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Study of the Potential Value of *Ilex affinis* (Aquifoliaceae) as a Novel Source for the Food and Pharmaceutical Industries

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Abstract: *Ilex paraguariensis* St. Hilaire (Aquifoliaceae) is processed industrially to produce the commercial product “yerba mate” which is used as a tea-like beverage. It is one of the most commercialized plants of South America. It is exported to the US, Europe and Asia as vegetal drug or extracts used in complementary and alternative medicine and in formulations for functional foods due to its properties as a CNS stimulant, diuretic, weight reducing, antioxidant and antihypercholesterolemic, among others. *Ilex affinis* grows in the same habitat and is used as substitute or adulterant of *I. paraguariensis*. This species was never investigated before. The objective of this work was to assess the phytochemical composition and to determine the pharmacological activity, according with the major compounds present in it. The results showed small quantities of caffeine and theobromine, but a considerable amount of polyphenols, especially chlorogenic acid and isochlorogenic acid. *I. affinis* extracts presented scavenging activity on free radical DPPH in a concentration-dependent manner. Antiproliferative action on lymphoma cell line exerting both cytostatic and cytotoxic activities was also demonstrated.

Key words: *Ilex affinis*, *Ilex paraguariensis*, polyphenols, chlorogenic acid, antiproliferative activity.

1. Introduction

Ilex paraguariensis St. Hilaire (Aquifoliaceae) is a plant which grows naturally in NE Argentina, SE Brazil, E Paraguay and Uruguay and it is cultivated in the first three countries [1]. This species is industrially processed to produce the commercial product “yerba mate”, used as a tea-like beverage [2]. It is one of the most commercialized plants of South America where approximately 30% population drink more than 1 L/day of this beverage [3]. It is exported to the US, Europe and Asia as vegetal drug or extracts used in complementary and alternative medicine and in formulations for functional foods due to its properties as a CNS stimulant, diuretic, weight reducing, antioxidant

and antihypercholesterolemic, among others [4].

Some related species of the genus *Ilex* from the same habitat, are used as substitutes or adulterants of *I. paraguariensis*. They are: *I. affinis*, *I. dumosa*, *I. brevicuspis* and *I. brasiliensis*, among others [5]. *I. affinis* has never been reported in Argentina. Collection campaigns conducted recently in the province of Misiones allowed to find this species in our country [6]. According to our knowledge, no phytochemical or pharmacological researches on this species were performed previously.

The presence of caffeoyl derivative compounds (caffeic acid, chlorogenic and isochlorogenic acids) in *I. paraguariensis*, *I. dumosa*, *I. brevicuspis* and *I. brasiliensis* has been reported [7-9]. *I. paraguariensis* also contain considerable amounts of methyl xanthines [2].

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Nowadays, *I. dumosa* is cultivated and it has recently been included in the Argentina Food Code, as an herb for infusions, to be used mixed with *I. paraguariensis* [10].

I. brevicuspis showed antioxidant, choleric and intestinal propulsion activity in rats [7]. In previous works, we demonstrated that *I. brasiliensis* exerted antiproliferative and apoptosis activity on a lymphoma cell line and chlorogenic acid proved to be one of the compounds associated to this activity [9].

There is an increasing interest in the antioxidant effects of compounds derived from herbs that could be relevant in relation to their nutritional effects and their role in health diseases.

Wine is one of the most important social beverages among Europeans. Many studies showed the cardioprotective properties of red wine. Resveratrol has been identified as one of the more powerful bioactives, but many other compounds including cinnamic acid derivatives, tannins and other polyphenols have been related to its beneficial effects [11, 12].

Low incidence of diabetes was linked to consumers of products containing chlorogenic acids. Green coffee beans may contain up to 55% of chlorogenic acids [13].

In a previous work, *L. paraguariensis* showed 2-times higher antioxidant activity compared to red wine [14]. The antioxidant properties of *I. paraguariensis* have been linked to the health benefits of yerba mate.

Taking into account the results of our previous investigations on *Ilex* spp., the objective of this work was to assess the phytochemical composition of *I. affinis* and to determine the pharmacological activity, according with the major compounds present in it.

2. Material and Methods

2.1 Plant Material

I. affinis was collected in San Ignacio, province of Misiones, Argentina and a sample was taken from an abundant specimen—leg. Keller & Keller 9588—kept at the herbarium BAF.

2.2 Preparation of Plant Extracts

Dried leaves were ground to fine powder. Decoctions extracts were prepared in order to compare with preparations commonly used by local people. One gram was boiled with 10 mL of water during 20 min, then it was cooled to 40-45 °C, filtered and the volume adjusted to 5 mL.

2.3 Determination of Caffeoyl Derivative Compounds, Flavonoids and Methylxanthines by HPLC

Previously validated methods were used for the analysis of caffeoyl derivative compounds, flavonoids and methylxanthines [2, 7]. A reverse phase column applying two different gradients, using as the mobile phase: Solvent A: water:acetic acid (98:2); solvent B: methanol:acetic acid (98:2). For the analysis of caffeoyl derivative compounds, the gradient used was: 15% B to 40% B, 30 min; 40% B to 75% B, 10 min; 75%B to 85% B, 5 min. Flow rate: 1.2 mL/min. For the analysis of methylxanthines the gradient used was: 17% B to 20% B, 10 min; 20% B isocratically, 5 min; 20% B to 23% B, 10 min; 23% B to 100% B, 5 min. Flow rate: 1.0 mL/min. The separation column was IB-SIL RP 18 (5 µm, 250 × 4.6 mm I.D.) Luna. Detection was carried out by UV Varian 9050 UV Detector and Varian 9065 Photodiode-Array Detector. UV: 325 nm (caffeoyl derivatives); 255 nm (flavonoids); 273 nm (methylxanthines). The equipment had a Rheodyne injector, fitted with a 100 µL loop. Quantification was achieved by the external standard method using standards compounds (Sigma-Aldrich Argentina, Buenos Aires) The amount of 3,4-dicaffeoylquinic, 3,5-dicaffeoylquinic and 4,5-dicaffeoylquinic isomer acids were calculated and expressed as cynarin (1,5-dicaffeoylquinic acid).

2.4 Determination of the Free Radical Scavenging Activity by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) Free-Radical Scavenging Assay

Scavenging activities of *I. affinis* extracts on the stable free radical DPPH were assayed using the

modified Bloi's method in which the bleaching rate of DPPH is monitored at a characteristic wavelength in presence of the sample [15]. Briefly a volume of 0.1 mL of an aqueous dilution of the extracts were mixed with 0.5 mL of a 500 M DPPH solution in absolute ethanol and 0.4 mL of a 0.1 M Tris-ClH buffer pH 7.4. The mixture was kept for 20 min in the darkness and then the absorbance was read at 517 nm. The percentage of decrease of DPPH bleaching was calculated by measuring the absorbance of the sample and applying the following equation:

$$\text{Inhibition (\%)} = [1 - (As/Ao)] \times 100$$

where: *As* is absorbance of sample (*I. affinis* extracts) and *Ao* is the absorbance of the DPPH solution. A standard ascorbic acid solution 100 µg/mL was used as positive control for antioxidant activity.

2.5 Proliferation, Viability and Apoptosis Assays

A tumoral cell line called EL4 was used. EL4 cells (ATCC) are a T cell lymphoma induced in a C57BL mouse by 9,10-dimethyl-1,2-benzanthracene. The cells were cultured at optimal concentrations in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and antibiotics: 100 U/mL penicillin and 100 µg/mL streptomycin. The effect on proliferation was evaluated by the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl tetrazolium bromide (Sigma, Buenos Aires, Argentina) method. Cells were incubated alone or in presence of different concentrations of *I. affinis* extracts (from 0.01 to 1,000 µg/mL) for 24 h. Then, MTT was added and the purple formazan formed was solubilized by addition of acidic iso-propanol. The absorbance was measured at 570 nm and results were expressed as percentage of proliferation. The same method was employed to determine cell viability and in this case results were expressed as percentage of viability relative to control [16].

2.6 Statistical Analysis

Data were expressed as means ± SD or SEM of two

or three independent experiments carried out in duplicate. A one-way ANOVA with a posteriori the Dunnett's test were used to evaluate the significancy of results. A probability (*P*) value < 0.01 was considered significant [17].

3. Results and Discussion

The identification of compounds present in the extracts was carried out using validated HPLC methods and determined by the coincidence of their retention times with those of reference compounds and the UV/Vis spectra provided by the DAD detector.

The phytochemical study showed traces of methylxanthines (caffeine and theobromine) and a considerable amount of polyphenols. The following compounds were isolated and quantified and the results are expressed as % on dried weight: a) caffeoyl derivative compounds (chlorogenic acid: 0.0398 ± 0.0004; 3,4-dicaffeoylquinic acid: 0.0166 ± 0.0001; 3,5-dicaffeoylquinic acid: 0.0296 ± 0.0003 and 4,5-dicaffeoylquinic acid: 0.0392 ± 0.0004); b) flavonoids (rutin: 0.134 ± 0.001 and quercetin: 0.0066 ± 0.0001). Kaempferol was not detected. Detection limit: 0.2 ppm. Quantification limit: 1.0 ppm.

There is evidence that plant-derived compounds may have beneficial effects on human health and that some of them (caffeoyl derivatives and flavonoids) exert antioxidant activity [18, 19].

In this study, *I. affinis* extracts presented scavenging activity on free radical DPPH in a concentration-dependent manner. The concentration of 100 µg/mL exerted an scavenging effect similar to that exerted by the antioxidant control ascorbic acid (100 µg/mL) (Fig. 1).

I. affinis showed antiproliferative activity on a lymphoma cell line. One drug can decrease cell proliferation by a cytostatic or a cytotoxic action. Cytostatic effect is exerted when a drug decreases cell proliferation, but does not modify cell viability; really, the decrease in cell proliferation is not due to the

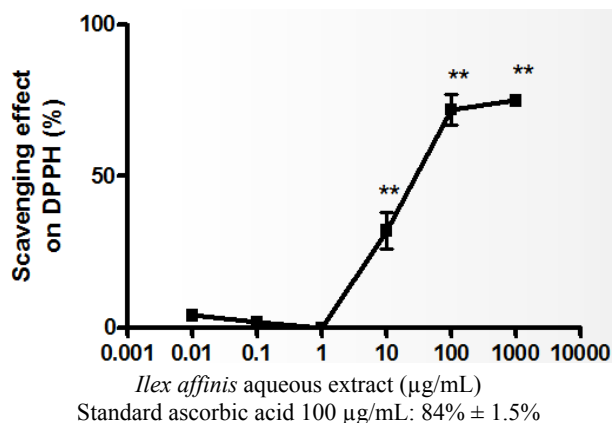


Fig. 1 Effect of different concentrations of *I. affinis* on free radical (DPPH) elimination. Results represent mean ± SEM of two experiments made by duplicate. ** $P < 0.01$ significantly differences between control (DPPH alone) and treatments accordingly with ANOVA + Dunnett's test.

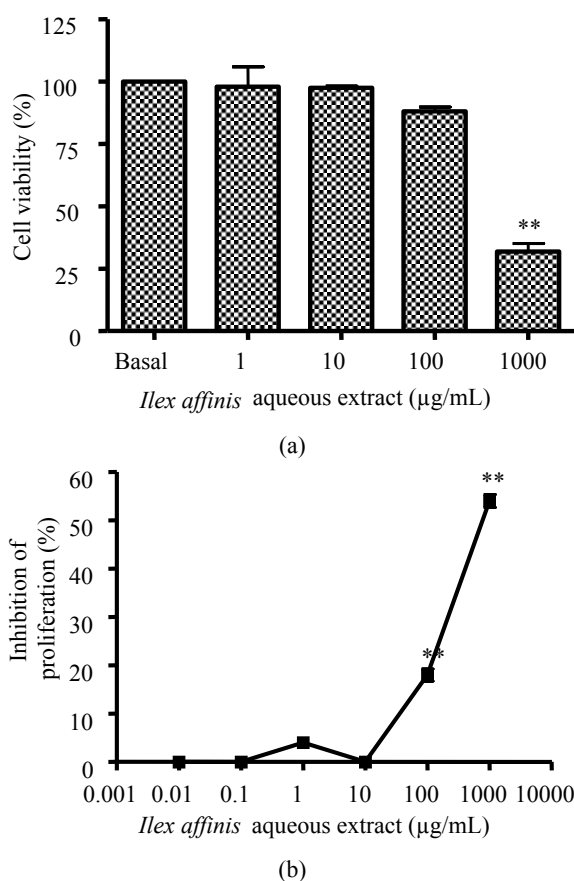


Fig. 2 Effect of different concentrations of *I. affinis* on lymphoma cell proliferation (A) and on cell viability (B). Cells were incubated during 24 h with the aqueous extract from 0.01 to 1,000 µg/mL. Results represent mean ± SEM of three experiments made by triplicate. ** $P < 0.01$ significantly differences between control and treatments in accord with ANOVA + Dunnett's test.

increase of cell mortality. Cytotoxic effect is exerted when a drug decreases cell proliferation, but a decrease in cell viability is also observed, so the drug decreases cell proliferation because it is killing the cells.

The activity displayed by *I. affinis* occurred by both, cytostatic and cytotoxic effects as it is shown in Fig. 2. The effect depended on the analyzed concentrations following a concentration-response relation-ship.

Antiproliferative action has been observed in other *Ilex* species, e.g., an *I. paraguariensis* extract was shown to exert an antiproliferative effect on an oral carcinoma by inhibition of topoisomerase II [20]; this specie was also reported to inhibit the growth of ras-transformed endothelial cells [21]. *I. brasiliensis* also exerted antiproliferative and apoptosis activity on a lymphoma cell line [9].

4. Conclusions

I. affinis aqueous extracts showed considerable amounts of polyphenols, especially chlorogenic acid and isochlorogenic acid and exerted antioxidant activity. Antiproliferative action on lymphoma cell line exerting both cytostatic and cytotoxic activities was also demonstrated. The results obtained in this work suggest the potential value of *I. affinis* for the development of novel products in the food and pharmaceutical industries.

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Study of the Potential Value of *Ilex affinis* (Aquifoliaceae) as a Novel Source for the Food and Pharmaceutical Industries

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