp. *pruinosa*.¹⁸ The possibility for other sugar fragments (i.e. robinoside) was considered, but it was not conclusive with the current information. In the literature, some quercetin glycosides are described for *Bauhinia* species. Pizzolati *et al.*⁹ have identified five glycosyl flavonoids in *B. forficata* Link, two of them being quercetin derivatives, 3,7-di-*O*- α -L-rhamnopyranosylquercetin and 3-*O*-[α -L-rhamnopyranosyl-(1-6)- β -D-glucopyranosyl]-7-*O*- α -L-rhamnopyranosylquercetin. In another review, the flavonoids, quercetin-3-*O*-L-rhamnopyranosido and kaempferol-3-*O*-L-rhamnopyranosido, were identified in *B. uruguayensis*.¹⁰

Compounds 2 and 4 were identified as kaempferol glycosides, kaempferol-3-*O*-(2-rhamnosyl)rutinoside and kaempferol-3-*O*-rutinoside, respectively. This identification was based on the residual fragment observed at *m/z* 286.8, which is characteristic for these derivatives. The monosaccharide units were determined by observing the fragment ions that are indicative of the loss of sugar residues and suggest the interglycosidic bond is broken. In this case, the rhamnosyl (146 u) and glucosyl residues (162 u) were seen, leading to the suggested chemical structures illustrated in Figure 3.

In terms of predominance, the kaempferol nucleus is very common in *Bauhinia* species, although the sugar and O-glycosylation site are variable. Recently, Ferreres *et al.*¹⁸ identified eight substances in *B. forficata* containing this aglycone structural. In respect to subsp. *forficata*, the derivatives are glycosylated simultaneously in positions 3 and 7, different from our results for subsp. *pruinosa*. Pizzolati *et al.*⁹ isolated the compounds kaempferitrin (kaempferol 3,7-dirhamnoside) and 3-*O*-[rhamnopyranosyl-(1-6)- β -glucopyranosyl]-7-*O*-rhamnopyranosylkaempferol from the leaves of *B. forficata* Link. In *B. candicans*, the presence of kaempferol 3-*O*- β -rutinoside was described.¹⁰ Rao *et al.*¹⁹ have also mentioned the presence of kaempferol 3-*O*- β -D-glucopyranoside in *B. variegata*.¹⁹

**Figure 3.** Chemical structures of flavonoid derivatives and the proposed MS fragmentation in positive ion mode. Hydroethanolic extracts obtained from *Bauhinia forficata* subsp. *pruinosa*

CONCLUSION

B. forficata subsp. *pruinosa* presents a chemical profile based on kaempferol and quercetin glycosides. The chromatographic assay proposed, involving detection by MS, allowed for rapid identification of the main components present in the hydroethanolic extracts and provides an option for the characterization of plant material.

REFERENCES

- Fuentes, O.; Arancibia-Avila, P.; Alarcón, J.; *Fitoterapia* **2004**, *75*, 527.
- Pepato, M. T.; Keller, E. H.; Baviera, A. M.; Kettelhut, I. C.; Vendramini, R. C.; Brunetti, I. L.; *J. Ethnopharmacol.* **2002**, *81*, 191.
- Cechinel Filho, V.; *Phytother. Res.* **2009**, *23*, 1347.
- Da Silva, K. L.; Biavatti, M. L.; Leite, S. N.; Yunes, R. A.; Monache, F. D.; Cechinel Filho, V.; *Z. Naturforsch., C: Biosci.* **2000**, *55*, 478.

5. Da Cunha, A. M.; Menon, S.; Menon, R.; Couto, A. G.; Bürger, C.; Biavatti, M. W.; *Phytomedicine* **2010**, *17*, 37.
6. Khalil, N. M.; Pepato, M. T.; Brunetti, I. L.; *Biol. Res.* **2008**, *41*, 165.
7. Lino, C. S.; Diógenes, J. P. L.; Pereira, B. A.; Faria, R.; Andrade Neto, M.; Alves, R. S.; Queiroz, M.; Souza, F. C. F.; Viana, G. S. B.; *Biol. Pharm. Bull.* **2004**, *27*, 125.
8. Pinheiro, T. S. D. B.; Johansson, L. A. P.; Pizzolatti, M. G.; Biavatti, M. W.; *J. Pharm. Biomed. Anal.* **2006**, *41*, 431.
9. Pizzolatti, M. G.; Cunha Jr, A.; Szpoganicz, B.; De Souza, E.; Braz-Filho, R.; Schripsema, J.; *Quim. Nova* **2003**, *26*, 466.
10. Da Silva, K. L.; Cechinel Filho, V.; *Quim. Nova* **2002**, *25*, 449.
11. Jorge, A. P.; Horst, H.; Sousa, E.; Pizzolatti, M. G.; Barreto Silva, F. R. M.; *Chem. Biol. Interact.* **2004**, *149*, 89.
12. Arygony, A. L. V.; *Dissertação de Mestrado*, Universidade Federal do Rio Grande do Sul, Brasil, 2005.
13. Sayago, C. T. M.; De Camargo, V. B.; Barbosa, F.; Gularte, C.; Pereira, G.; Miotto, S.; Cechinel Filho, V.; Puntel, R. L.; Folmer, V.; Mendez, A.; *Acta Biol. Hung.* **2013**, *64*, 25.
14. Volpato, G. T.; Damasceno, D. C.; Rudge, M. V. C.; Padovani, C. R.; Calderon, I. M. P.; *J. Ethnopharmacol.* **2008**, *116*, 131.
15. De Sousa, E.; Zanatta, L.; Seifriz, I.; Creczynski-Pasa, T. B.; Pizzolatti, M. G.; Szpoganicz, B.; Silva, F. R. M. B.; *J. Nat. Prod.* **2004**, *67*, 829.
16. Salgueiro, A. C. F.; Leal, C. O.; Bianchini, M. C.; Prado, I. O.; Mendez, A. S. L.; Puntel, R.; Folmer, V.; Soares, F. A.; Ávila, D. S.; Puntel, G. O.; *J. Ethnopharmacol.* **2013**, *148*, 81.
17. Cuyckens, F.; Claeys, M.; *J. Mass Spectrom.* **2004**, *39*, 1.
18. Ferreres, F.; Gil-Izquierdo, A.; Vinholes, J.; Silva, S. T.; Valentao, P.; Andrade, P. B.; *Food Chem.* **2012**, *134*, 894.
19. Rao, Y. K.; Fang, S. H.; Tzeng, Y. M.; *Phytother. Res.* **2008**, *22*, 957.