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Antioxidant and antiglycation properties of *Passiflora alata* and *Passiflora edulis* extracts

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Abstract

The leaves of *Passiflora alata* Dryander and *Passiflora edulis* Sims, traditionally used in American countries to treat both anxiety and nervousness by folk medicine, are rich in polyphenols, which have been reported as natural antioxidants. In this study, the antioxidant activities of *P. edulis* and *P. alata* hydroalcoholic leaf extracts were verified in in vitro and ex vivo assays. *P. alata* showed a higher total reactive antioxidant potential than did *P. edulis*. The antioxidant activities of both extracts were significantly correlated with polyphenol contents. In addition, both extracts attenuated ex vivo iron-induced cell death, quantified by lactate dehydrogenase leakage, and effectively protected against protein damage induced by iron and glucose. These findings demonstrate that the *P. alata* and *P. edulis* leaf extracts have potent in vitro and ex vivo antioxidant properties and might be considered as possible new sources of natural antioxidants.
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Keywords: *Passiflora alata*; *Passiflora edulis*; Antioxidant; Antiglycation; Polyphenols

1. Introduction

Species of the genus *Passiflora* (Passifloraceae), widely distributed throughout Latin America, are present as official drugs in pharmacopoeias of several countries (Parfitt, 1999). *Passiflora alata* Dryander (*P. alata*) is present at the Brazilian Pharmacopoeia (1977) and its leaf extract, as well as the leaf extract of *Passiflora edulis* Sims (*P. edulis*), is included as an active component in many phytopharmaceutical preparations. *Passiflora* species are very popular, not only because of their fruits (passion fruits), but also because the tea of their leaves has been largely used in American and European countries (in folk medicine) as a sedative, diuretic, tonic and also in the treatment of hypertension and skin diseases (Dhawan, Dhawan, & Sharma, 2004). The chemical composition of leaf extracts from *P. alata* and *P. edulis* has been extensively studied over the past few decades, showing a predominance of alkaloids (Lutomski & Malek, 1975), saponins (Reginatto et al., 2001; Yoshikawa, Katsuta, Mizumori, & Arihara, 2000a, Yoshikawa, Katsuta, Mizumori, & Arihara, 2000b) and mainly polyphenols (Pereira et al., 2004; Petry et al., 2001; Ulubelen, Oksuz, & Mabry, 1982).

A large number of studies have recently demonstrated that polyphenols possess antioxidant properties (Rice-Evans, Miller, & Paganga, 1996) and might play a role in the prevention of various pathophysiological processes associated with oxidative stress, such as cancer, neurodegenerative and cardiovascular diseases (Havsteen, 2002;

Abbreviations: AAPH, 2,2′-azobis (2-methylpropionamidine) dihydrochloride; AGEs, advanced glycation end products; BSA, bovine serum albumin; DNPH, 2,4-dinitrophenylhydrazine; LDH, lactate dehydrogenase; TAE, tannic acid equivalents; TBARS, thiobarbituric acid-reactive species; TEAC, trolox equivalent antioxidant capacity; TRAP, total reactive antioxidant potential.

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Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). Polyphenols may act as antioxidants by scavenging reactive oxygen and nitrogen species and chelating redox-active transition metal ions in vitro (Rice-Evans et al., 1996).

Despite the widespread use of the tea of *P. alata* and P. edulis leaves, in folk medicine, and the intense investigation of the chemical composition of the *P. alata* and *P. edulis* leaf extracts, few data on their pharmacological properties are available. To date, only the central nervous system depressant properties of *P. alata* and *P. edulis* leaf extracts have been investigated (Oga, de Freitas, Gomes da Silva, & Hanada, 1984; Petry et al., 2001). Since these extracts are enriched in polyphenols and have not been screened for antioxidant activity, the aim of the present study was to investigate the antioxidant properties of *P. alata* and *P.edulis* hydroalcoholic leaf extracts using in vitro and ex vivo models.

2. Materials and methods

2.1. Chemicals

Thiobarbituric acid, Folin–Ciocalteu reagent, dinitrophenylhidrazine (DNPH) and luminol (3-aminophthalhydrazide) were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were purchased from Aldrich Chemical (Milwaukee, WI). Glycine was purchased from Nuclear (Diadema, SP, Brazil).

2.2. Plant material

Leaves of *P. alata* Dryander were collected in Montenegro, Rio Grande do Sul, Brazil, and leaves of *P. edulis*. Sims were collected in Arroio do Sal, Rio Grande do Sul, Brazil. Voucher specimens are on deposit at the Herbarium of Institute de Ciências Biológicas, Universidade de Passo Fundo, Passo Fundo, Brazil (RSPF 7232) and Departamento de Botânica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil (ICN 114356), respectively.

2.3. Extracts

P. alata and *P. edulis* leaves were air-dried at 40 °C for 7 days. Ten grams of dry and powdered leaves were extracted, separately, using 100 ml of 40% ethanol (plant: solvent, 1:10, w/v) under reflux (80 °C) during 30 min. After cooling, the extracts were filtered and evaporated under reduced pressure, yielding a dry residue.

2.4. Determination of total phenolics

Total phenolic content of the extracts was determined using the Folin–Ciocalteau method (Waterman & Mole, 1994), employing tannic acid as standard. Briefly, a 100 μl aliquot of extracts was assayed with 100 μl of Folin reagent and 200 μl of sodium carbonate (35%, w/v). The mixture was vortexed and diluted with distilled water to a final volume of 2 ml. The absorbance was read at 725 nm and the total phenolic content was expressed as tannic acid equivalents (TAE μg/mg extract).

2.5. Total reactive antioxidant potential

The in vitro antioxidant activity of the *P. edulis* and *P. alata* leaf extracts was estimated by the total reactive antioxidant potential (TRAP) assay, as previously described (Polydoro et al., 2004). Briefly, the reaction mixture (4 ml), containing the free radical source (10 mM AAPH) in glycine buffer (0.1 M), pH 8.6, and luminol (4 mM) as an external probe for monitoring radical production, was incubated at 25 °C. The addition of 10 μl of trolox (200 nM) as the standard antioxidant, or different concentrations of the extracts (final concentrations of 0.1, 1 and 10 μg/ml) decreases the chemiluminescence proportionally to its antioxidant potential and measured in an out-of-coincident mode in a liquid scintillation counter (Wallac 1409) as counts per minute (CPM). The chemiluminescence emission was monitored for 80 min after the addition of the *Passiflora* leaf extracts or trolox. The antioxidant capacity of 1 μg of these extracts is expressed as trolox equivalent antioxidant capacity (TEAC, mM).

2.6. Ex vivo assay

The antioxidant activity of the *Passiflora* leaf extracts was also evaluated in an ex vivo assay using $FeSO_4$ as oxidative stress inducer. Rat liver slices (300 μm) were incubated for 90 min at 37 °C under 95% O_2/5% CO_2 in a shaking water bath (60 oscillations/min) in a medium of oxygen-equilibrated Krebs-Ringer phosphate buffer – 10 mM glucose, pH 7.4. Immediately prior to incubation, 0.1 mM $FeSO_4$, *P. alata* or *P. edulis* leaf extract alone (1 μg/ml), 0.1 mM $FeSO_4$ plus *P. alata* or *P. edulis* leaf extract (1 μg/ml), or water was added to different liver slice samples. Ferrous sulphate, *P. alata* and *P. edulis* leaf extracts were dissolved in distilled water. After incubation, the rat liver slices were removed and the medium was centrifuged at 12,000*g* for 10 min. The supernatant portion was used to measure lactate dehydrogenase activity using a commercial kit (LDH Liquiform^{TM}, Brazil). For lipid peroxidation and carbonyl protein assays, the rat liver slices were homogenized with phosphate buffer, pH 7.0 and kept at −75 °C prior to analysis.

2.7. Protein carbonyl determination

Protein carbonyl determination was assayed as previously described (Levine et al., 1990). First, 600 μl of the liver slices homogenates were centrifuged at 7000*g* for 15 min and 200 μl of supernatant were mixed with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl. The mixture was incubated at room temperature for 1 h,

followed by the addition of 100 μl 20% trichloroacetic acid and centrifugation at 3000*g* for 3 min. The protein pellets were washed three times with 500 μl ethanol:ethyl acetate (1:1, v/v) and dissolved in 1 ml 6 M guanidine (pH 2.3). The absorbance was read at 370 nm to quantify protein carbonyls and the data are expressed as nmol of carbonyls/mg protein.

2.8. Lipid peroxidation

Thiobarbituric acid-reactive species (TBARS) formation was used to evaluate lipid peroxidation (Draper & Hadley, 1990). First, 600 μl 10% trichloroacetic acid were added to 300 μl of the liver slices homogenates and centrifuged at 7000*g* for 10 min. Then, 400 μl supernatant were mixed with 400 μl 0.67% thiobarbituric acid. The reaction mixture was incubated in a boiling water bath for 30 min, cooled to room temperature and the absorbance read at 532 nm. The data are expressed as MDA equivalents (nmol/mg protein).

2.9. Protein determination

Protein concentration in the liver slices homogenates was measured by the Lowry method, employing bovine serum albumin as standard (Lowry, Rosebrough, Farr, & Randall, 1951).

2.10. Non-enzymatic glycation of protein

According to the method previously described (Vinson & Howard, 1996), bovine serum albumin (BSA, 10 mg/ml) in phosphate buffer (50 mM, pH 7.4) containing 0.02% (w/v) sodium azide was preincubated with the *Passiflora* extracts at final concentrations of 1, 5 and 10 μg/ml for 30 min at room temperature (25 °C). Glucose (25 mM) and fructose (25 mM) solutions were added to the reaction mixture. After incubating at 37 °C for 3 days, the fluorescent reaction products were assayed in a fluorescence spectrophotometer with an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Results were expressed as percentage inhibition of formation of the glycated protein.

2.11. Statistical analysis

Data were expressed as means ± standard deviation (SD) of triplicates from three independent experiments. Differences between treatments were compared by one-way ANOVA, followed by Tukey's test for approximately normally distributed variables or non-parametric Kruskal–Wallis ANOVA, followed by Dunn's procedure for variables with skewed distribution. Pearson's correlation coefficient was used to test correlation between polyphenol content and TEAC. Data analyses were performed using the SPSS 8.0 software package (SPSS Inc., Chicago, IL) and the statistical significance was set at the 0.05 level (two-tailed).

3. Results

3.1. Total phenol content (TAE), antioxidant capacity (TEAC) and correlation between TEAC and TAE

The total phenolic content determination showed that *P. alata* leaf extract possessed higher phenolic content than did *P. edulis* leaf extract (Table 1).

When three different concentrations of each extract (0.1, 1 and 10 μg/ml) were used in the TRAP assay to assess the antioxidant activity, we verified that both extracts presented significant antioxidant capacity in vitro only at final concentrations of 1 and 10 μg/ml. Furthermore, the antioxidant capacity (expressed as TEAC) was proportional to the amount of extract added and gave a linear response. As also shown in Table 1, the *P. alata* leaf extract showed higher antioxidant activity than did the *P. edulis* leaf extract. As depicted in Fig. 1, TEAC values of the extracts and their total phenolic contents were well correlated for both *P. alata* ($r = 0.997$, $p < 0.01$) and *P. edulis* ($r = 0.996$, $p < 0.01$) leaf extracts, suggesting that the phenolic compounds make a major contribution to the antioxidant capacity of the extracts. Nevertheless, it is worth mentioning that the Folin–Ciocalteu phenol reagent gives only an approximate estimate of the total phenolic content.

3.2. Ex vivo assay

In order to evaluate the ex vivo antioxidant effects of the *P. alata* and *P. edulis* extracts at the lowest concentration found as significant in the TRAP assay (1 μg/ml), oxidative stress was induced in rat liver slices using iron. Whereas the addition of an oxidative stress inducer (0.1 mM $FeSO_4$) in the incubation medium substantially increased cell death, the co-incubation with *P. alata* or *P. edulis* extracts prevented the iron-induced increase on cell death, evidenced by significantly decreased LDH leakage when compared to control slices (Fig. 2).

To better characterize the protective effects of the *P. alata* and *P. edulis* extracts against iron-induced oxidative damage, the levels of protein carbonyls and TBARS in the rat liver slices were determined. While both *Passiflora* extracts failed to show statistically significant influence on TBARS levels (Fig. 3), the levels of protein carbonyls were significantly lower in the slices co-incubated with *P. alata* or *P. edulis* extracts than in the control slices (Fig. 4).

Table 1
Total phenolic content and antioxidant capacity of *Passiflora* extracts

	Extract	
	P. alata	*P. edulis*
TAE (μg/mg extract)	171 ± 1.6	92.5 ± 2.2
TEAC	0.52 ± 0.012	0.23 ± 0.02

The results are expressed as means ± SD of triplicates from three independent experiments.

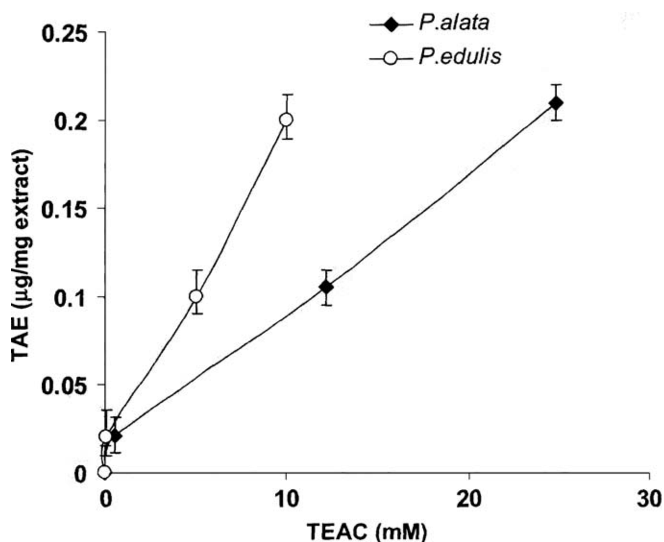

Fig. 1. Correlation of TEAC values with total phenolic content (TAE) of the *P. alata* and *P. edulis* leaf extracts. Data are representative of at least three independent experiments performed in triplicate ± SD.

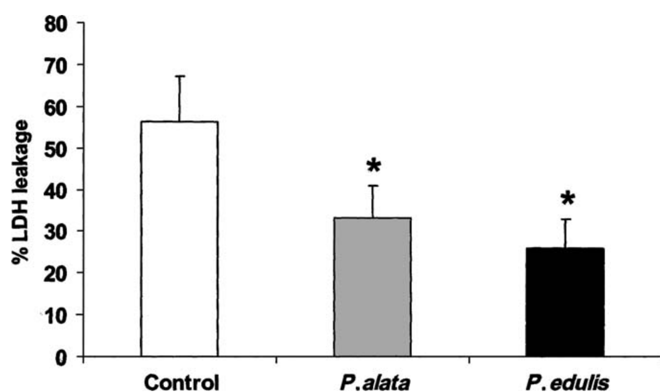

Fig. 2. The effect of the *P. alata* and *P. edulis* leaf extracts on LDH leakage. Results are expressed as average percentage leakage of the enzyme into the incubation medium compared to a homogenized fresh rat liver slice ± SD of triplicates from three independent experiments. $^{*}p < 0.05$ compared to control.

3.3. Non-enzymatic glycation of protein

We examined the protective effect of the *Passiflora* extracts on advanced glycation end-products (AGEs) formation (Fig. 5). The *P. alata* extract showed a significant inhibition of AGEs formation at concentrations of 5 and 10 μg/ml. In contrast, the *P. edulis* extract showed significant inhibition of AGEs formation only at a concentration of 10 μg/ml.

4. Discussion

Many studies carried out over the past few years have shown that polyphenols found in dietary and medicinal plants inhibit oxidative stress (Manach et al., 2004; Rice-Evans et al., 1996). The *P. alata* and *P. edulis* leaf extracts are rich in polyphenols, especially C-glycosyl derivatives of

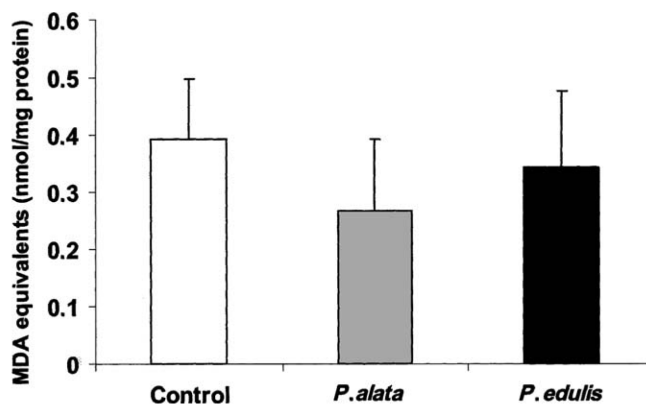

Fig. 3. The effect of the *P. alata* and *P. edulis* leaf extracts on TBARS levels. Results are expressed as means ± SD of triplicates from three independent experiments.

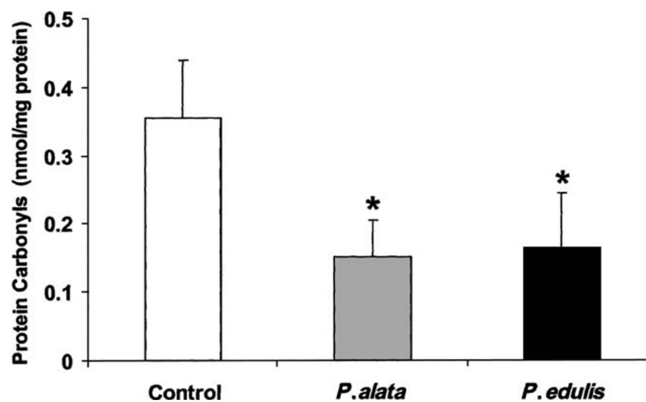

Fig. 4. The effect of the *P. alata* and *P. edulis* leaf extracts on protein carbonyls content. Results are expressed as means ± SD of triplicates from three independent experiments. $^{*}p < 0.05$ compared to control.

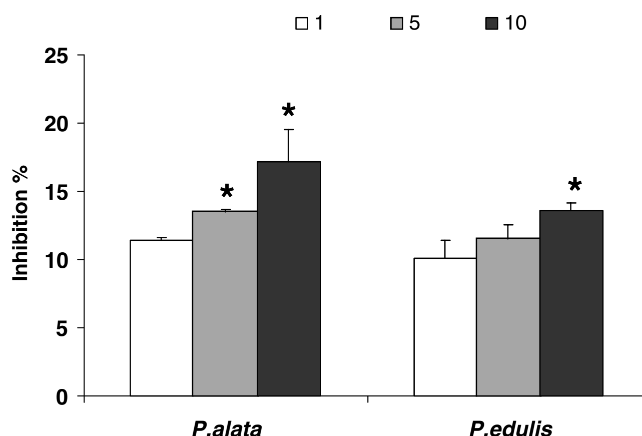

Fig. 5. Inhibition of protein glycation (AGE formation) by *Passiflora* extracts. Results are expressed as average percentage of inhibition of AGE formation ± SD of from three independent experiments performed in quintuplicate. $^{*}p < 0.05$ compared to control.

apigenin and luteolin, such as vitexin, isovitexin, orientin and isoorientin (Pereira et al., 2004; Petry et al., 2001; Ulubelen et al., 1982). In this study we showed, for the first

time, both antioxidant and antiglycation properties of *P. alata* and *P. edulis* hydroalcoholic leaf extracts. As in other studies evaluating antioxidant activity of leaf extracts from medicinal plants (Zainol, bd-Hamid, Yusof, & Muse, 2003) and fruits (Banerjee, Dasgupta, & De, 2005), we found a direct linear relationship between the total phenolic content and total antioxidant activity in both the *P. alata* and *P. edulis* leaf extracts, indicating that the phenolic compounds might be the major contributors to the antioxidant activities of these extracts.

Iron is a well-described initiator of free radical oxidations, stimulating lipid peroxidation and inhibiting the function of various membrane proteins in vitro (Qian & Buettner, 1999). In our model, the inclusion of iron in the incubation medium of rat liver slices substantially increased cell death, generation of TBARS and protein carbonyl content. However, the co-incubation with *P. alata* or *P. edulis* leaf extracts at concentration of 1 μg/ml provided significant antioxidant protection to the rat liver slices, as evidenced by decreased LDH leakage. In addition, while several studies investigating the antioxidant activity of extracts rich in polyphenols have focussed on protective effects against lipid peroxidation (Polydoro et al., 2004; Zainol et al., 2003), our findings demonstrate that both *P. alata* and *P. edulis* leaf extracts have significant protective effects against carbonyl protein formation. This is of particular importance, since oxidized proteins are often functionally inactive and oxidative stress may affect the activity of enzymes, receptors, and membrane transporters (Stadtman, 2001). Moreover, oxidized proteins are suggested to play a toxic role in the pathogenesis of several diseases, especially in neurodegenerative diseases (Dean, Fu, Stacker, & Davies, 1997).

Proteins are also modified by glucose through the glycation reaction, resulting in the formation of AGEs (Ulrich & Cerami, 2001). The contribution of AGEs to diabetes, aging and Alzheimer's disease has received considerable attention in recent years (Chace, Carubelli, & Nordquist, 1991; Jakus & Rietbrock, 2004), and free radicals have been shown to participate in AGEs formation (Halliwell, 2001). It has been reported that antioxidants and radical scavengers inhibit these processes (Nakagawa, Yokozawa, Terasawa, Shu, & Juneja, 2002). In this study, the *P. alata* and *P. edulis* leaf extracts showed protective effects against glucose-induced protein modifications, significantly inhibiting the AGEs formation. However, there is evidence demonstrating that antioxidant activity might not be the unique mechanism required to protect against early stage glycation for all reactants (Vasan et al., 1996). Thus, the mechanism of inhibition of AGEs formation by *Passiflora* extracts requires further investigation.

In conclusion, the data presented here indicate that the *P. alata* and *P. edulis* hydroalcoholic leaf extracts possess in vitro and ex vivo antioxidant activity against oxidative protein damage and should be considered as new sources of natural antioxidants. Further studies are needed to examine the potential use of these extracts in the prevention of pathologies, such as diabetes mellitus and neurodegenerative diseases, where oxidative stress damage to protein seems to play a major role.

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