Antioxidant and free radical scavenging effects in extracts of the medicinal herb *Achyrocline satureioides* (Lam.) DC. ("marcela")

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Abstract

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Achyrocline satureioides (Lam.) DC. (Compositae) is a medicinal herb used in Argentina, Uruguay, Brazil and Paraguay for its choleretic, antispasmodic and hepatoprotective properties. The presence of the flavonoid quercetin and its derivatives, and of different phenolic acids such as caffeic, chlorogenic and isochlorogenic acids in the aerial parts of this plant has led us to study the antioxidant activity of its extracts using different bioassays. The inhibition of luminol-enhanced chemiluminescence by the aqueous and methanolic extracts was used to show that their total reactive antioxidant potential index (TRAP; in μM Trolox equivalents) was 91.0 ± 15.4 and 128.1 ± 20.1 μM, respectively, while the total antioxidant reactivity index (TAR) was calculated to be 1537 ± 148 and 1910 ± 171 μM. Only the methanolic extract was capable of reducing iron (II)-dependent DNA damage. Lipid peroxidation was assessed by two different methods. The aqueous extract reduced hydroperoxide-initiated chemiluminescence in rat liver homogenates at all concentrations in a dose-dependent manner, with a calculated $IC_{50} = 225$ μg/ml, while the methanolic extract was only effective at higher concentrations (100 and 1000 μg/ml). Both aqueous and methanolic extracts were capable of reducing the production of thiobarbituric acid reactive substances (TBARS) in rat liver homogenates, with an $IC_{50} > 1000$ μg/ml. The results obtained suggest that the extracts of *A. satureioides* possess significant free radical scavenging and antioxidant activity *in vitro*, a fact that should encourage future *in vivo* studies.

Introduction

Recent years have witnessed a renewed interest in plants as pharmaceuticals. This interest has been focused not only on the discovery of new biologically active molecules by the pharmaceutical industry, but also on the adoption of crude extracts of plants, such as infusions, for self-medication by the general public (1). Within this context, considerable interest has arisen in the possibility that the impact of several diseases may be either ameliorated or prevented by improving the dietary intake of natural nutrients with antioxidant properties, such as vitamin E, vitamin C, ß-carotene and plant phenolics such as tannins and flavonoids (2).

Achyrocline satureioides (Lam.) DC. (Compositae), known as "marcela" or "yateí caá", is a medicinal plant widely used in Argentina, Uruguay, Brazil and Paraguay for its choleretic, antispasmodic and hepatoprotective properties (3). Pharmacological evaluations of different extracts of this plant concerning their possible antispasmodic, anti-inflammatory, analgesic and sedative activities, together with their effects on intestinal transit and acute toxicity, have been reported (4,5), and phytochemical studies have confirmed the presence of the flavonoid quercetin and its derivatives. Similarly, the presence of caffeic, chlorogenic and isochlorogenic acids has also been reported in the aerial parts (6).

Different studies have demonstrated that flavonoids present interesting levels of anti-inflammatory activity (7,8). Likewise, caffeic acid and its derivatives have shown a choleretic and hepatoprotective action in different pharmacological studies (9). The high content of polyphenolic compounds in the aerial parts of *A. satureioides* has led us to study the antioxidant properties of the extracts of this medicinal plant. For this purpose the aqueous and methanolic extracts were submitted to different bioassays in or-der to determine their free radical scavenging activity and their capacity to reduce lipid peroxidation and iron (II)-dependent DNA damage.

Material and Methods

Chemicals

tert-Butyl hydroperoxide (t-BOOH), catechin, thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), phosphotungstic acid, bovine serum albumin acid, trichloroacetic acid (TCA), calf thymus DNA (Sigma Chemical Co., St. Louis, MO), sodium dodecyl sulfate (SDS) (Mann Research Laboratories Inc., New York, USA), dichloromethane (Dorwill, Buenos Aires, Argentina), methanol, ethanol and ferrous ammonium sulfate (Mallinckrodt, New York, USA), dimethylsulfoxide (DMSO), n-butanol and trichloroacetic acid (Merck, Buenos Aires, Argentina) and 2,2'-azo *bis*(2-amidinopropane) (ABAP) and luminol (Acros, Geel, Belgium) were of analytical grade.

Plant material

Plant material was collected in the province of Entre Ríos, Argentina. Botanical identification was made by Dr. A.A. Gurni in the Cátedra de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, where a voucher specimen is deposited.

Plant extracts

Extracts were prepared following the recommendations of CYTED (10). Infusions were made by pouring 100 ml of boiling water on 5 g of powdered plant material placed in a stoppered flask. The mixture was left to stand for 20 min and then filtered. The resulting infusion was freeze-dried in a Gamma A lyophilizer (Chriss, Germany). The resulting powder was considered to be

the aqueous extract.

Methanolic extracts were prepared by previously defatting 5 g of dry powdered plant material for 24 h at room temperature in a stoppered flask containing 50 ml of dichloromethane. The resulting marc was extracted with methanol under the same conditions as described for dichloromethane, and the extract was filtered and concentrated under reduced pressure at 43ºC in a Savant Speed Vac Plus SC210A concentrator. DMSO was used to pre-solubilize the methanolic extract (1% v/v).

Total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR)

TRAP and TAR indices were measured by luminol-enhanced chemiluminescence (11). For TRAP determinations, the reaction medium consisted of 100 mM sodium phosphate buffer, pH 7.4, 20 mM ABAP, 10 µM luminol, and increasing volumes (2-10 µl) of the extracts (0.1 mg/ml). Light intensity was measured at room temperature in a Wallac WinSpectral 1414 liquid scintillation counter with the circuit coincidence out of mode. ABAP is a source of free radicals which reacts with luminol yielding chemiluminescence. The system was calibrated using the α-tocopherol analog Trolox (150 µM). A comparison of the induction times after the addition of known concentrations of Trolox and antioxidants allows to obtain TRAP values as equivalents of Trolox concentration necessary to suppress the emitted luminescence by employing the following equation:

$$\text{TRAP (µM Trolox)} = \mu l_{\text{total}}/\mu l_{\text{sample}} \times \delta_{i\ \text{sample}}/\delta_{i\ \text{Trolox}} \times \delta_{i\ \text{Trolox (1 µM)}}$$

where μl_{total} is the final volume (3000 µl), μl_{sample} is the volume of the sample added to the reaction, $\delta_{i\ \text{sample}}$ is the induction time observed for the different volumes of the sample, $\delta_{i\ \text{Trolox}}$ is the induction time observed for the reference compound (Trolox), and $\delta_{i\ \text{Trolox (1 µM)}}$ is the induction time ob-

served for the reference compound at a 1 µM final concentration.

TAR values were determined by measuring the initial decrease of the luminol luminescence after addition of small aliquots of the sample at room temperature using a Wallac WinSpectral 1414 liquid scintillation counter with the circuit coincidence out of mode. The conditions of the experiment were the following: 100 mM phosphate buffer, pH 7.4, 30 mM ABAP and 50 µM luminol. Trolox (150 µM) was used as a standard.

The TAR index is defined by:

$$\text{TAR (µM Trolox)} = \mu l_{\text{total}}/\mu l_{\text{sample}} \times \text{Io/I}_{\text{sample}}/\text{Io/I}_{\text{Trolox}} \times \text{Io/I}_{\text{Trolox (1 µM)}}$$

where μl_{total} is the final volume (3000 µl), μl_{sample} is the volume of the sample added to the reaction, Io is the initial emission of chemiluminescence (before the addition of the antioxidant), and I is the instantaneous chemiluminescence after the addition of the different aliquots of the sample or the reference compound (Trolox). TAR indices reflect the capacity of the additive to engage in the electron transfer processes to luminol-derived radicals.

Assay of deoxyribose damage

Deoxyribose damage was assayed by the method of Halliwell and Gutteridge (12), with modifications. The reaction mixture in a total volume of 1.2 ml contained 0.5 ml calf thymus DNA (1 mg/ml of 0.15 M NaCl), 0.5 ml 0.1 M sodium phosphate buffer, pH 7.4, 0.2 ml 4.8 mM ferrous ammonium sulfate and 1000, 100 and 10 µg/ml of the methanolic plant fraction. The reaction mixture was incubated for 1 h at 37ºC in a water bath shaker. After incubation, 1 ml 1% TBA (w/v) plus 1 ml 2.8% TCA (w/v) were added to the reaction mixture which was kept in a boiling water bath for 15 min. The thiobarbituric acid-reactive substances (TBARS) thus generated were extracted into 2 ml n-bu-

tanol. After centrifugation, the fluorescence of the butanol layer was measured as described above. The iron (II)-dependent deoxyribose damage inhibition values are expressed as the ratio of TBARS in the presence of plant extracts to that in their absence (control). Catechin was used as a positive control, and Finney's (13) statistical method of Probit analysis was used to calculate the IC$_{50}$.

Preparation of rat liver homogenates

Adult Wistar rats weighing 180-200 g fed on a standard laboratory diet and receiving water *ad libitum* were used. The livers were excised, perfused and homogenized with 120 mM KCl, 50 mM phosphate buffer, pH 7.4 (1:10 w/v). The samples were centrifuged at 700 g for 10 min at 0-4°C. The supernatant fraction was kept at -20°C until the time for use. Protein concentration was measured by the method of Lowry et al. (14) using bovine serum albumin as a standard.

Hydroperoxide-initiated chemiluminescence

Hydroperoxide-initiated chemiluminescence (QL) of liver homogenates (15) was measured in a Wallac WinSpectral 1414 liquid scintillation counter with the circuit coincidence out of mode, at room temperature. Rat liver homogenates adjusted to a final protein concentration of 0.5 mg/ml in 120 mM KCl, 50 mM sodium phosphate buffer, pH 7.4, and to a final volume of 2 ml were placed in 10 x 35-mm glass tubes, which were inserted into low potassium 25 x 50-mm glass vials. Plant extracts were solubilized in distilled water and adjusted to the reaction medium at 10, 100 and 1000 µg/ml. Chemiluminescence was initiated by the addition of 3 mM *tert*-butyl hydroperoxide, and was expressed as total counts. Chemiluminescence inhibition is reported as the ratio of the maximum emission in the presence of plant extracts to that in their absence (con-

trol). Catechin was used as a positive control or standard due to its high antioxidant activity (16). Finney's (13) statistical method of Probit analysis was used to calculate the concentration of the extract that inhibited chemiluminescence by 50%, i.e., the IC$_{50}$.

Thiobarbituric acid-reactive substance assay

TBARS were determined as described by Fraga et al. (17). Rat liver homogenates, adjusted to 10 mg protein/ml in 120 mM KCl, 50 mM phosphate buffer, pH 7.4, were incubated with 1000, 100 and 10 µg dry weight/ml of plant extract at 37°C for 15 min. Sodium dodecyl sulfate (0.2 ml of 3% (w/v)) and 0.05 ml of 4% BHT in ethanol were added. After mixing, 2 ml of 0.1 N HCl, 0.3 ml of 10% (w/v) phosphotungstic acid and 1 ml of 0.7% (w/v) 2-thiobarbituric acid were added. The mixture was heated for 60 min in boiling water, and TBARS were extracted into 5 ml n-butanol. After centrifugation, the fluorescence of the butanol layer was measured at 515-nm excitation and 555-nm emission using a Hitachi F-3010 fluorescence spectrophotometer. The values are reported as the ratio of the amount of TBARS formed in the presence of plant extracts compared to control. Catechin was used as standard. The extract concentration that would inhibit by 50% the production of thiobarbituric acid-reactive substances, i.e., the IC$_{50}$ with a 95% confidence interval, was calculated using Finney's (13) statistical method of Probit analysis.

Results and Discussion

Total reactive antioxidant potential and total antioxidant reactivity

In order to determine the TRAP and TAR indices of the extracts of *A. satureioides*, a simple method based on luminol-enhanced chemiluminescence was used, which is based on the measurement of induction times in the

oxidation of ABAP, a free radical source (11). This method is based on the trapping of peroxyl radicals (ROO•), and is capable of detecting most of the significant compounds with antioxidant activity present in complex mixtures of antioxidants such as plant extracts. Since TRAP measurements indicate the quantity of antioxidants present in the plant extracts, the TAR was also determined in order to measure the quality (given by the reactivity) in those extracts which showed antioxidant activity (11).

The addition of increasing concentrations of the aqueous and methanolic extracts to the reaction medium resulted in a reduction of luminol-enhanced chemiluminescence, indicating their capacity of scavenging peroxyl radicals. The induction times observed were almost proportional to the concentration of the additive (Figure 1), allowing to determine the TRAP and TAR indices for the extracts expressed as µM Trolox equivalents (Table 1). The results obtained indicate that TAR values are considerably higher than TRAP values. This may be due to the presence of antioxidants of relatively high reactivity, thus suggesting that some of the compounds present in *A. satureioides* could be considerably more reactive than Trolox.

Inhibition of deoxyribose damage

The effect of *A. satureioides* on the inhibition of free radical-mediated deoxyribose damage was assessed by means of the iron (II)-dependent DNA damage assay. The Fenton reaction generates hydroxyl radicals (OH•) which degrade DNA deoxyribose, using Fe^{2+} salts as an important catalytic component (12). Oxygen radicals may attack DNA either at the sugar or the base, giving rise to a large number of products. Attack at a sugar ultimately leads to sugar fragmentation, base loss, and strand break with a terminal fragmented sugar residue (18). Addition of low concentrations of transition metal

ions such as iron to DNA causes degradation of the sugar into malondialdehyde and other related compounds which form a chromogen with TBARS. Table 2 shows the effect of the extracts on the iron (II)-dependent deoxyribose damage. The methanolic extract was the only one capable of reducing DNA damage at all concentrations. Catechin, used as a standard, was highly effective in inhibiting the oxidative DNA damage, showing an IC_{50} = 5 µg/ml.
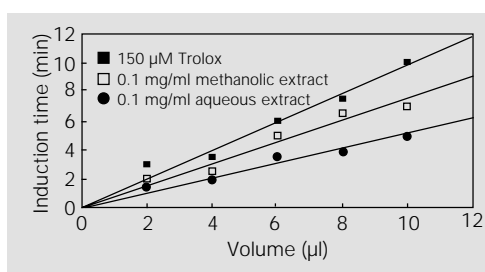

Figure 1 - Induction times as a function of additive volumes (µl) of two extracts of A. satureioides and the reference compound Trolox. Initial concentrations of the extracts were 150 µM for Trolox (closed squares), 0.1 mg/ml for the methanolic extract (open squares), and 0.1 mg/ml for the aqueous extract (closed circles). The initial reaction mixture before addition of the samples was 3 cc of 0.1 M sodium phosphate buffer, pH 7.4, containing 10 µM luminol and 20 mM ABAP. N = 2.

Table 1 - Total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR) of A. satureioides extracts, expressed as µM Trolox equivalents.

The values are means of 5 determinations.

Extract	TRAP (µM)	TAR (µM)
Aqueous	91.0 ± 15.4	1537 ± 148
Methanolic	128.1 ± 20.1	1910 ± 171

Table 2 - Antioxidant activity of A. satureioides extracts measured as the inhibition of free radical-mediated deoxyribose damage induced by the presence of Fe (II) salts.

ND: Not determined. Inhibition percentage of iron (II)-mediated deoxyribose damage was calculated by the following equation: (1 - Dtm/Dcm) x 100; Dtm: fluorescence of test samples, Dcm: fluorescence of controls. Catechin was used as a standard. The values are means of 2 determinations.

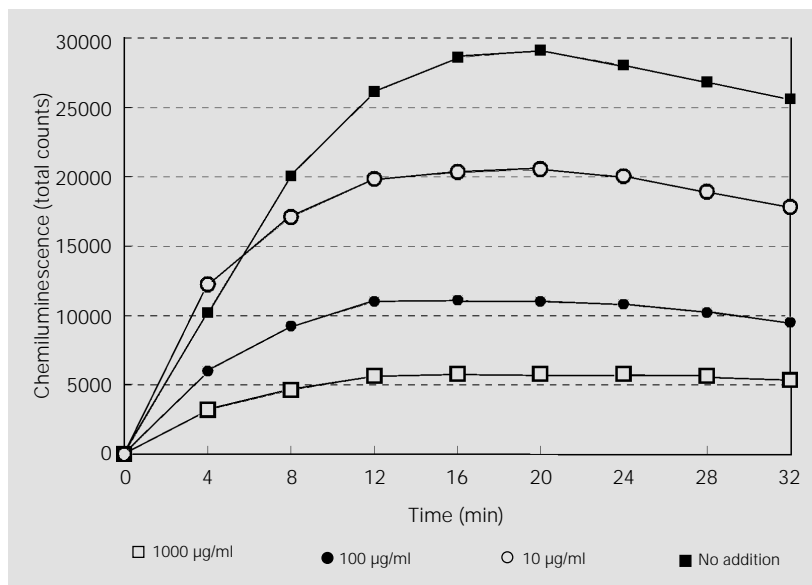| Extract	Inhibition (%)			IC_{50}	
	100 µg/ml	10 µg/ml	1 µg/ml	µg/ml	µM
Catechin (control)	74	59	40	5 (13-1)	17
Aqueous	5	-	-	ND	-
Methanolic	33	33	15	>1000	-

Figure 2 - Effect of A. satureioides (aqueous extract) on the time course of hydroperoxide-initiated chemiluminescence of rat liver homogenates. Zero time was the moment of tert-butyl hydroperoxide addition. The reaction mixture was a 2-ml final volume of rat liver homogenate (final protein concentration, 0.5 mg/ml) in 120 mM KCl, 50 mM phosphate buffer, pH 7.4. Plant extracts were adjusted to the reaction medium at 1000 (open squares), 100 (closed circles), 10 (open circles) µg/ml, and control (no addition) (closed squares). Chemiluminescence was initiated by the addition of 3 mM tert-butyl hydroperoxide and is reported as total counts. The values are means of 2 determinations.
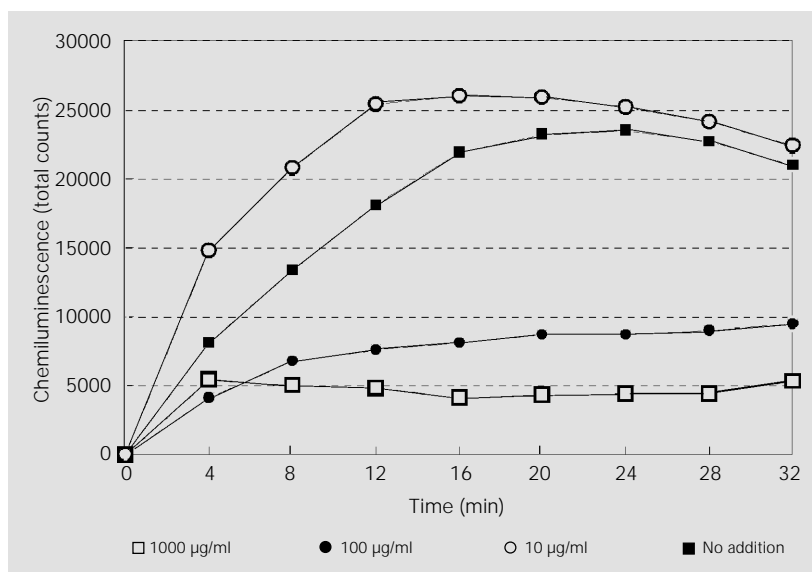

Figure 3 - Effect of A. satureioides (methanolic extract) on the time course of hydroperoxide-initiated chemiluminescence of rat liver homogenates. No addition (closed squares), 1000 (open squares), 100 (closed circles) and 10 (open circles) µg/ml. Experimental conditions are as described in the legend to Figure 2. Zero time was the moment of tert-butyl hydroperoxide addition. The values are means of 2 determinations.

Inhibition of lipid peroxidation

Peroxyl and hydroxyl radicals are important agents that mediate lipid peroxidation, thereby damaging cell membranes (19). In order to determine if the extracts of *A. satureioides* are capable of reducing *in vitro* oxidative stress, these were further tested using the hydroperoxide-initiated chemiluminescence assay in rat liver homogenates (15). The results obtained for the aqueous and methanolic extract are shown in Figures 2 and 3. Table 3 shows the antioxidant activity expressed as percentage of inhibition evaluated at maximum emission (20), while negative values are the effect of increasing chemiluminescence. The aqueous extract was able to reduce the emission of chemiluminescence at all concentrations in a dose-dependent manner, thus allowing to calculate an $IC_{50} = 225$ µg/ml. On the other hand, the methanolic extract was only effective at higher concentrations (100 and 1000 µg/ml), showing an increase in chemiluminescence at lower concentrations (10 µg/ml). Hydroperoxide-initiated chemiluminescence in rat liver homogenates is a qualitatively different method, since it is used to determine oxidative stress associated with the existence of an imbalance between antioxidant and prooxidant compounds present in the reaction mixture (15). The prooxidant activity observed at a lower concentration of the methanolic extract could therefore be due to the gradual disappearance of antioxidant compounds in the reaction mixture, in contrast to the persistence of substances with prooxidant activity. Furthermore, it has been determined that the lipid peroxidation that occurs in the hydrophobic domains of the membranes accounts for a large part of the emission of chemiluminescence (15). In contrast, the major antioxidant components of *A. satureioides* are mostly hydrophilic, and this could reduce their ability to reach these domains.

Because hydroperoxide-initiated chemiluminescence in rat liver homogenates de-

termines the free radical trapping activity in a chain reaction that occurs both in the hydrophilic and the hydrophobic domains of the biological membranes (15), a second assay that determines the production of malondialdehyde and related compounds was performed. TBARS are produced as by-products of lipid peroxidation that occur only in the hydrophobic core of biomembranes (17). The results obtained suggest that both the aqueous and methanolic extracts of *A. satureioides* are capable of reducing the lipid peroxidation that occurs in these domains (Table 4).

Catechin, used as a reference compound in the determination of the inhibition of lipid peroxidation, was highly effective in reducing both chemiluminescence and TBARS, showing an $IC_{50} = 1$ µg/ml in the first assay, and an $IC_{50} = 46$ µg/ml in the second.

Based on the results described, we may conclude that the aqueous and methanolic extracts of *A. satureioides* possess significant free radical scavenging and antioxidant activity *in vitro*. It has been stated that chemiluminescence methods are more sensitive than TBARS production in the determination of antioxidant activity (21-23). This could explain the absence of activity observed in the aqueous extract in inhibiting DNA damage. In a similar way, the methanolic extract was less active when tested by fluorescence methods. These results should

encourage future *in vivo* studies, which could ultimately lead to the inclusion of this medicinal herb in different antioxidant pharmaceutical formulations.

Table 3 - Antioxidant activity of A. satureioides extracts using the hydroperoxide-initiated chemiluminescence assay.

ND: Not determined. Inhibition of chemiluminescence was calculated by the following equation: [1 - (Esm/Ecm)] x 100; Esm: emission intensity of test samples at maximum emission; Ecm: emission intensity of the control at maximum emission. Catechin was used as a standard. A negative value for inhibition indicates an increase in light emission compared with controls. The values are means of 2 determinations.

Extracts	Inhibition of chemiluminescence (%)				IC_{50} and 90% confidence interval (µg/ml)
	1000 µg/ml	100 µg/ml	10 µg/ml	1 µg/ml	
Catechin (control)	85	81	73	49	1.00 (0.1-2.7)
Aqueous	63	50	12	-	225 (404-139)
Methanolic	84	69	-11	-	ND

Table 4 - Inhibition of the production of thiobarbituric acid-reactive substances (TBARS) by extracts of A. satureioides.

Inhibition percentage was calculated by the following equation: (1 - Ptm/Pcm) x 100; Ptm: production of TBARS by test samples, Pcm: production of TBARS by controls. Catechin was used as a standard. The values are means of 2 determinations.

Extracts	Inhibition (%)			IC_{50} and 95% confidence interval (µg/ml)
	1000 µg/ml	100 µg/ml	10 µg/ml	
Catechin (control)	65	50	42	46 (78-22)
Aqueous	33	17	11	>1000
Methanolic	38	21	20	>1000

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