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CHEMICAL COMPOSITION AND *IN VITRO* ANTIOXIDANT ACTIVITY OF HYDRO-ETHANOLIC EXTRACTS FROM *BAUHINIA FORFICATA* SUBSP. *PRUINOSA* AND *B. VARIEGATA*

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Bauhinia species are known to have hypoglycemiant and antioxidant activities. Here, hydro-ethanolic leaf extracts from *Bauhinia forficata* subsp. *pruinosa* and *Bauhinia variegata,* collected in a Pampa biome region of Brazil, were investigated to characterize their chromatographic profile, flavonoid content and *in vitro* antioxidant activity (TBARS and DPPH assays). The extracts were obtained from dried and fresh leaves. The total flavonoid content was assessed by spectrophotometric determination, and the results ranged between 572.08 and 1,102.99 μg mL^{-1}. Moreover, flavonoids were more predominant in *B. variegata* than in *B. forficata* subsp. *pruinosa.* HPLC analysis detected a complex profile of phenolic compounds, being the flavonoid kaempferitrin founded *B. forficata* subsp. *pruinosa;* in addition, other kaempferol and quercetin derivatives were present. *In vitro* antioxidant assays demonstrated a different behavior depending on the species, leaf treatment and extract concentration. In general, *B. variegata* extracts obtained from fresh material presented higher antioxidant potential, which can be attributed to the predominance of flavonoids in their chemical composition.

Keywords: Bauhinia species – flavonoids – HPLC analysis – antioxidant activity

INTRODUCTION

The genus *Bauhinia* (Fabaceae – Caesalpinioideae) comprises approximately 300 species distributed in tropical areas [19]. They are popularly known in Brazil as "pata de vaca" (cow's hoof) due to the large and bilobed aspect of the leaves, a characteristic that is distinctive to this genus [4, 5, 8, 21]. Interestingly, the aerial parts are widely used in folk medicine for diabetes treatment, which has led many authors to perform pharmacological studies on leaf extracts in search of their hypoglycemiant activity [13, 30, 32]. *B. forficata* subsp. *forficata* is the most well characterized with respect to its pharmacological aspects, biochemical properties and phytochemical

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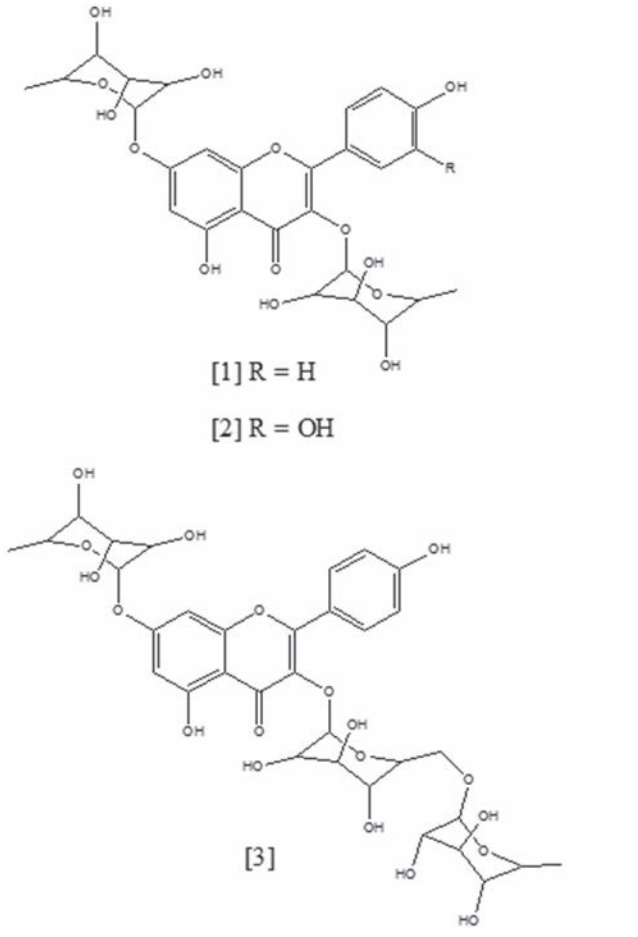

Fig. 1. Chemical structure of glycosil-flavonoids present in *Bauhinia*. [1] kaempferitrin; [2] quercetin 3,7-di-O-α-L-rhamnopyranoside; [3] kaempferol-3-robinoside-7-rhamnoside

analysis, where the presence of glycosides kaempferol and quercetin O-glycoside derivatives, such as kaempferol 3,7-dirhamnoside (kaempferitrin) and quercetin 3,7-di-O-α-L-rhamnopyranoside was observed (Fig. 1) [7, 16, 21, 22].

In southern Brazil, there is an important biome that has been recently explored, the Pampa biome. This biome extends from eastern Argentina and Uruguay through the southern half of Rio Grande do Sul state in Brazil [12, 31]. Many species are being studied to elucidate the composition of the flora. Here, we evaluate two *Bauhinia* species, namely, *B. forficata* subsp. *pruinosa* and *B. variegata*. Despite of being different from *B. forficata* subsp. *forficata* and have few studies about, *B. forficata* subsp. *pruinosa* is indicated by local populations to treat diabetes. *B. variegata* is

used as an ornamental tree. Both studied species are evergreen trees with white flowers that grow to a height of 5 to 10 m.

Several investigations have confirmed the hypoglycemiant potential of *B. forficata* subsp. *forficata* extracts [3, 9, 17, 19]. Lino et al. [17] verified this effect in the *n*-butanol fraction of *B. forficata* leaves in alloxan-induced diabetic rats. Similarly, aqueous and ethanolic extracts demonstrated a potential to reduce glucose, triglycerides, total cholesterol and HDL cholesterol levels. A study on a streptozotocin-induced diabetes model was conducted with decoction treatment and, as a result, there was an improvement in carbohydrate metabolism [19]. For *B. forficata* subsp. *pruinosa*, the literature is scarce. Arygony [2] studied the leaf extracts and detected a major compound identified as kaempferol-3-robinoside-7-rhamnoside (Fig. 1).

B. variegata has been studied because of its use in several countries, such as India and China, as astringent tonic as well as to treat skin diseases and ulcers [23]. The studies of the pharmacological effects were mainly conducted using infusions of stems, bark and roots [1, 23]. Likewise, ethanolic and aqueous extracts from these parts have been assayed, and their antioxidant potential has been confirmed by testing their ability to scavenge DPPH, nitric oxide and superoxide radical [1]. The chemical composition of *B. variegata* includes flavonoid glycosides (ramnopyranoside derivatives), kaempferol-3-galactoside and kaempferol-3-ramno-glycoside [9, 14, 33].

Considering the importance of investigating phytochemical profiles and *in vitro* activities of medicinal plants, here, we present a study on hydro-ethanolic leaf extracts from *B. forficata* subsp. *pruinosa* and *B. variegata* collected in a Pampa biome region of Brazil. A qualitative analysis of flavonoids, using kaempferitrin, quercetin and kaempferol as references, and an investigation of the potential against free radical production were carried out.

MATERIALS AND METHODS

Chemicals

The 3,7-di-O-α-L-rhamnopyranosylkaempferol glycosil-flavonoid (kaempferitrin) was isolated from *B. forficata* according to Pizzolatti et al. [22]. Quercetin and kaempferol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile was purchased from Tedia (Fairfield, OH, USA). Phosphoric acid, acetic acid, tris[hydroxymethyl]aminomethane, and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate, thiobarbituric acid, malondialdehyde, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and iron sulfate ($FeSO_4$) were purchased from Sigma-Aldrich. 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 and *Bauhinia variegata* L. were collected in September of 2010 in Uruguaiana city (Rio Grande do Sul, Brazil), located near to Argentina's western border with Brazil. The plants were identified by Dr. Silvia T. S. Miotto, Universidade Federal do Rio Grande do Sul, and voucher specimens (ICN 167491 and ICN 167492) were deposited at the ICN Herbarium (Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Brazil).

Extracts

Air-dried (at 35 °C) and crushed (fresh) leaves of *B. forficata* and *B. variegata* were reduced to a powder and a quantity of 25.0 g was submitted to extraction by percolation (1 : 1, w/v) using ethanol-water (1 : 2) [11, 21]. These hydro-ethanolic extracts were then filtered and stored in a refrigerator until the analysis.

For a chromatographic assay, the extracts were diluted in a solution of 50% methanol in a proportion of 1 : 10 (v/v). All solutions were filtered through a 0.45 μm membrane filter from Millipore (Milford, MA, USA) before injection.

Concentration of extracts and total flavonoid content

The percentage of dried extractives (dry residue) in extracts was determined according to the Farmacopéia Brasileira [11]. Samples of 2.0 ml each were used, and each analysis was done in triplicate. The determination of the total flavonoid content was performed based on a previously reported methodology, which uses $AlCl_3$ [20]. The dilution factor used was 1 : 100. The extracts were assayed in triplicate, and the rutin flavonoid was used as a reference. UV-Vis absorbance was measured in a Perkin Elmer® Lambda 35 UV/Vis Double array Spectrophotometer (Norwalk, CT, USA) with 1 cm quartz cells.

LC apparatus

An LC analysis was performed in a Prominence Liquid Chromatograph Shimadzu equipped with a LC-20AT pump, a SIL-20A auto sampler, a SPD-20AT PDA detector and a CTO-20A column oven (Kyoto, Japan). LC Solution V. 1.24 SP1 system software was used to control the equipment and to obtain data and responses from the LC system.

Chromatographic conditions

The method included using a reversed phase technique. The analyses of the leaf extracts and of flavonoids references were performed in a gradient elution mode with a 0.8 mL min^{-1} flow according to Pinheiro et al., i.e. 1–23 min, 10–40% solvent B (acetonitrile) in A (H_2O–H_3PO_4, 100 : 0.05, pH 2.88) [21]. An equilibration period of 10 min was used between runs. The wavelength of the DAD detector was set to 340 nm. The mobile phase was prepared daily, filtered through a 0.45 μm membrane filter from Millipore (Milford, MA, USA) and sonicated before use. An ODS-Hypersil Thermo Scientific C18 column (250 × 4.6 mm i.d., 5 μm particle size) (Bellefonte, United States) was used. The HPLC system was operated at 25 ± 1 °C. The injection volume was 20 μL.

For kaempferitrin, quercetin and kaempferol presence evaluation, the standards were prepared by dilution in 50% methanol at a concentration of 100 μg mL^{-1}. A co-injection was done by adding the kaempferitrin standard solution to the extract sample solution to a final concentration of 50 μg mL^{-1}. The UV spectra obtained for each chromatographic peak were compared to references.

Radical-scavenging activity: DPPH assay

The antioxidant activity of the extracts was evaluated by monitoring their ability for quenching stable free radicals using DPPH assays according to Choi et al. [6] with minor modifications. Three different volumes of each extract (25 μL, 2.5 μL and 0.25 μL), which corresponded to the concentrations of extracts obtained from dried or fresh samples, were mixed with 1.0 mL of a 0.3 mM DPPH ethanol solution. The absorbance was measured at 518 nm after 30 min of reaction at 25 °C. Tests were carried out in duplicate.

In vitro *antioxidant activity assay*

Animals

Adult male Swiss mice (25–35 g) from our own breeding colony were used in this study. The animals were housed in plastic cages with water and food provided *ad libitum* at 22–23 °C, 56% humidity, and 12 h of light–dark cycle. The animals were used kept in accordance with guidelines of the Committee on Care and use of Experimental Animal Resources, Universidade Federal de Santa Maria, Brazil.

Tissue preparation

Animals were anesthetized with ether and killed by decapitation. The whole brain was quickly removed, placed on ice, and homogenized within 10 min in 10 volumes of a cold solution of 150 mmol/L saline. The homogenate was centrifuged at 4,000 *g* at 4 °C for 10 min to yield a low-speed supernatant fraction (S1) that was used immediately for a TBARS assay.

Lipid peroxidation assay

Lipid peroxidation was determined by measuring thiobarbituric acid- reactive substances (TBARS), as described by Ohkawa et al. [18], in the presence of the indicated concentrations of plant extracts. Brain homogenates were prepared by homogenization as described above. In addition, an adapted TBARS method was used to measure the antioxidant capacity of the extracts using egg yolk homogenate as a lipid-rich substrate [27]. Briefly, egg yolk was homogenized (1 : 10 v/v) in 100 mM Tris buffer (pH 7.4). The mixtures were incubated at 37 °C in a water bath for 60 min. The reaction was stopped by adding 0.5 mL of acetic acid buffer, and the lipid peroxidation products were measured by adding 0.5 mL of 0.6% TBA and 0.2 mL of 8.1% SDS. A color reaction was developed by incubating tubes in boiling water for 60 min. TBARS levels were measured at 532 nm using a standard curve of MDA. Solutions of $FeSO_4$ were made just before use with distilled water. Values are expressed as % of control.

Statistical analysis

Data from the lipid peroxidation experiment were analyzed with a two-way ANOVA followed by a Duncan's Multiple Range Test when appropriate. Differences between groups were considered to be significant when $P \leq 0.05$.

RESULTS

The concentration of extractives (dry residue) of *B. forficata* subsp. *pruinosa* and *B. variegata* was 0.92 and 1.16% (w/w) (fresh leaves) and 1.88 and 2.19% (w/w) (dried leaves), respectively. In both cases, *B. variegata* had a higher content of extracts; however, for both species, the extracts were more concentrated when prepared from dried leaves. The results of total flavonoid content determination are described in Table 1. A comparison of both species showed a variation in the range of 572.08 and 1,102.99 μg mL^{-1} (expressed as rutin equivalent) with a greater predominance of flavonoids in *B. variegata*.

Table 1
Total flavonoid content of hydro-ethanolic leaf extracts from *Bauhinia forficata* subsp. *pruinosa* and *B. variegata*

Sample	Total flavonoids[a]		RSD (%)
	(μg mL^{-1})	(g 100g leaf^{-1})[b]	
B. forficata subsp. *pruinosa*			
Fresh sample	572.08	0.6135	5.95
Dried sample	876.30	0.9447	1.82
B. variegata			
Fresh sample	873.56	0.9671	2.52
Dried sample	1102.99	1.1990	5.71

[a] Assay in triplicate.
[b] Density and dilution factor were considered.

The results from chromatographic analysis are illustrated in Figures 2 and 3. Based on values of dry residue and water content, the concentration of samples for injection was 0.0203 g mL^{-1} and 0.0237 g mL^{-1} for *B. forficata* subsp. *pruinosa* and *B. variegata,* respectively. For both species, the analytical profile is similar in the range of retention times of 16.0–19.0 min, being observed the same four components at times of 16.4, 17.3, 18.1 and 18.3 min. Observing the standard chromatogram (Fig. 2A), kaempferitrin, quercetin and kaempferol were detected at 17.0, 25.3, and 29.3 min, respectively. The glycosil-flavonoid kaempferitrin was detected (17.03 min) in hydro-ethanolic extracts from *B. forficata* subsp. *pruinosa* (Figs 2B, 3B), being absent in extracts from other species. To confirm this result, a co-injection was

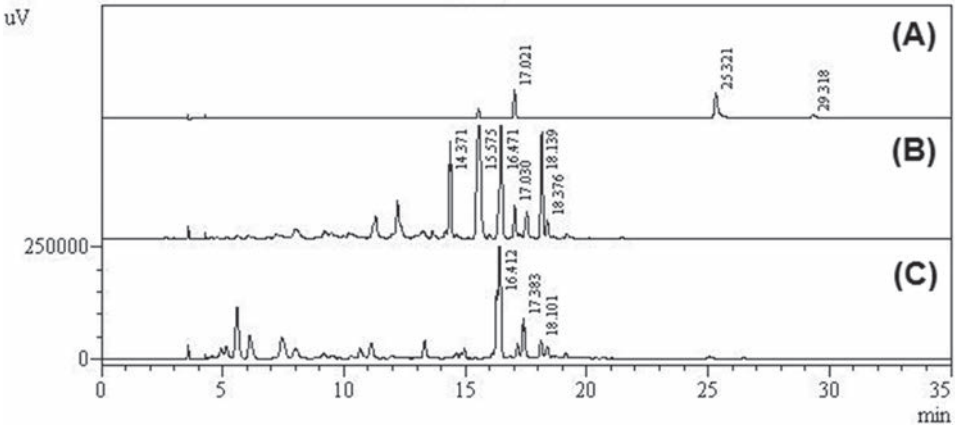

Fig. 2. Chromatographic profile of kaempferitrin, quercetin and kaempferol standards (retention time of 17.02, 25.32 and 29.31 min) (A) and hydro-ethanolic leaf extracts from *B. forficata* subsp. *pruinosa* (B) and *B. variegata* (C)

performed adding kaempferitrin standard in the extract, which resulted in a peak increased at the same retention time. A qualitative assay was performed comparing the UV spectra of the chromatographic peaks correspondent to kaempferitrin in standard solution and kaempferitrin in *B. forficata* subsp. *pruinosa* (Fig. 3A1, B1). The UV profile and both maximum wavelength values (264 and 342 nm) indicated the same identity of the analyzed substance.

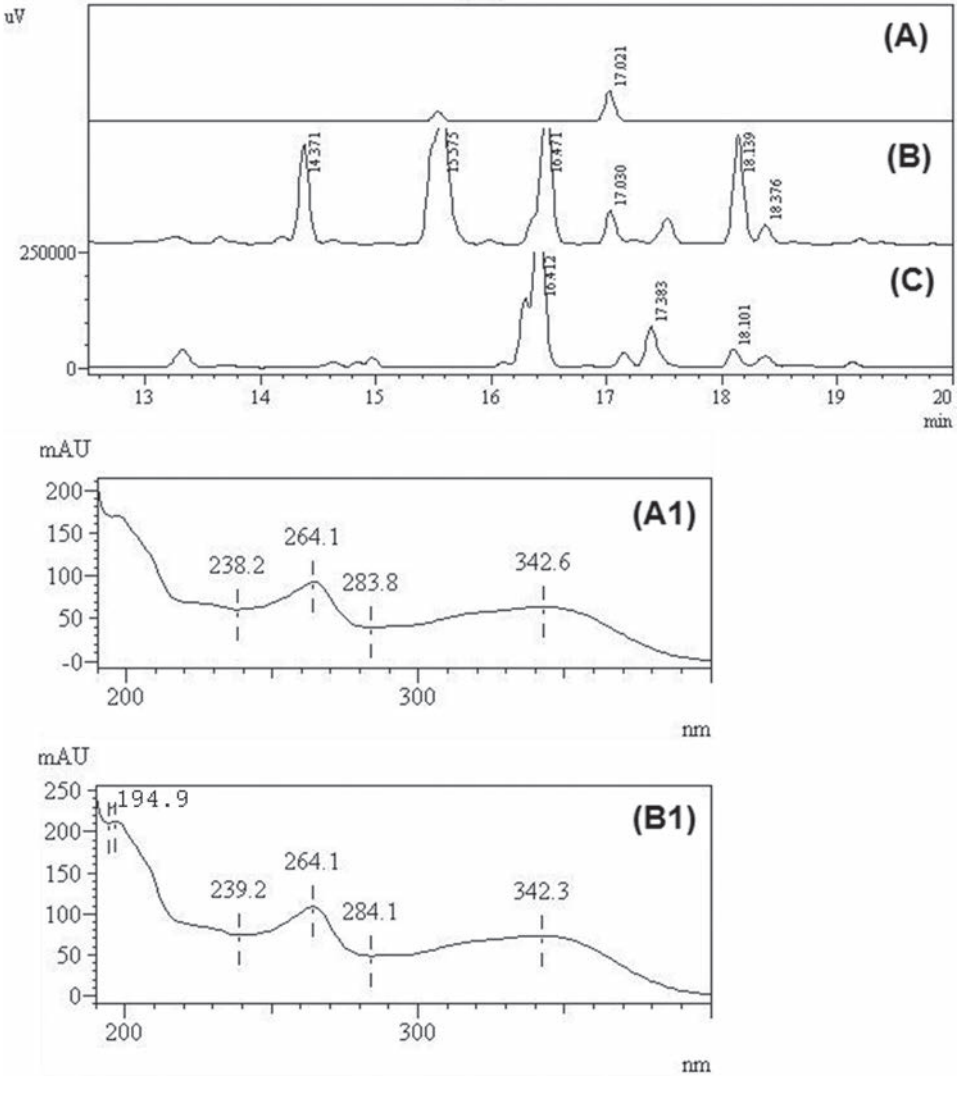

Fig. 3. Detailed chromatographic profile of kaempferitrin standard (A) and hydro-ethanolic leaf extracts from *B. forficata* subsp. *pruinosa* (B) and *B. variegata* (C). UV spectra of the chromatographic peaks correspondent to kaempferitrin standard (A; A1) and kaempferitrin in *B. forficata* subsp. *pruinosa* (B – retention time of 17.03 min; B1)

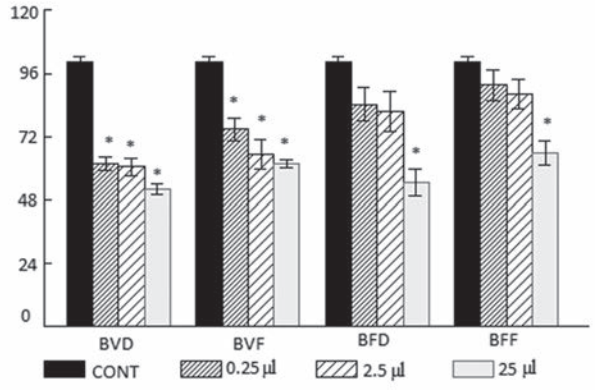

Fig. 4. Effects of extracts from *B. forficata* subsp. *pruinosa* and *B. variegata* on radical-scavenging activity-DPPH. BVD, *B. variegata* dried sample; BVF, *B. variegata* fresh sample; BFD, *B. forficata* subsp. *pruinosa* dried sample; BFF, *B. forficata* subsp. *pruinosa* fresh sample. Values are means ± S.E.M., $n = 7$. *Indicates significant difference from control ($P < 0.05$)

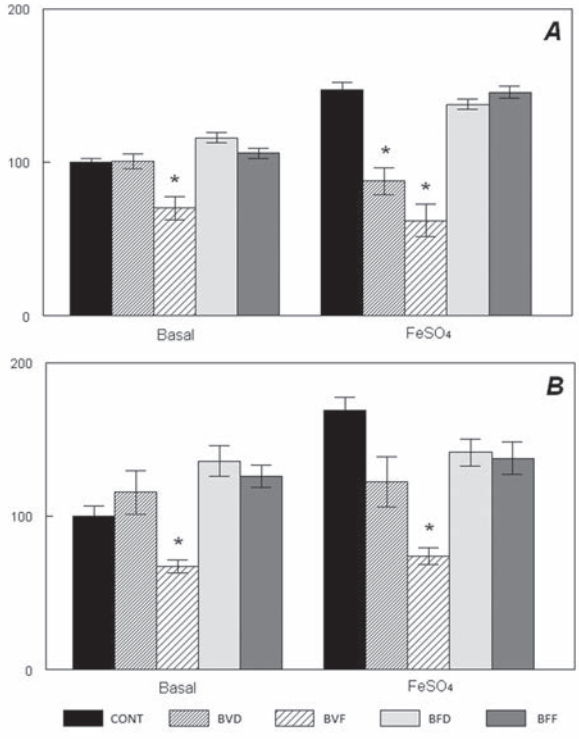

Fig. 5. Effects of extracts from *B. forficata* subsp. *pruinosa* and *B. variegata* on Fe^{2+}-induced lipid peroxidation. (A) TBARS production in phospholipids system; (B) TBARS production in brain homogenate. BVD, *B. variegata* dried sample; BVF, *B. variegata* fresh sample; BFD, *B. forficata* subsp. *pruinosa* dried sample; BFF, *B. forficata* subsp. *pruinosa* fresh sample. Values are means ± S.E.M., $n = 5–8$. *Indicates significant difference from control ($P < 0.05$)

In this work the radical-scavenging activity was investigated with a DPPH assay and a lipid peroxidation assay by measuring TBARS. As shown in Figure 4, *B. variegata* (both fresh and dried extracts) significantly prevented DPPH oxidation, which reflects a strong antioxidant potential with all tested concentrations. In contrast, *B. forficata* extracts (dried and fresh) presented antioxidant activity only at the highest concentration. As shown in Figure 5A, Fe^{2+} induced a significant increase in TBARS production by the phospholipid system. However, a fresh extract of *B. variegata* significantly inhibited the basal TBARS production. In addition, both fresh and dried extracts of this species were able to inhibit Fe^{2+}-induced TBARS production in the phospholipid system (Fig. 5A). *B. forficata* subsp. *pruinosa* (fresh and dried extracts) did not exert any significant effect (neither on basal or Fe^{2+}-induced TBARS production). Figure 5B shows the effect of *Bauhinia* extracts on TBARS production using brain tissue. Likewise, Fe^{2+} (10 μM) was able to cause a significant increase in TBARS production *per se*. Statistical analyzes revealed that only the fresh extract of *B. variegata* was able to significantly inhibit TBARS production in brain homogenates, both in basal or iron-induced conditions.

DISCUSSION

In the present work, *B. forficata* subsp. *pruinosa* and *B. variegata,* collected in a region of Pampa biome in Brazil, were investigated. Through a literature survey, we found that *B. forficata* subsp. *forficata,* a native species of southern Brazil, has hypoglycemiant and antioxidant activities. Here, in this region, the *Bauhinia* species indicated for diabetes treatment is the subspecies *pruinosa*. The genus *Bauhinia* has many flavonoid derivatives that have known antioxidant potential with hydrogen-donating radical scavenger properties [24, 25, 28, 29].

Considering the chemical evaluation done, higher values for total flavonoid content were observed in extracts obtained from dried leaves (Table 1). A recent study investigated the hypoglycemiant activity of dried extracts from *B. forficata* (subspecies *forficata*), where the total flavonoid content of fluid extracts was 720 μg mL^{-1} [7]. In another study, the obtained values were 863.35 and 2,759.95 μg mL^{-1} for aqueous and hydro-ethanolic extracts [21]. Thus, our results are in accordance with the flavonoid content of other species, although the values founded were higher in *B. variegata*.

In the chromatographic analysis, the results suggest high complexity of the phenolic compounds. According to Cechinel Filho [5], kaempferol and quercetin derivatives are common in several species of this genus. Considering the flavonoids investigated (kaempferitrin, quercetin and kaempferol), only kaempferitrin was detected in *B. forficata* subsp. *pruinosa,* while on *B. variegata* none of those was found. Pinheiro et al. [21] and Da Cunha et al. [7] found a higher quantity of kaempferitrin in *B. forficata* subsp. *forficata*. According to literature, kaempferitrin could be responsible for the known hypoglycemiant activity of this species [8, 10, 15, 21].

So, our results are very important and constitute a relevant contribution to understand the popular use for the diabetes treatment in this region. In order to confirm the identity suggested, it is important to perform the isolation and characterization in another study.

An additional evaluation of chromatograms may indicate the presence of other derivatives. The comparison of the results obtained to those from literature suggests that the major compound of *B. forficata* subsp. *pruinosa,* detected at 15.5 min (Fig. 3B), is kaempferol-3-robinoside-7-rhamnoside (Fig. 1), recently studied [2]. The peak with a retention time of 16.4 min (Fig. 3B, C), present in both studied species and detected as majority compound in *B. variegata,* could be classified as quercetin-3,7-di-O-α-L-rhamnopyranoside (Fig. 1), which was already cited for *B. forficata* subsp. *forficata* [7, 21].

Considering the results obtained regarding the antioxidant activity (Figs 4, 5), they revealed that the *in vitro* antioxidant capacity of *Bauhinia* extracts varied depending on the extract. Indeed, considering the different systems used (brain tissue and phospholipids), fresh *B. variegata* extract was the most active, while *B. forficata* subsp. *pruinosa* (dried and fresh) extracts were ineffective in the TBARS assay. Similarly, *B. variegata* was more effective than *B. forficata* for avoiding damage associated with lipid peroxidation and in preventing DPPH oxidation. We suggest that the most pronounced antioxidant effects of *B. variegata* may be due to the highest content of antioxidant compounds. Despite having higher water content, the greatest effect of *in vitro* antioxidant activity was obtained with fresh extracts. This outcome could be due to the maintenance of plant composition in a material that is not subject to dehydration, thus, avoiding the loss of active molecules. Indeed, differences in the amount of flavonoids found in extracts (see Table 1), that indicates higher content in *B. variegata*, can help to understand the differences in antioxidant capacity among them. Thus, the lack of an antioxidant effect of *B. forficata* subsp. *pruinosa* extracts could be attributed to the chemical composition of them, the flavonoid content and the active molecules. According to the literature, aerial parts of *B. variegata* show an antioxidant activity quantified by the DPPH assay [26]. For *B. forficata* subsp. *pruinosa,* the antioxidant activity was determined by measuring DPPH reduction, and the results indicated a high activity in the buthanolic fraction obtained from hydro-ethanolic extracts [2].

In conclusion, hydro-ethanolic extracts prepared from *Bauhinia* species showed differences in qualitative and quantitative flavonoid composition. A complex phytochemical profile was verified and the glycosil-flavonoid kaempferitrin was found only in *B. forficata* subsp. *pruinosa*. These profiles might contribute to the *in vitro* antioxidant activity observed, mainly, in extracts obtained from fresh leaves. In particular, the radical-scavenging activity (DPPH) and lipid peroxidation assays showed a behavior that varied depending on the tissue used and the plant material tested, which demonstrates the complexity involved in chemical and biochemical systems.

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