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RESEARCH ARTICLE

Antioxidant and cytotoxic properties of an aqueous extract from the Argentinean plant *Hedeoma multiflorum*

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Abstract

Context: *Hedeoma multiflorum* Benth. (Lamiaceae) is widely used in Argentinean popular medicine for digestive and anti-spasmodic purposes. However, knowledge about its pharmacological properties has been poorly investigated.

Objective: The antioxidant and cytotoxic properties of an aqueous extract from the plant were investigated for the first time.

Material and methods: Scavenging of stable free radicals of 2,2-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS$^+$) and 2,2-diphenyl-1-picryl hydrazyl (DPPH), reducing of ferric (III) iron of ferric reducing ability of plasma (FRAP) reagent, and inhibition of lipid peroxidation (LP) of human plasma and rat brain homogenates were assessed. Cytotoxicity was tested on human polymorphonuclear (PMN) cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cytotoxicity was assessed by flow cytometric techniques.

Results: Extract scavenged ABTS$^+$ and DPPH (1.78 and 0.78 µmol Trolox equivalent/mg dry extract, respectively) and reduced FRAP reagent (0.66 µmol ascorbic acid equivalent/mg dry extract). LP of human plasma and rat brain was also inhibited in a dose-dependent way (inhibitory concentration 50%=27.0 and 86.0 µg/mL, respectively). Extract is rich in polyphenol compounds (0.96 ± 0.08 µmol equivalent caffeic acid/mg dry matter). Treatment of PMN decreased significantly the cell ability to reduce the MTT salt and increased the hypodiploid nuclei from 4 to 18% quantified using propidium iodide (PI). In the annexin V-Fluorescein isothiocyanate (annexin V-FITC) assay, 26% of treated cells were annexin V-FITC positive and PI negative. Using the 3,3'-dihexyloxacarbocyanine iodide uptake method, the negative fraction of cells was calculated as 29%.

Discussion and conclusion: *H. multiflorum* extract was found to have a significant antioxidant and pro-apoptotic activities, and a great potential as a source of healthy products.

Keywords: *Hedeoma multiflorum*, scavenger, lipid peroxidation, apoptosis, polymorphonuclear neutrophils

Introduction

There is growing evidence that consumption of certain foods, dietary supplements, or traditional beverages leads to a reduction in some parameters of oxidative damage in biological systems (Aruoma et al., 2003; Juan et al., 2006). Oxidative damage is produced by reactive oxygen species (ROS) that are continuously produced during the aerobic life, and they seem to be involved in the pathogenesis of several human diseases such as cancer, diabetes, cardiovascular diseases, inflammation, ageing, etc. (Gutteridge & Halliwell, 1994). Aerobic organisms are protected from oxygen toxicity by a natural antioxidant defense system constituted by enzymatic and nonenzymatic mechanisms. If this endogenous system is not appropriate for scavenging the ROS completely, there is oxidative damage of important macromolecules (Halliwell, 1999).

The role of herbal teas in disease prevention and cure has been attributed, in part, to antioxidant properties of

liposoluble and water soluble vitamins (E and C), and other compounds such as the amphipatic polyphenols can act as reducing agents, hydrogen donators, singlet oxygen quenchers, and metal chelators (Morel et al.,1998; Ulrich-Merzenich et al., 2009).

On the other hand, extracts from fruits, herbs, vegetables, and other plant materials rich in phenolics are of increasing interest for the pharmaceutical and food industries, because they retard oxidative degradation of the final products (Javanmardi et al., 2003).

The genus *Hedeoma* Pers. (Lamiaceae) includes about 43 species of annual or perennial herbs distributed in America, from the United States to Argentina. A species of this genus is used in Mexico and North America to prepare herbal tea or as a spice (Viveros-Valdez et al., 2008). Methanol extracts of their aerial parts exhibited a strong antioxidant effect attributable to the high content of the major active constituents, chlorogenic, caffeic, and rosmarinic acids. There are three species in Argentina: *Hedeoma multiflorum* Benth., *Hedeoma medium* Epling, and *Hedeoma mandonianum* Wedd. (Slanis & Bulacio, 2005).

H. multiflorum Benth. is widespread in Argentina, where it is known as "tomillo serrano" (mountain thyme) (Toursarkissian, 1980). Infusion of the herb is used as a medicine for digestive and anti-spasmodic purpose (Goleniowski et al., 2006). Because of its desirable flavor, flowers, leaves, and stems of the plant are blended with other species, particularly *Ilex paraguariensis*, in beverages. Despite the widespread consumption of Argentine people, this species has received little attention, and almost nothing is known about its pharmacological and toxicological properties.

In this work, we assessed the *in vitro* antioxidant activity of an aqueous extract of *H. multiflorum* using different experimental models. The extract was also tested for cytotoxicity using human polymorphonuclear (PMN) cells. The nature of cytotoxicity was assessed by flow cytometric techniques.

Material and methods

Plant material and preparation of the extract
Herbal bags of *H. multiflorum* were purchased in January 2008 at the market Prama, Villa de las Rosas, Córdoba, Argentina. Samples were identified by Dr. E. Spegazzini (Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina) and they were free of preservative and artificial flavoring. A voucher specimen was deposited at the Cátedra de Farmacobotánica of that institution. The bags were kept dry in darkness and were processed for 3 months after the purchase. The plant extract was obtained from infusions prepared according to the popular use. Briefly, herbal samples (10 ± 1 g) were weighed in 250 mL Erlenmeyer flasks and 200 ml of boiling distilled water was added and left to cool down to 40°C. Each infusion was filtered through paper (Whatman No 1), lyophilized, and the dry matter was maintained at −20°C until use. The aqueous extract yielded 15.3% (w/w

of dry material) and it was standardized in total phenolics (0.96 ± 0.08 μmol equivalent of caffeic acid/g dry material). For bioassay analyses, samples were dissolved in dimethyl sulphoxide (DMSO). Stock solutions were serially diluted with the solvent to obtain different concentrations (1–250 μg/mL). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ascorbic acid, and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT) were used as positive control for the antioxidant-activity tests.

Chemicals
2,2-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS$^+$); 2,2-diphenyl-1-picryl hydrazyl (DPPH); 2,4,6-tripyridyl-*s*-triazine (TPTZ); DMSO; (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); 3,3′-dihexyloxacarbocyanine iodide (DIOC$_6$(3)); propidium iodide (PI); and caffeic acid (CA) were obtained from Sigma–Aldrich de Argentina S.A. FITC Annexin V was obtained from BD Pharmingen™. All other chemicals used were of the highest analytical grade.

Antioxidant activity
DPPH· radical scavenging activity
DPPH· radical scavenging was carried out according to the study by Cos et al. (2000). For the assay, 10 μL of the plant extract was added to 990 μL of a 0.04 mg/mL DPPH· solution in methanol. A series of concentrations ranging from 1 to 100 μg dried extract/mL were tested. Mixtures were shaken vigorously and incubated in the dark for 20 min, then the reduction of DPPH· absorption was measured at 517 nm. A calibration curve was obtained by measuring the reduction in absorbance of the DPPH solution in the presence of different concentrations of Trolox (0–400 μM). Results were expressed as micromoles of Trolox equivalents (TE) per milligram of dried extract. All determinations were performed in triplicate. DMSO was used as a negative control.

ABTS$^+$ radical scavenging activity
The antioxidant capacity of extracts of *H. multiflora* was also determined by the TEAC assay (scavenging of the radical ABTS$^+$) (Re et al., 1999) with some modifications. ABTS$^+$ radical was generated by reacting 7 mM ABTS$^+$ solution in water with 2.45 mM potassium persulfate in the dark for 12–16 h. Absorbance of the reactant was later adjusted to 0.700 ± 0.02 with phosphate-buffered saline (PBS) at a wavelength of 734 nm. Radical scavenging reaction was started by the addition of 10 μL of appropriately diluted extracts to 990 μl of the ABTS$^+$ solution. The absorbance of the mixture was recorded at 734 nm, 25 min after addition of the sample. A Trolox calibration curve (0–200 μM) was obtained. Results were expressed as micromoles of TE per milligram of dried extract. All determinations were performed in triplicate.

Ferric reducing activity
The ferric reducing activity of plant (FRAP) extracts was estimated by the FRAP assay (Benzie & Strain, 1996). The

solutions for this assay consisted of 300 mmol/L acetate buffer, 10 mmol/L TPTZ in 40 mmol/L of HCl, and 20 mmol/L $FeCl_3 \cdot 6H_2O$. Reagent for this assay was prepared on the same day of assay by mixing 25 mL acetate buffer with 2.5 mL TPTZ solution and 2.5 mL $FeCl_3 \cdot 6H_2O$. The assay was performed as follows: 990 µL of the FRAP reagent was added to 10 µL of appropriately diluted extracts or buffer. Absorbance readings at 593 nm were recorded 20 min after starting the reaction. The change in absorbance was related to absorbance changes of a standard solution of ascorbic acid tested in parallel. Results were expressed as micromole equivalent of ascorbic acid per milligram of dry weight of the extract.

Lipid peroxidation assays
Human plasma
Human plasma was oxidatively modified by a non-enzymic method. Heparin plasma (100 µL) from healthy donors (200 ± 20 µg total cholesterol) was diluted with 350 µl PBS, and the oxidation was started by adding 50 µL of 10 mM $CuSO_4$. After 180 min of incubation at 37°C, the reaction was stopped by adding EDTA. These incubations and relevant controls were performed in the presence of *H. multiflora* extract (10–100 µg/mL). Only one time point at 3 h of incubation was selected. This was in accordance with the previous studies which indicated that this was the optimal period for oxidative modification of human plasma (Schinella et al., 2007). Thiobarbituric acid-reactive substances (TBARS) production was used as an indicator of lipid peroxidation (LP; Pompella et al., 1987). Results are expressed as percentage of inhibition related to controls without the extract. BHT was used as a positive control.

Rat brain homogenates
Brain of normal rats was dissected and homogenized in ice-cold 20 mM Tris–HCl buffer pH 7.4 to produce a 1/10 homogenate. After centrifugation at 25,000g for 15 min, aliquots of the supernatant were incubated with the extract (10–100 µg/mL) in the presence of 10 µM $FeSO_4$ and 100 µM ascorbic acid at 37°C for 1 h. The reaction was stopped with trichloroacetic acid, and the TBARS production was determined as indicated. Results were expressed as percentage of inhibition related to controls without the extract. BHT was used as the positive control.

Determination of total phenol content
Total phenolic content (TPC) of the extract was determined using Folin Ciocalteu reagent (Singleton & Rossi, 1965) and caffeic acid as standard. Results were expressed as micromole equivalent of caffeic acid per milligram of dry extract.

Cytotoxicity assay
Cells
Human anticoagulated peripheral blood from healthy donors was collected and neutrophils were isolated by sequential sedimentation in dextran 2% in saline and centrifuged in Histopaque-1077. Residual erythrocytes were removed by hypotonic lysis (cold water). The neutrophils were washed twice in PBS pH 7.4 and then resuspended in the same buffer containing 1 mg/mL glucose, 0.4 mM Mg^{2+}, and 1.20 mM Ca^{2+}. The preparations contained 85–90% neutrophils with viability higher than 95% established by exclusion with trypan blue.

Measure of cytotoxic activity
The MTT assay (Mosmann, 1983) was used as a criterion of cytotoxicity for human PMN cells. Briefly, 2.5×10^6 cells were incubated at 37°C for 3 h with PBS pH 7.4 containing the extract at concentration 200 µg/ml. Controls received vehicle and corresponded to 100% viability. The water insoluble dark-blue formazan produced by living cells was dissolved in 10% Sodium Dodecyl Sulfate in 0.01 M HCl and measured at 490 nm in a Beckman DU 640.

Nature of cytotoxicity
Analysis of apoptotic (hipodiploid) nuclei
PI staining was assessed according to Nicoletti et al. (1991) with slight modifications. Cells (2.5×10^6 per tube) were suspended in ice-cold 70% ethanol and stored at –20°C for at least 30 min, after which they were washed twice in PBS at 4°C and resuspended in 500 µL of DNA staining solution (20 µg/mL PI plus 0.2 mg/mL RNAse A in PBS). The resuspended cells were then incubated in the dark at room temperature for 30 min and the fluorescence intensity of individual nuclei was measured using a FACScan, Becton Dickinson flow cytometer. A minimum of 20,000 events were counted per sample. The percentage of apoptotic cell nuclei (hypodiploid DNA peak) was calculated.

Analysis of phosphatidylserine externalization
Phosphatidylserine exposure was measured by annexin V-FITC binding (Homburg et al., 1995). After the appropriate incubations with the extract (100 µg/ml), cells (2.5×10^6) were washed twice with cold PBS and then resuspended in binding buffer solution (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Then, 1×10^5 cells were transferred to a 5-ml culture tube where 5 µl of Annexin V-FITC and 10 µl of PI solution (50 µg/ml in PBS) were added. The tubes were then incubated for 15 min at room temperature in the dark. After incubation, 400 µl of binding buffer was added to each tube and analyzed by flow cytometry.

Detection of the mitochondrial membrane potential ($\Delta\Psi_m$)
$DiOC_6(3)$ is a lipophilic cationic cyanine dye that strongly labels mitochondria. A decrease in $\Delta\Psi_m$ in apoptotic cells is related with a reduction of $DiOC_6(3)$ uptake (Macho et al.,1997). PMN cells were treated with the extract (100 µg/ml) for 3 h at 37°C, then incubated with $DiOC_6(3)$ (40 nM) for 30 min at 37°C. After treatment, cells were washed with cold PBS. Fluorescence intensities of $DiOC_6(3)$ were analyzed by flow cytometry.

Statistics

Data were expressed as means ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Turkey–Kramer multiple comparison test. Differences were considered significant at p < 0.05. The inhibitory concentration 50% (IC_{50}) was calculated from concentration/effect regression line.

Results

Antioxidant activity

The antioxidant capacity of plant extracts largely depends on their composition and conditions of the test system and they are influenced by several factors, which cannot be fully described with one single method. The antioxidant activity of the *H. multiflorum* extract was then evaluated on the basis of different methods: scavenging of the stable $ABTS^{+}$ and DPPH free radicals, the ability to reduce ferric (III) iron to ferrous (II) iron in the FRAP reagent, and the capacity to inhibit LP using two biological systems: the copper-induced human plasma oxidation and the rat brain homogenates incubated in the presence of Fe^{2+}/ascorbate.

Scavenging of free radicals and reducing activity

H. multiflorum extract exhibited potent antioxidant activity against both $ABTS^{+}$ and DPPH radicals. It was found to be more potent against the $ABTS^{+}$ radical (1.78 μmol TE/mg dry extract vs 0.78 μmol TE/mg dry extract for the DPPH radical) (Figure 1). The extract also showed capacity to reduce the iron present in the FRAP reagent (0.66 μmol equivalent of ascorbic acid/mg dry matter)

Inhibition of lipid peroxidation

There is an evidence that metal ions at micromolar concentrations play a role in human plasma lipoprotein oxidation. It has been suggested that loe–density lipoprotein oxidation induced *in vitro* in whole plasma is expected to reflect the oxidation *in vivo* more adequately than *in vitro* oxidation of the isolated lipoprotein (Spranger et al., 1998). For that reason, we assessed the antioxidant capacity of *H. multiflorum* extract for lipoproteins protection using human plasma. In this system, the extract showed a dose–response relationship of anti-peroxidative activity with IC_{50} values of 27.0 μg/mL (Figure 2).

Similar to the inhibiting human plasma LP, the *H. multiflorum* extract also showed an inhibitory effect when the peroxidative process was induced in rat brain homogenates by a nonenzymatic mechanism using Fe^{2+}.

Total phenol content

TPC was compared with standard caffeic acid and the results were expressed in terms of micromole equivalent per milligram of dry matter. The TPC value for *H. multiflorum* extract was 0.96 ± 0.08 μmol equivalent caffeic acid/mg dry extract.

Cytotoxic activity

In this work, we also assessed the cytotoxic activity of the *H. multiflorum* extract on PMN cells. The MTT assay revealed that the ability of cells to reduce the MTT salt to formazan decreased significantly in the presence of the extract. The viable treated cells were 66 ± 7% of control cells. This effect was observed at concentrations greater than those with antioxidant effect (100 μg/ml).

Two distinct modes of cell death, apoptosis and necrosis, can be distinguished based on differences in morphological and biochemical changes of dying cells. A characteristic pattern of structural changes in cytoplasm and nucleus can be detected in cells undergoing apoptosis including at, different times, cell shrinkage, loss of integrity of the mitochondrial membrane, loss of mitochondrial membrane potential, phosphatidylserine translocation to outer membrane, caspase activation,
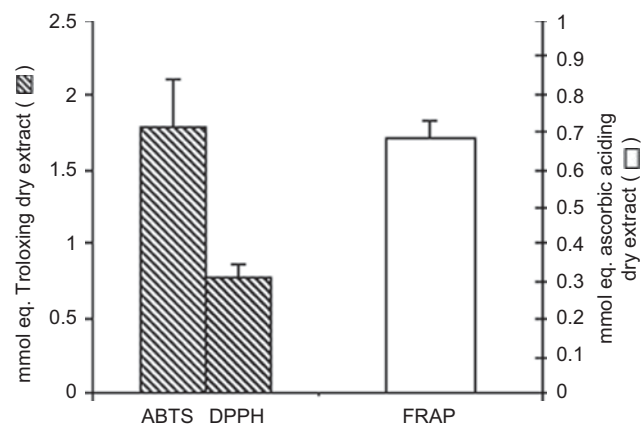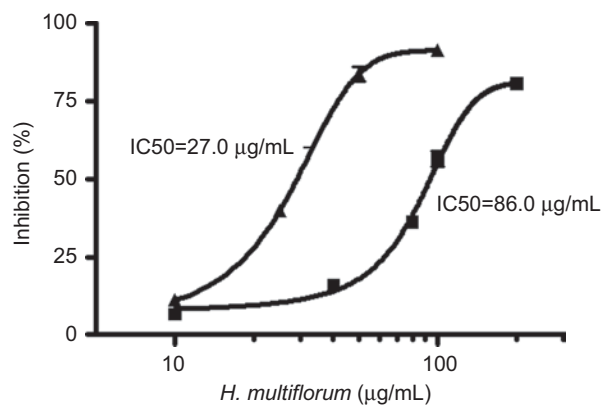

Figure 1. Scavenging of free radicals and reducing activities of *H. multiflorum* extract. Scavenging activities are expressed as micromole equivalents of Trolox per milligram of dry matter. Reducing activity is expressed as micromole equivalents of ascorbic acid per milligram of dry matter. The values represent the mean ± SE (*n* = 5).

Figure 2. Inhibition of lipid peroxidation by *H. multiflorum* extract. Peroxidation was induced by treatment with 10 μM FeSO$_4$ and 100 μM ascorbic acid for rat brain homogenate and 1 mM CuSO$_4$ for human plasma. Each value represents the mean ± SD (*n* = 5). IC_{50} values were obtained from the concentration/effect regression line.

nuclear fragmentation, and formation of apoptotic bodies (Mower et al., 1994).

Nature of cytotoxicity

To know the nature of *H. multiflorum* cytotoxic effect, we used three different assays based on flow cytometric techniques (Figure 3A). Using PI, we quantified the development of hypodiploid nuclei in PMN leukocytes in the presence of *H. multiflorum*. Treatment of cells (3 h in the presence of 100 µg/mL of the extract at 37°C) increased the apoptotic nuclei from $4.0 \pm 2.2\%$ of control cells to $18.2 \pm 3.2\%$ observed in the treated cells (Figure 3B). In the annexin V-FITC binding assay to measure phosphatidylserine exposure, PMN cells were treated in the same conditions as before. Cells were then incubated with annexin V-FITC in a buffer containing PI and analyzed by means of flow cytometry. Although a small proportion ($5.7 \pm 1.7\%$) of PMNs in the untreated population underwent apoptosis (annexin V-FITC positive, PI negative), the proportion of apoptotic cells was significantly greater after treatment ($26.1 \pm 5.1\%$; Figure 3C). Early during apoptosis, there is a reduction in mitochondrial transmembrane potential. In $DIOC_6(3)$ uptake method, $DIOC_6(3)$ positive fraction represents viable cells. Although the $DIOC_6(3)$ negative fraction of control cells was $5.6 \pm 1.9\%$, in treated cells with *H. multiflorum* extract, the $DIOC_6(3)$ negative fraction was $29.9 \pm 8.2\%$, characteristic of apoptotic cells and/or dead cells (Figure 3D).

Taken together, these results suggest that the cytotoxic effect of *H. multiflorum* extract is carried out by an apoptotic mechanism.

Discussion

The implication of redox mechanisms in human diseases has led to the fact that antioxidants, in particular, plant diet-derived antioxidants, might have health benefits as therapeutic agents (Aruoma et al., 2003). Extracts showing potent antioxidant activity as well proapoptotic capacity could exert protective effects against oxidative damage occurring in some human diseases.

The inflammatory process is a complex series of cellular and biochemical events in which neutrophils play an important role. During inflammation, activated neutrophils and macrophages release important amounts of ROS, mediators that provoke or sustain the inflammatory process. However, the over production of ROS leads to tissue injury by damaging macromolecules and LP of membranes (Halliwell, 2006). ROS neutralization by natural antioxidants could attenuate the oxidative damage of tissue (Geronikaki & Gavalas, 2006).

Large amounts of phenolic compounds are found in teas, fruits, and vegetables being polyphenols the most common and widely distributed group of this kind of phytochemicals. Polyphenols have shown different pharmacological properties (Yonathan et al., 2006; Wu et al., 2007; Del Rio et al., 2010). Recent studies support the notion of tea polyphenols as chemopreventive agents against the development of colon cancer in humans (Kumar et al., 2007). Some antioxidant phenolics have also been found to suppress growth and proliferation of transformed or malignant cells through induction of apoptosis (Rao et al., 2007). However, more clinical trials
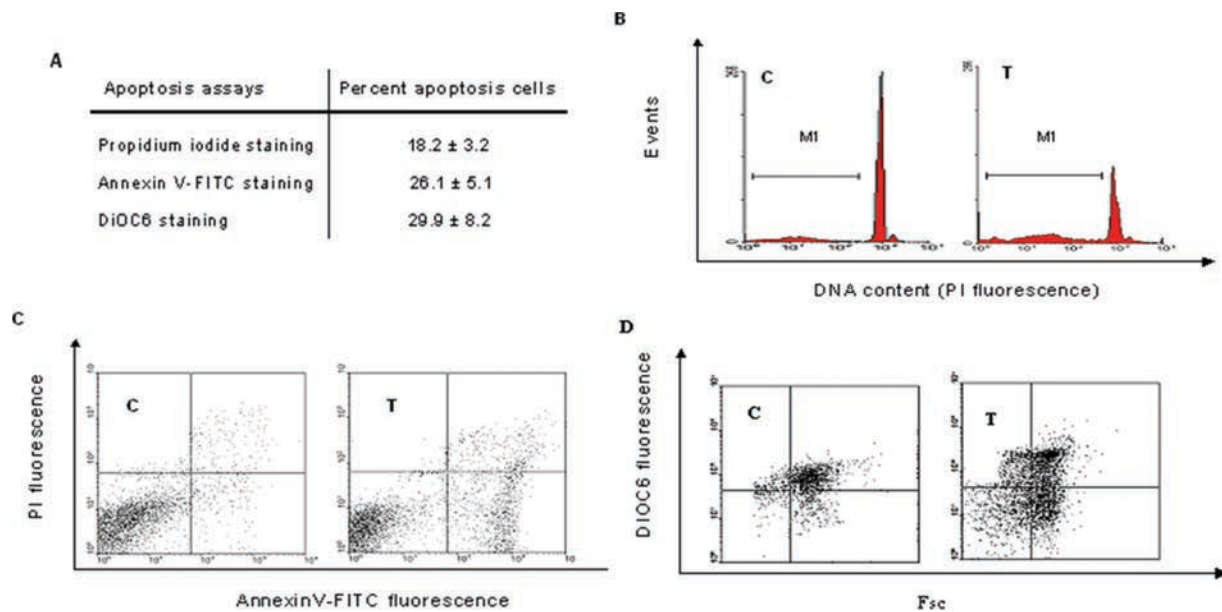

Figure 3. Promotion of PMN cells apoptosis by *H. multiflorum* extract (T). (A) Apoptosis was measured by three distinct methods: analysis of apoptotic (hypodiploid) nuclei, measurement of phosphatidylserine exposure, and quantitation of uptake and retention of $DIOC_6(3)$. All values are represented as means ± SD of four separate experiments. (B) Typical histograms showing the percentage (M1) of nuclei with hypodiploid DNA content. (C) Representative dot plots of Annexin V/PI staining are shown. The lower left quadrant contains the vital (double negative) population. The lower right quadrant contains the apoptotic (Annexin V+/PI+) population. Finally, cells in the top right quadrant (Annexin V+/ PI+) are in later stages of apoptosis and necrosis. (D) PMNs were loaded with $DIOC_6(3)$ and analyzed by FACS.

are needed to assess that antioxidants are beneficial in human diseases (Halliwell, 2009).

Concerning inflammation, the resolution of the process depends on apoptosis of inflammatory cells and their subsequent clearance by macrophage phagocytosis (Hallett et al., 2008). A reduction in neutrophils apoptosis has already been linked to several inflammatory conditions, including rheumatoid arthritis and acute respiratory distress syndrome (Chilvers et al., 2000), and there is some evidence that pharmacological manipulation can reduce or increase the inflammatory cell apoptosis (Ward et al., 1999). Recently, it was also reported that some antioxidant may revert the inhibition of human PMN neutrophil apoptosis, via caspas-3, a principle apoptosis executor, induced by reactive oxygen species (Zielinska-Przyjemska & Ignatowicz, 2008). Previously, we have shown that caffeoyl conjugates exert a marked notable activity on some of the biological functions of PMN implicated in the initiation and maintenance of inflammation (Góngora et al., 2002). The high content of phenolic compounds in *H. multiflorum* extract could play an important role in its proapoptotic activity.

The potent antioxidant activity and the mechanism of its cytotoxic effect suggest that *H. multiflorum* extract may be an excellent source of compounds with protective effects against oxidative damage occurring in different pathological conditions. Further investigations are necessary to determine which components of *H. multiflorum* extract are responsible for such antioxidant and apoptotic capacities and whether they actually work *in vivo*, diminishing oxidative damage at the correct site of action.

Conclusion

Extracts of vegetables rich in phenolics are of increasing interest in several fields. These are used in pharmaceutical and food industries because they could retard oxidative degradation of different compounds, and thereby improve the quality or nutritional value of final product. From the medical point of view, supplementation of antioxidants would be useful for diseases associated with oxidative stress.

In this work, we demonstrated for the first time that an aqueous extract of *H. multiflorum*, herb widely used in Argentina, has strong antioxidant capacity in the different models used. The extract also showed cytotoxic activity on PMN cells and this effect, observed at higher concentration than that responsible for the antioxidant activity, seems to be produced by apoptotic mechanisms. *H. multiflorum* extract deserves further investigation as it might be used in the field of pharmaceutical products and functional foods.

Acknowledgement

Declaration of interest

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