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Antioxidant effects of crude extracts from *Baccharis* species: inhibition of myeloperoxidase activity, protection against lipid peroxidation, and action as oxidative species scavenger

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Abstract: The objective of this study was to show a comparison of the antioxidant properties of aqueous and ethanolic extracts obtained from *Baccharis articulata* (Lam.) Pers., *Baccharis trimera* (Less.) DC., *Baccharis spicata* (Lam.) Baill. and *Baccharis usterii* Heering, Asteraceae, by several techniques covering a range of oxidant species and of biotargets. We have investigated the ability of the plant extracts to scavenge DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radical, action against lipid peroxidation of membranes including rat liver microsomes and soy bean phosphatidylcholine liposomes by ascorbyl radical and peroxynitrite. Hydroxyl radical scavenger activity was measured monitoring the deoxyribose oxidation. The hypochlorous acid scavenger activity was also evaluated by the prevention of protein carbonylation and finally the myeloperoxidase (MPO) activity inhibition. The results obtained suggest that the *Baccharis* extracts studied present a significant antioxidant activity scavenging free radicals and protecting biomolecules from the oxidation. We can suggest that the supposed therapeutic efficacy of this plant could be due, in part, to these properties.

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Introduction

Myeloperoxidase (MPO) figures prominently in the antimicrobial action of neutrophils, the dominant cell effectors of the innate host defense response and also in inflammatory tissue damages. This enzyme found in the azurophilic granules, converts hydrogen peroxide and oxygen chloride to hypochlorous acid, a powerful oxidant that reacts readily with many important biological molecules (Barbior, 2000) which contributes to both microbial killing, and subsequent oxidative injury of host tissue triggering severe inflammatory disorders (Fernandes et al., 2008).

Clinicians and biomedical scientists are interested in antioxidants because they could retard the oxidative damage of a tissue by increasing natural defenses. There is an increasing interest in the antioxidant effects of compounds derived from herbs which could be relevant in relation to their nutritional incidence and their role in health and disease (Sarkar & Bhaduri, 2001).

The *Baccharis* genus, Asteraceae, is native of South Brazil, Paraguay, Uruguay and Argentine, commonly known as "carqueja". The infusions of its aerial parts are used in the popular medicine as anti-inflammatory, diuretic, and digestive (Zardini et al., 1984). Phytochemical studies have been reviewed and reported the identification of flavonoids, phenolic acids and diterpenes as major constituents of *Baccharis* species (Verdi et al., 2005).

Phenolic compounds as phenylpropanoids and flavonoids posses a variety of biological properties *in vitro* and *in vivo*. These biological effects are attributed mainly to the property of protection against lipid peroxidation by free radicals scavenging or chelating metal ions responsible for the generation of reactive species, which are capable of damaging a wide range of essential biomolecules (Madsen et al., 2000).

Several diseases including rheumatoid arthritis,

inflammatory bowel disease, cystic fibrosis, and gastrointestinal disorders seem to be induced by oxidative stress. This fact suggests that the utilization of *Baccharis* species in the popular medicine could be associated with their antioxidant properties (Oliveira et al., 2003; Oliveira et al., 2004; Simões-Pires et al., 2005).

In this work we analyzed for the first time an inhibitory effect against MPO activity and scavenger activity against hypochlorous acid of crude extracts of four Baccharis species. The scavenger properties against hydroxyl and DPPH radicals, inhibition of lipid peroxidation induced by ascorbyl radical and by peroxynitrite in two models of lipid membranes, liposomes, microsomes were also evaluated.

Materials and Methods

Reagents

Hexadecyl trimethyl-ammonium bromide (HTMAB), ɵ-dionisine, 2-deoxy-D-ribose, 1,1-diphenyl-2-picryl-hydrazil (DPPH), thiobarbituric acid (TBA), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT), bovine serum albumin (BSA), 2,4-dinitrophenylhydrazine (DNPH), 5,5´-dithio-bis(2-nitrobenzoic acid) (DTNB), cholic acid, deoxycholic acid were purchased from Sigma® Chemical Company (EUA). Soy bean phosphatidylcholine from Fluka® (Germany). All others reagents were of analytical grade.

Plant material

Aerial parts of *Baccharis articulata, B. trimera, B. spicata*, and *B. usterii*, Asteraceae, were collected in Porto Alegre, State of Rio Grande do Sul, Brazil. The plants were botanically identified by the doctor Sérgio Bordignon (Unilasalle) and were deposited at the Herbarium of the Botany Department of Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. Each plant material was air dried and powdered separately.

Extraction

Plant material (1 g,) was macerated in ethanol (plant-solvent, 1:10, w/v) (2 x 10 days). The crude ethanol extract was obtained after filtration and evaporation of the ethanol under vacuum. The aqueous extract was obtained by decoction of the plant material (1 g, 2 x 100 mL) (Oliveira et al., 2003).

Myeloperoxidase activity

Rat lungs were homogenized in an ice-cold 50 mM phosphate buffer at pH 6.0, containing 0.5% (HTMAB) as previously described and freeze-thawed three times (Rao et al., 1994). The samples were centrifuged at 12000 x *g* at 4 ºC for 20 min. The supernatant was assayed in a reaction medium containing 50 mM phosphate buffer, pH 6.0 at 25 ºC, 1-dianisidine (0.167 mg/mL) and H_2O_2 (0.0006%). The enzyme activity was determined by the slope of the absorption curve set at 450 nm, following the changes in absorption for the first 30 s in each sample concentration of the extracts. The major peroxidase activity in the supernatant is mainly of the MPO, although other peroxidases such eosinophil peroxidase may be present (Rao et al., 1993; 1994; Teixeira et al., 2003). A standard curve of myeloperoxidase activity was obtained previously with a commercial enzyme batch. Sodium azide at 50 µM was used to inhibit the myeloperoxidase as a control of the enzymatic activity. Considering 100% of MPO activity 4.0±1.2 U/min/mg.

DPPH radical scavenging activity

Radical scavenging activity of the extracts was measured by slightly modified method (Vivot et al., 2001). The assay is based on the incubation of reaction medium for 30 min at 37 ºC in an ethanolic solution of 150 µM DPPH and the optical density is measured afterwards at 515 nm. The antioxidant activity of the plants extracts was expressed as IC50, which was defined as the concentration of extract required to reduce 50% DPPH free radicals.

Liposomes preparation

Bilayer liposomes were prepared by cholate dialysis as described previously (Sone et al., 1977; Creczynski-Pasa & Gräber, 1994). Briefly, the method consists of the solubilization of the phospholipids at 50 mg/mL in a buffer containing 10 mM tricine, 20 g/L cholic acid, 10 g/L deoxicholic acid at pH 8.0 followed by a dialysis procedure at 30 ºC for 5 h.

Microsomes preparation

Microsomes were prepared from the livers of Wistar rats weighing 200-250 g by differential centrifugation with calcium aggregation (Schenkmann & Cinti, 1978). The fractions obtained were stored in a freezer at -84 ºC. The protein concentration was determined according to Lowry's method.

Production and detection of hydroxyl radical

Hydroxyl radical was produced by a variation of Fenton reaction, through the mixture of hydrogen peroxide with $FeCl_3$-NTA system. The reaction medium contained 25 µM $FeCl_3$, 100 µM nitrolotriacetic acid (NTA), 100

mM phosphate buffer pH 7.4, 2.8 mM deoxyribose and 1.4 µM hydrogen peroxide was incubated at 37 ˚C for 20 min in different concentrations of extracts. After that 2.8% TCA and 1 % TBA were added to the reaction and heated to 100 ˚C for 15 min followed by ice bath immersion. Products of deoxyribose oxidation were determined spectrophotometrically at 532 nm. For deoxyribose oxidation studies, the extracts were dissolved in 0.05 M NaOH and the pH was adjusted to 7.4 with 0.1 M HCl. Organic solvents were not used for preparing solutions since they interfere with hydroxyl radical determination (Gutteridge & Halliwell, 1988).

Lipid peroxidation induced by ascorbyl radical and peroxynitrite

Lipid peroxidation was induced by the addition of 25 µM $FeSO_4$ and 500 µM ascorbate, for ascorbyl radical or 2.7 mM peroxynitrite in a reaction medium containing 2 mg microsomal protein/mL or liposomes (lipids at 12.5 mg/mL), and 0.1 M Tris-HCl, pH 7.4. The samples were incubated for 30 min at 37 °C. Next, 4% TCA and 0.3 % TBA were added to the reaction medium. The samples were then heated to 100 ˚C for 15 min in and centrifuged at 5000 *g*. The extent of lipid peroxidation was determined by the thiobarbituric acid (TBA) method (Bird & Draper, 1984). The amount of TBARS (thiobarbituric acid reactive substances) was calculated using an extinction coefficient of 1.56 x 105 $M^{-1}cm^{-1}$. In all cases, a blank run with the same amount of the organic solvent only, to consider its interference in the assays. Lipid peroxidation inhibitory activity was expressed as IC50. Results were expressed as percentage of lipid peroxidation. Considering 100% of microsomes peroxidation induced by ascorbyl radical and peroxynitrite 23.9±1.0 µmol TBA/mg of protein and 15.4±1.6 µmol TBA/mg of protein, respectively.

Protein carbonyl assay

BSA (1 mg/mL) was used as a protein to be oxidized by 200 µM of hypochlorous acid in 10 mM phosphate buffer, pH 7.4 at 37 ºC for 30 min. After, 10 mM DNPH in 2.5 M HCl was added and the mixture was incubated at room temperature for 1 h followed by addition of 30% TCA. Protein pellets were washed three times with ethanol/ethyl acetate (1:1, v / v) and dissolved in 10 mM phosphate buffer (pH 6.8). Carbonyl content was determined from the absorbance at 360 nm using a molar absorption coefficient of 22.000 M^{-1} cm^{-1} (Yan et al., 1996). Protein carbonyl groups formation inhibitory activity was expressed as IC50. Considering 100% of carbonyl groups 6.4±0.7 nmol/mg of protein.

Statistical analysis

The results were presented as mean±SEM of triplicates from three independent experiments. When necessary a t-test or ANOVA followed by Dunnet's analysis were applied.

Results and Discussion

In this work, eight extracts from *Baccharis* species were studied for their activity as inhibitors of MPO, scavengers of reactive species in vitro and inhibitors of lipid peroxidation by using different systems.

Myeloperoxidase activity

Previous studies have demonstrated that some anti-inflammatory drugs are able to inhibit MPO activity and this inhibition may account for their anti-inflammatory effect (Ramos et al., 1995). The Table 1 shows the effects of plant the extracts on the peroxidative activity of MPO. Each extract reduced the level of MPO activity in a concentration-dependent manner. In this condition, the ethanolic extracts of *B. articulata* and *B. spicata* showed stronger inhibition than the aqueous extracts. In the case of the *B. trimera* the aqueous extract was more effective inhibiting MPO activity, and the extract of *B. usterii* was the most effective, although both extracts showed statistically similar inhibitory potential. The Figure 1 shows the inhibition of myeloperoxidase in the presence of aqueous extract of *B. usterii* at a concentration range of 10 to 300 µg/mL, reaching a $K_{0.5}$ of 66±4 µg/mL. This procedure was performed for the analysis of all extracts.

Phenolic acids derivatives as quercetin, curcumin, ferulic, caffeic and gallic demonstrated a strong MPO inhibition (Kato et al., 2003). The activity observed herein could be related to the presence of these compounds in the extracts, since the TLC profile of our extracts showed the caffeoyl derivatives as the major compounds, such as (4'-*O*-β-D-glucopyranosyl-3',5'-dimethoxybenzyl-caffeate) and caffeoylquinic derivatives (Oliveira et al., 2003; Simões-Pires et al., 2005).

DPPH radical scavenger assay

DPPH assay evaluates the ability of antioxidants to scavenge free radicals. DPPH is a free radical, stable at room temperature, which presents violet-color. It is reduced in the presence of an antioxidant molecule, the absorption decreases and the resulting decoloration is stoichiometrically related to the number of electron captured. Recent studies demonstrated that the interaction of a potential antioxidant with DPPH depends on configuration and conformation of chemical compounds. The number of DPPH molecules that are reduced seems to be correlated with the number of available hydroxyl groups (Brand-Williams et al., 1995). The screening

Table 1. *Bacharis* sp. extracts as antioxidants and as MPO inhibitors.

Plant extracts	DPPH IC50 (μg/mL)	Inhibition of lipoperoxidation induced by ascorbyl radical (IC50 μg/mL)		Inhibition of lipoperoxidation induced by peroxynitrite (IC50 μg/mL)		Inhibition of deoxyribose oxidation (IC50 μg/mL)	Inhibition of protein carbonylation (IC50 μg/mL)	MPO inhibition $K_{0.5}$ (μg/mL)
		liposomes	microsomes	liposomes	microsomes			
B. articulata								
aqueous	26±2	118±1#	95±2#	45±3#	87±2#	6±1	237±4#	148±2#
ethanolic	50±5*,#	60±4***,#	130±5**,#	36±2	73±4*,#	17±1**,#	205±3**,#	126±2**,#
B. trimera								
aqueous	30±1	85±3#	59±4#	23±3	85±3#	9±1	213±4#	136±2#
ethanolic	29±2	122±5**,#	81±8#	31±4	93±3#	15±3#	245±4**,#	178±5**,#
B. spicata								
aqueous	36±4#	61±5#	32±3	47±4#	89±5#	12±2	184±2#	165±3#
ethanolic	78±5**,#	70±2#	75±5**,#	51±4#	66±4*,#	18±2#	153±3**,#	123±2***,#
B. usterii								
aqueous	18±3	24±3	31±4	25±4	44±2	7±1	119±3	71±3
ethanolic	27±3	18±2	45±4	32±2	56±2*	12±1*	148±3**,#	66±4

*Indicates the differences among the aqueous and ethanolic extracts of each fraction, after analysis by t-test ; *p<0.05; **p<0.01 and ***p<0.001; #Indicates the differences in relation to the lower value of IC50 of each column, after analysis of ANOVA followed by Dunnett's test; #p<0.05

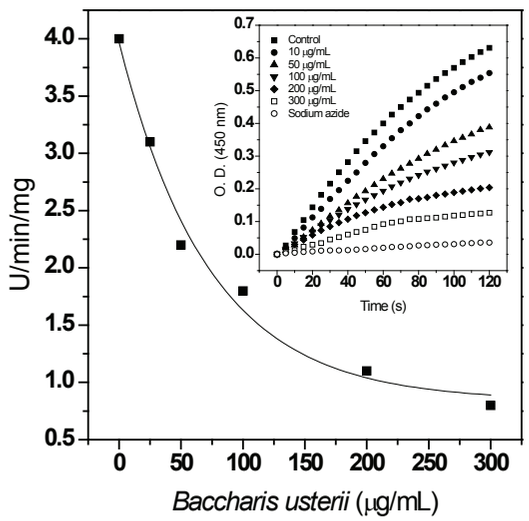

Figure 1. Inhibition of myeloperoxidase by the aqueous extract of *Baccharis usterii*. The results with the others extracts are summarized in the Table 1. The values of the enzyme activity were calculated from the curves showed in the figure inset. The enzyme activity was measured spectrophotometrically following the oxidation of ɵ-dianisidine by HOCl produced by the enzyme. More details are described in materials and methods. 100% of enzyme activity is 4.0±1.0 U/min/mg. This is the most representative of three experiments.

of the *Baccharis* extracts using the DPPH free radical method showed to be effective for the selection of those which could have an antioxidant activity. Hydrogen-donating ability is an index of the primary antioxidants (Mensor et al., 2001). These extracts may be rich in radical scavengers, such as flavonoids, phenolic acids derivatives known as antioxidants. The DPPH radical scavenging activities of the investigated extracts from Baccharis species are summarized in Table 1. Significant DPPH radical scavenger activity was evident for all extracts tested. The extracts of *Baccharis usterii* showed the highest DPPH radical scavenging activity. Only *B. spicata* showed values for IC50 statistically significant higher for ethanolic extract. Although, the thin layer chromatography profile of both extracts was found to be similar (Oliveira et al., 2006). Quercetin, used as a positive control showed very low IC50 for DPPH radical (0.02 μg/mL), comparing with the plant extracts. However, this result must be seen carefully because we are comparing an isolated compound with samples with a mixture of them. It is expected that the electron transfer between the isolated compounds and DPPH occurs easily.

Lipid peroxidation

In biological systems, lipid peroxidation generates a number of degradation products, such as malondialdehyde, and is found to be an important cause of cell membrane destruction and cell damage (Dotan et al., 2004). We assessed all the plants extracts to inhibit lipid peroxidation induced by ascorbyl radical and peroxynitrite in rat liver microsomes and Soy bean PC liposomes. Microsomes were used as lipid source because of their high concentration in polyunsaturated fatty acids, in which the major element is endoplasmic reticulum membrane containing phosphatidylcholine, cholesterol, sphigomyelin, phosphatidylethanolamine and phosphatidylinositol. Soy bean lipossomes is a lipid mixture of phosphatidylethanolamine, phosphatidylinositol and phosphadidylcholine (Leikin et

al., 1988; Gourley et al., 1983). The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities.

Table 1 shows the results of lipid peroxidation in liver microsomes and soy bean liposomes by ascorbyl radical and peroxynitrite. All extracts protected against the action of these reactive species on two kinds of lipid membrane preparation. The overall lipid peroxidation inhibitory activity of the samples used in this study revealed similar activity compared to DPPH radical scavenging. The aqueous extracts showed to be more efficient than ethanolic extracts concerning microsomes lipid peroxidation induced by ascorbyl radical. However, were observed significant differences only between the actions of the *B. articulata* and *B. trimera* extracts in liposomes protection against damages induced by ascorbyl radical, comparing aqueous and ethanolic extracts. Aqueous extract of *Baccharis usterii* showed a tendency to be more potent again protecting microsomes and liposomes against peroxidation, being the IC50 for TBARS of 31±4 µg/mL and 24±3 µg/mL, respectively although not statistically significant (Table 1). This result is consistent with those described by Oliveira et al (2004) that observed the protective effect of these extracts on lipid peroxidation induced by hydrogen peroxide possibly by the presence of the phenolic compounds predominant in these extracts able to transfer e-to peroxyl radical. (Wu et al., 2004).

Hydroxyl radical scavenger

The extracts were also found to be potent scavengers of hydroxyl radical, one of the most aggressive oxidants formed from Haber-Weiss and Fenton reactions. In this regard, the plant extracts were effective in avoiding the oxidation of deoxyribose in very low concentration, see in the Table 1. In a general way all aqueous extracts showed to be stronger than the ethanolic extracts in this task. The Figure 2 shows the effect of aqueous extract of *B. usterii*. The concentration dependence of deoxyribose on its oxidative degradation by Fenton reagents with or without 0.2 mg/mL extracts was investigated. To verify this effect, the concentration of deoxyribose in the reaction medium was gradually increased and the effect of the extracts in preventing the deoxyribose oxidation by •OH decreased in a dose dependent way. This result indicates that the extracts and deoxyribose are competing by •OH trapping.

Although we did not address the site-specificity of the extracts, is whether the plant extracts only scavenges •OH or if it also acts as an Fe^{2+} chelator (Bird & Draper, 1984) we observed a competition between the adductor (deoxyribose) and the extracts, suggesting an action as scavenger. However, the confirmation of this hypothesis requires further investigations.

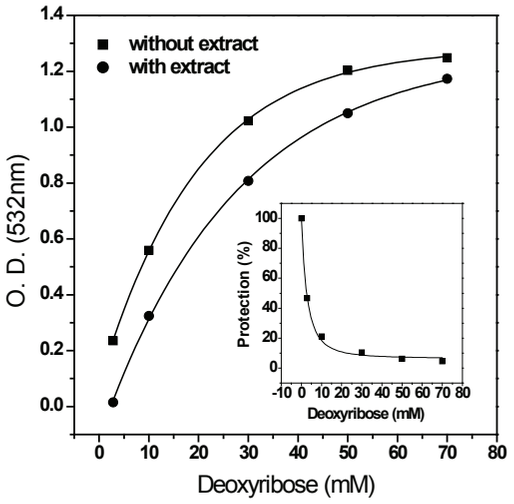

Figure 2. Inhibition of deoxyribose oxidation by hydroxyl radical in by the aqueous extract of *Baccharis usterii*. The hydroxyl radical was generated by Fe(III)-NTA and H_2O_2 and monitored by the deoxyribose method. Inset: decrease of protection against deoxyribose degradation by the increase of deoxyribose concentration. Replot of the data obtained directly in the presence of the extract. The results are the means±SEM of triplicate determinations from three independent experiments. The results with the others extracts are summarized in Table 1.

Protein carbonyl assay

During hypochlorite oxidation, amino acids residues of proteins are directly modified. Higher doses of hypochlorite (>50 µM) have been reported to lead to oxidation of thiol groups, such as methionine and tryptophan residues, and formation of carbonyl protein (Schraufstatter et al., 1990). Using the protein carbonyl assay, we evaluated the ability of the extracts to scavenge HOCl. 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, including neurodegenerative and inflammatory process (Dean et al., 1997). In the present study, we demonstrate at first time an inhibition of carbonyl protein formation by *Baccharis* species extracts. The Table 1 shows the comparison of the potency of *Baccharis* extracts in inhibiting HOCl-induced BSA carbonyl formation. The higher inhibition potential of carbonyl protein formation was observed for both extracts of *B. usterii* in comparison with the extracts of other *Baccharis* species. These results suggest that the ability of these extracts to scavenge HOCl could be associated with the higher MPO inhibition potential.

In summary, our results further support the view that extracts of *Baccharis* are promising sources

of potential antioxidants They have the combination of requisites to be good antioxidants in hydrophilic and in hydrophobic phases which are the facility to donate e-, and the capability to protect different lipid membranes. Possibly this property is responsible for the part of the benefits that this medicinal plant continue to play in the traditional medicine of many modern cultures. Further studies are needed to examine the potential use of these plant materials, taking the advantage of the possible synergism between the molecules present in the extracts covering the protection against most dangerous oxidative species. These properties may facilitate the prevention of pathologies induced by oxidative stress, including inflammatory disorders, several disease of the gastrointestinal tract, and neurodegenerative diseases.

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