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Free Radical Scavenging Profile and Myeloperoxidase Inhibition of Extracts from Antidiabetic Plants: *Bauhinia forficata* and *Cissus sicyoides*

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ABSTRACT

There is abundant evidence that reactive oxygen species are implicated in several physiological and pathological processes. To protect biological targets from oxidative damage, antioxidants must react with radicals and other reactive species faster than biological substrates do. The aim of the present study was to determine the in vitro antioxidant activity of aqueous extracts from leaves of *Bauhinia forficata* Link (Fabaceae - Caesalpinioideae) and *Cissus sicyoides* L. (Vitaceae) (two medicinal plants used popularly in the control of diabetes mellitus), using several different assay systems, namely, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) decolorization, superoxide anion radical (O_2 •-) scavenging and myeloperoxidase (MPO) activity. In the ABTS assay for total antioxidant activity, *B. forficata* showed IC_{50} = 8.00±0.07 μg/mL, while *C. sicyoides* showed IC_{50} = 13.0±0.2 μg/mL. However, the extract of *C. sicyoides* had a stronger effect on O_2•- (IC_{50} = 60.0±2.3 μg/mL) than the extract of *B. forficata* (IC_{50} = 90.0±4.4 μg/mL). *B. forficata* also had a stronger inhibitory effect on MPO activity, as measured by guaiacol oxidation, than *C. sicyoides*. These results indicate that aqueous extracts of leaves of *B. forficata* and *C. sicyoides* are a potential source of natural antioxidants and may be helpful in the prevention of diabetic complications associated with oxidative stress.

Key terms*:* ABTS decolorization assay, antioxidant activity, superoxide anion scavenging activity.

INTRODUCTION

Leaves of the *Bauhinia forficata* Link (Fabaceae - Caesalpinioideae) tree (1,2) and *Cissus sicyoides* L. (Vitaceae) (3), plants known popularly as Cows Hoof and Plant Insulin, respectively, are used in folk medicine to treat diabetes mellitus, either together with or in place of conventional treatment (4). Our earlier studies showed that long-term oral treatment with leaf decoction of either *B. forficata* or *C. sicyoides* significantly reduced the levels of blood glucose, urinary glucose and urinary urea (5,6), as well as both the food and fluid intake and the volume of urine excreted, in streptozotocin-diabetic rats (5).

Hyperglycemia, as occurs in diabetes mellitus, can induce oxidative stress by several mechanisms, such as glucose autoxidation, the formation of advanced glycation end-products (AGE) and activation of the polyol pathway. Such mechanisms generate reactive oxygen species (ROS) (7). Besides this, the increased production of ROS by mitochondria in hyperglycemic conditions is recognized as a major cause of the clinical complications associated with diabetes and obesity (8).

Some researchers have demonstrated that patients with increased levels of circulating markers of free radical-induced damage also exhibit an associated decline in their antioxidant defenses (9,10).

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Medicinal plants, especially those that contain flavonoids, exhibit multiple pharmacological activities (11). Beltrame *et al*. identified two flavonoids in leaves of *C. sicyoides*: kaempferol 3-α-rhamnoside and quercetin 3-α-rhamnoside (4). Pizzolatti *et al*. also identified four different flavonoids in *B. forficata* leaves, with kaempferitrin predominating (12). It is a well-established fact that flavonoids possess antioxidant properties (13). Considerable interest has gathered around the role and use of antioxidants as a means of preventing damage due to the oxidative imbalance found in diabetes. This is because they may help to protect the body against damage by free radicals and ROS (14). Some of the most important ROS produced *in vivo* are: superoxide anion radical (O_2•-), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and hydroxyl radical (HO•) (15).

Superoxide anion is one of the ROS formed during the oxidative burst that occurs in numerous pathological events, such as diabetes, atherosclerosis, cardiovascular illnesses, and cancer (16).

Myeloperoxidase (MPO), an enzyme present in polymorphonuclear neutrophil granulocytes and monocytes, has been found in human atherosclerotic tissue and specific products of MPO-catalyzed reactions have been detected during all stages of the development of atherosclerosis and other disorders (17,18,19).

In light of these observations, we continued our investigations on extracts of *B. forficata* and *C. sicyoides* leaves in this study, by testing the possibility that both plants contain appreciable levels of antioxidant activity, available under conditions similar to their normal use, in folk medicine, namely in prepared decoctions. To this end we assessed the O_2•- scavenging activity and the total antioxidant activity, by the ABTS decolorization assay, as well as the effect of these extracts on MPO activity, in leaf decoctions of both plants. Another question explored was the potential usefulness of these tests for the characterization of such extracts.

METHODS

Plant material

Leaves of both *B. forficata* and *C. sicyoides* were collected from the Medicinal Plants Garden of the School of Pharmacy, UNESP, Araraquara, São Paulo State, Brazil. Leaves of *B. forficata* were collected between April and May (the end of Autumn in the Southern Hemisphere) and leaves of *C. sicyoides* were collected in August and September (at the end of Winter). The two plants were identified, authenticated and deposited under accession nos. 119 and 120, respectively, in the Herbarium of the Department of Industrial Pharmacy, Federal University of Santa Maria, Rio Grande do Sul, Brazil, by Dr Gilberto Dolejal Zanetti.

Decoction preparation

The decoction of leaves of *B. forficata* was prepared by a method typically used in the Araraquara region, i. e. washing, then boiling 150 g of fresh leaves in 1 L of distilled water for 5 min, allowing the decoction to stand for 30 min and filtering it through filter paper. The final yield was 87% by volume. The method utilized for *C. sicyoides* was similar, with a yield of 94% by volume. The dry extracts were prepared by lyophilizing 36 mL of each decoction, which was the mean volume ingested daily by rats during a one-month trial (6). This procedure was carried out in a Freezone 6l L Benchtop lyophilizer, and the yields were 0.0053 g/mL (3.5%) for *B. forficata* and 0.0021 g/mL (1.4%) for *C. sicyoides*.

Reagents

Potassium persulfate, phenazine methosulfate (PMS), nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), trolox, uric acid, quercetin and MPO were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical grade and commercially available.

ABTS Radical Cation Decolorization Assay

The total antioxidant activities of extracts were evaluated by the ABTS radical cation decolorization assay (20). Briefly, a mixture of 7mM ABTS and 2.45 mM potassium persulfate was prepared and allowed to stand at room temperature for 12 h in the dark. The resulting ABTS•+ solution was diluted to an absorbance of 0.70 at 734 nm in 50 mM sodium phosphate buffer, pH 7.4. Phosphate buffer without (0% scavenging control) or with various concentrations of *B. forficata* and *C. sicyoides* were added to the ABTS•+ solution. The absorbance at 734 nm was read after 30 minutes. The percentage of scavenging activity (%) was calculated as follows:Scavenging activity = [1-(absorbance of sample at 734 nm)/ absorbance of control at 734 nm)] x 100. Trolox and uric acid were used as positive controls in this assay.

O_2•- Scavenging Activity in the NADH/ PMS/NBT System

The O_2•- scavenging activity of *B. forficata* and *C. sicyoides* was determined by the method described by Kakkar et al. (21), slightly modified. Solutions containing 156 μM NBT dissolved in 50 mM phosphate buffer, pH 7.4, 468 μM NADH and various concentrations of crude extracts were mixed and the reaction started by adding 100 μL of 60 μM PMS solution. The reaction mixture was incubated at 25ºC for 5 min and absorbance at 560 nm was measured against control samples (without NADH). All tests were performed in triplicate. The percentage of scavenging activity (%) was calculated as follows: Scavenging activity = [1-(absorbance of sample at 560 nm)/ absorbance of control at 560 nm)] x 100. Trolox and uric acid were used as positive controls in this assay.

Activity of MPO

Activity of MPO was also determined spectrophotometrically, with guaiacol as substrate (22). The reaction mixture contained 2.5 nM MPO, 1 mM guaiacol and 0.5 mM H_2O_2 in 1.0 mL of 50 mM phosphate buffer, pH 7.4. The mixture was incubated at 37 ºC without (control) or with *B. forficata* or *C. sicyoides* and the reaction was initiated by the addition of H_2O_2, after which the increase of absorbance at 470 nm was recorded for 3 min. MPO activity was calculated from the initial rate of reaction (in s^{-1}). % inhibition = [1-(activity of test)/ activity of control] x 100.

We used a Diode Array 8452 A spectrophotometer from Hewlett Packard, equipped with a thermostatic cell, for all determinations. Quercetin was used as positive control in this assay.

Statistic Analysis

Results are expressed as means ± SD. Statistical significance was determined by Student's *t* test ($p<0.05$).

RESULTS AND DISCUSSION

Several total antioxidant assays have been used to determine the antioxidant scavenging activity of plant-based foods (23) and clinical fluids and tissues (24). The ABTS•+ assay has several advantages: it is simple, colorimetric, does not require sophisticated analytical equipment (25) and provides a good estimate of the antioxidant activity of pure compounds and complex matrices. The rate of the decrease in absorbance at 734 nm depends on the type and amount of antioxidants and the results are thus expressed as antioxidant standard equivalents (20,25). Thus, initially we used the ABTS•+ assay to estimate the total antioxidant activity of the extracts. In Figure 1, the suppression of the absorbance of ABTS•+ in a concentration-dependent manner is typically shown by *B. forficata* and *C. sicyoides* extracts. In the tested conditions, *B. forficata* showed a 50% inhibitory concentration of IC_{50} = 8.00±0.07 μg/mL, while *C. sicyoides* showed IC_{50} = 13.0±0.2 μg/mL. *B. forficata* exhibited higher antioxidant activity than *C. sicyoides* ($p<0.05$). One hundred percent ABTS scavenging activity was reached only when 25.0 μg/mL of *C. sicyoides* was used.

Uric acid, an endogenous antioxidant present in extracellular fluids (26), and trolox were used as standard antioxidants, respectively showing IC_{50} = 1.20 ± 0.03 μg/mL and IC_{50} =0.600 ± 0.006 μg/mL. To obtain these IC_{50} values, it was necessary to use concentrations of the plant extracts in the range 0 a 25 μg/mL.

Since the results of the ABTS•+ radical cation assay indicated a potential antioxidant activity in the extracts, we decided to test this effect on the superoxide anion. Figure 2 shows the effects of the *B. forficata* and *C. sicyoides* decoctions on the superoxide anion generated by the NADH/PMS/NBT system. In this assay four different concentrations of each decoction were tested (25; 50; 75; 100 μg/mL). The extract of *C. sicyoides* had a stronger effect on the superoxide anion (IC_{50} = 60.0±2.3 μg/mL) than the extract of *B. forficata* (IC_{50} = 90.0±4.4 μg/mL). At the lowest tested concentration (25 μg/mL), to the *C. sicyoides* the inhibition was ~ 37%, whereas *B. forficata e*xhibited an inhibition of ~15.0 %. *C. sicyoides* exhibited stronger antioxidant activity than *B. forficata* ($p<0.05$). The generation of superoxide anions was markedly inhibited by superoxide dismutase, with an IC_{50} of 3.7 μg/mL. In this assay, 0-100 μg/mL of the each plant extract had to be used to obtain the IC_{50} values. Uric acid and trolox were used as standard antioxidants and showed IC_{50} higher than 100 μg/mL.

Figure 3 shows the effects of *B. forficata* and *C. sicyoides* on guaiacol oxidation by MPO. *B. forficata* had a stronger inhibitory effect on MPO (61% inhibition at 25 μg/mL and 85% at 50 or 100 mg/mL) than did *C. sicyoides* (48% inhibition at 100 μg/mL). Quercetin, which is an effective antioxidant and inhibitor of MPO (27), exhibited an IC_{50} of 1.2±0.3 μg/mL. Although the IC_{50} of the extracts with the MPO/H_2O_2/guaiacol system was not determined, it can be estimated that, compared to quercetin, the extracts showed only weak inhibition of this enzyme *in vitro*. Further research is necessary to elucidate the different mechanisms involved in the interaction of *B. forficata* and *C. sicyoides* with MPO.

The human body possesses a series of defenses against ROS formed in vivo, but those defenses are not enough in certain conditions, such as liver cirrhosis, atherosclerosis, cancer and diabetes, in which there is a higher rate of formation of ROS (28).

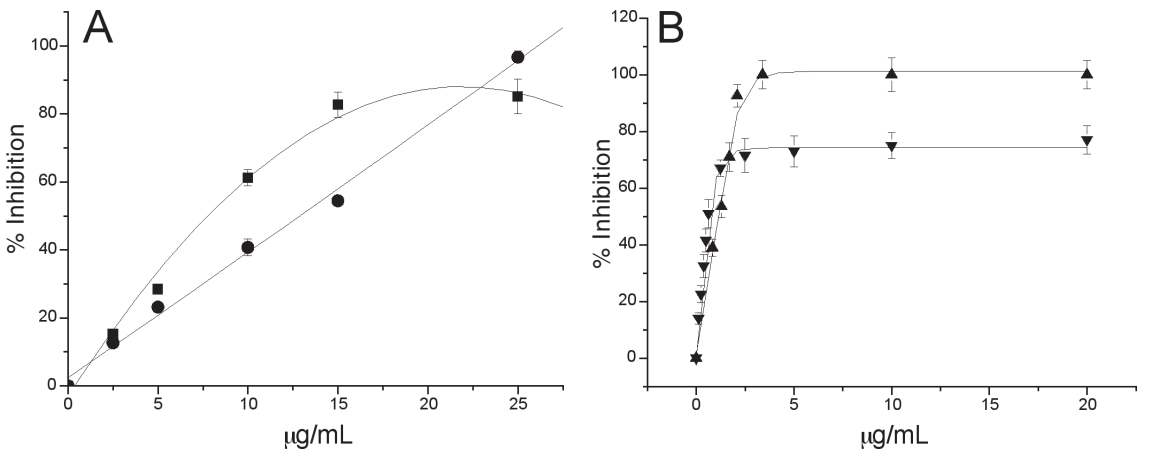

Figure 1: Reduction of the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation in (A) the presence of *Bauhinia forficata* (■) and *Cissus sicyoides* (●); (B) in the presence of uric acid (▲) and trolox (▼). Values are expressed as mean percent inhibition of ABTS in 4 experiments.

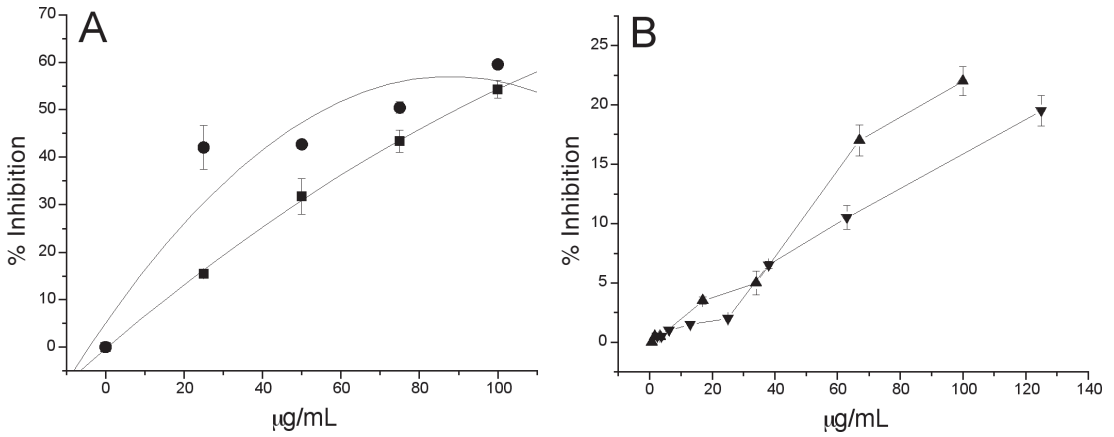

Figure 2: Effect of (A) *Bauhinia forficata* (■) and *Cissus sicyoides* (●) and (B) uric acid (▲) and trolox (▼) on reduction of NBT by superoxide anions generated by PMS and NADH. Values are expressed as mean percent inhibition of NBT reduction in 4 experiments.

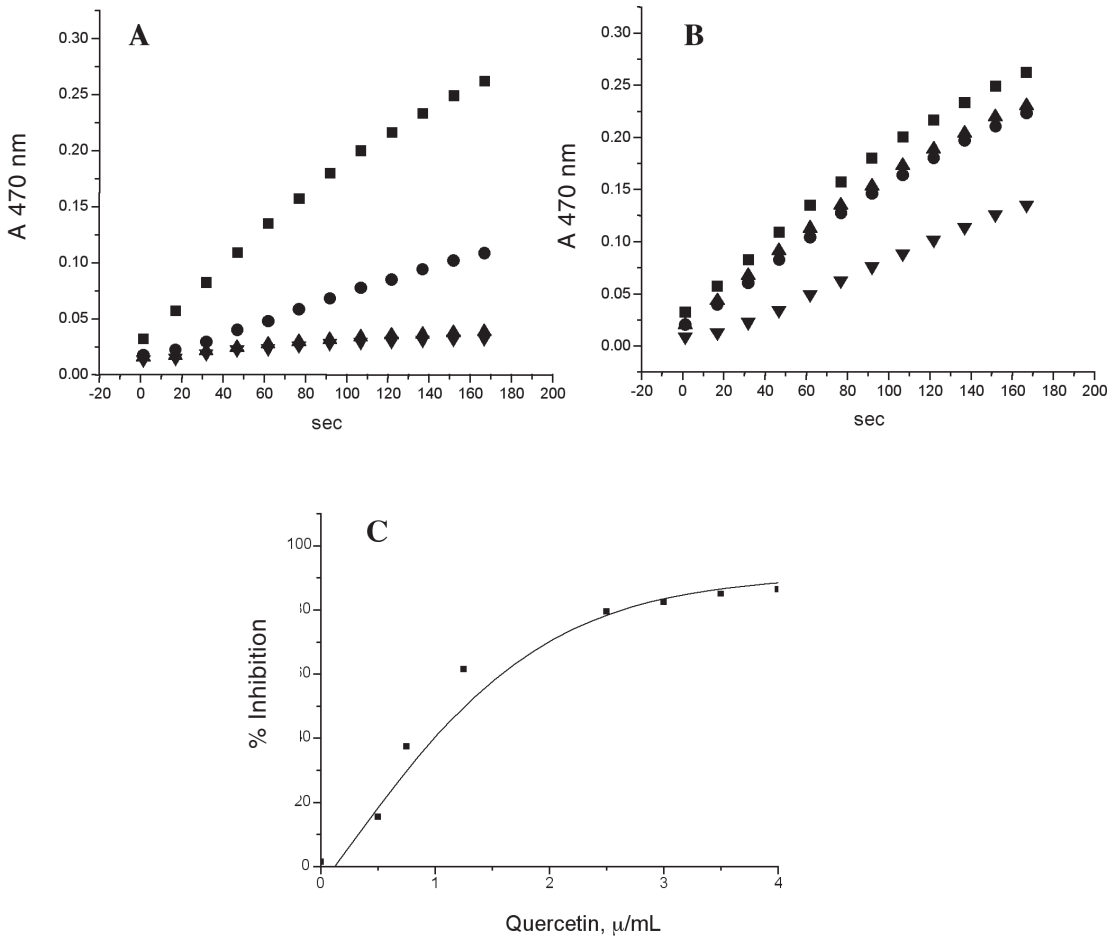

Figure 3: Time course of oxidation of guaiacol (1 mM) by MPO (2.5nM) / H_2O_2 (0.5 mM) system in 50 mM sodium phosphate buffer (pH 7.4) at 37ºC in the absence (■) or presence of *Bauhinia forficata* (A) or *Cissus sicyoides* (B) at 25 (●), 50 (▲) and 100 μg/mL (▼). C) Percent quercetin inhibition on MPO. Values show the mean from 3 experiments.

It has been found that some plant extracts used in traditional medicines for human diabetes also have a significant antioxidant activity (29). However, the mechanism of action of these drugs in reducing diabetes is not known.

A quite different question that our results may help to illuminate is the standardization of herbal medicines demanded in a recent ruling published by Anvisa, the Brazilian National Health and Sanitation Authority (RDC 48/04, dated 16-04-2004). This ruling has led pharmaceutical companies to use only standard extracts and to set up strict Quality Control in plant-based products. However, it is only rarely that the active principles of plants used medicinally are known; in the case of *B. forficata* and *C. sicyoides*, for example, the compounds acting in the treatment of diabetes are unknown. In this situation, the law permits quality control to be applied to any substance that is regularly present in a defined concentration range in the extract. So far, however, no such compound is known in either plant, which is regularly found and could serve as a molecular marker. Hence, we believe that the tests performed on these plants and the results reported here could be employed in industrial quality control.

In summary, the recognized benefits of antioxidants in the prevention of the complications of diabetes supports the use of *B. forficata* and *C. sicyoides,* tested in this study. The continuation of these studies will involve the isolation and identification of the compounds responsible for the beneficial effects on diabetes and/or antioxidant activity and in vivo tests. Meanwhile, the assays carried out here could potentially be used in the quality control of extracts of these plants, until such time as their respective active principles are discovered.

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