Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 97 (2006) 459–464
Food Chemistry
www.elsevier.com/locate/foodchem

Peroxidase-like activity of *Ilex paraguariensis*

Claudia Anesini *, Graciela Ferraro, Rosana Filip

Institute of Drug Chemistry and Metabolism (IQUIMEFA-UBA-CONICET), National Research Council, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Junin 956, Buenos Aires, Argentina

Received 23 August 2004; received in revised form 10 May 2005; accepted 10 May 2005

Abstract

Ilex paraguariensis St. Hil. (Aquifoliaceae) is widely exploited in northeastern Argentina, southern Brasil and eastern Paraguay. The commercial product named "Mate" or "*Yerba Mate*" is used to prepare a tea like beverage (infusions or decoctions).

Reactive oxygen species (ROS) are well known to have an active role in different disorders such as rheumatoid arthritis, diabetes mellitus, vascular, neurodegenerative and periodontal disease. Normally, enzymes such as peroxidases, superoxide dismutase and catalase eliminate ROS. In this study, the peroxidase-like activity of aqueous extracts from *I. paraguariensis* and *I. paraguariensis* commercial sample "*Yerba Mate*" was determined. Caffeoyl derivatives (chlorogenic and caffeic acid) and total polyphenols content were determined in order to establish a relationship between their content and their peroxidase activity.

Both aqueous extracts showed peroxidase-like activity, but *I. paraguariensis* was significantly more active than "*Yerba Mate*".

The antioxidant activity expressed as peroxidase-like activity was evidently related to total polyphenol content.

Keywords: Peroxidase activity; *I. paragauariensis*; Yerba Mate; Antioxidative action

1. Introduction

Reactive oxygen species (ROS) are well known to induce cellular and tissue pathogenesis leading to numerous disorders, including rheumatoid arthritis (McCord, 1974), diabetes mellitus, vascular, neurodegenerative (Halliwell, Gutterdge, & Cross, 1992) and periodontal disease (Shapira, Borinski, Sela, & Soskolne, 1991) and contribute to the normal cellular ageing. Hydrogen peroxide (H_2O_2) is a ROS that is a substrate for the generation of more active biologically ROS, especially the hydroxyl radical ($^{\cdot}OH$) and hypochlorous acid (HOCl). In normal health conditions, antioxidant enzymes, enzymes cofactors and antioxidant substances naturally scavenge these free radicals. The most important antioxidant enzymes are peroxidases, superoxide dismutase and catalase. Low levels of these enzymes are implicated in diseases such as cancer, immune system related diseases and cardiovascular disorders.

Different polyphenols have also been shown to be potent antioxidants interfering with the oxidative/antioxidative potential of the cell or acting as free radicals scavengers (Lodovici et al., 2001). This antioxidant activity was related to their ability to prevent liposomes oxidation. There are reports of the antioxidant activity of caffeoyl derivatives and flavonoids (Wang, Nair, Strasburg, Booren, & Gray, 1999) supported by further studies which proved their role as protective agents against cardiovascular diseases and breast, gastrointestinal and skin cancer (Carbonaro, Grant, & Pusztai, 2001).

Many synthetic antioxidant compounds have shown toxic or mutagenic effects, which have shifted the attention to naturally occurring antioxidants. Due to

* Corresponding author. Tel.: +54 11 4964 8247; fax: +54 11 4508 3642.
E-mail address: canesini@yahoo.com.ar (C. Anesini).

0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.05.025

the low content of antioxidants in the normal daily diet, interest has greatly increased recently in finding naturally occurring antioxidants for use in foods and dietary supplements.

Ilex paraguariensis St. Hil. (Aquifoliaceae) is widely used in northeastern Argentina, southern Brasil and eastern Paraguay (Giberti, 1979). The commercial product made with it, named "Mate" or "*Yerba Mate*" is used to prepare a tea-like beverage (infusions or decoctions) appreciated for its particular flavour. This "*Yerba Mate*" is obtained through an industrial process that includes a "zapecado" or roasting at 750 °C during 30 s. This tea is used against mental and physical fatigue (due to the xanthines: caffeine and theobromine content) (Filip, López, Coussio, & Ferraro, 1998) and nutritional properties. It is also used in folk medicine for gastrointestinal disorders as eupeptic and choleretic drugs (Alonso Paz, Bassagoda, & Ferreira, 1992; Gorzalczany et al., 2001). Caffeoyl derivatives have been isolated from this species (Clifford & Ramirez-Martinez, 1990; Filip, López, Giberti, Coussio, & Ferraro, 2001). "*Yerba Mate*" is recognized worldwide for its nutritional and medicinal value being included in important national food codes such as the Argentine Food Code, Latinamerican Food Code and Pharmacopoeias such as Martindale, British Herbal Pharmacopoeia, and German Commission E Monographs.

A previous paper reported the choleretic and antioxidant properties of another *Ilex* species, *I. brevicuspis,* a substitute or adulterant of "*Yerba Mate*" (Filip & Ferraro, 2003).

In view of these findings, the evaluation of the peroxidase-like activity of extracts from natural *I. paraguariensis* and *I. paraguariensis* commercial sample "*Yerba Mate*" was undertaken.

The caffeoyl derivatives, chlorogenic acid and caffeic acid were detected and quantified by HPLC with a diode array detector and the total polyphenol content was determined by spectrophotometry. Caffeine content was also determined and the peroxidase-like activity of these compounds was assayed in order to relate the antioxidant activity with the presence of these compounds in the analysed extracts.

2. Materials and methods

2.1. Chemicals

3-3′-Diaminobenzidine tetrahydrochloride (DAB), horseradish peroxidase type IV were purchased from the Sigma Chemical Co, St. Louis, MO, USA. An stock solution, in distilled water, of peroxidase (37.5 U/ml) were used to prepared different concentrations. The others drugs were freshly prepared in distilled water to achieve the final concentrations cited below (see 2.6).

2.2. Plant material

Leaves of *I. paraguariensis* were collected from their original habitat and identified employing morphological, anatomical and histochemical techniques. Voucher specimens were deposited in CEFYBO-CONICET under the BACP number: 502. "*Yerba Mate*" derived from a commercial sample.

2.3. Preparation of plant extracts

The dried material was ground to a fine powder; 10 g of each sample was boiled with 200 ml of water for 20 min and left to cool at room temperature to 40–45 °C. After filtration with (Whatman No. 1) filter paper, extracts were lyophilized using a flexi-dry FTS Systems, yielding the following residues of aqueous crude extracts: *I. paraguariensis,* 1.25 g; "*Yerba Mate*", 1.24 g.

2.4. High performance liquid chromatography

A Varian™ series 9000 instrument equipped with a binary Varian 9012 pump was used. Quantitation of caffeoyl derivatives and caffeine was achieved using validated HPLC external standard method (Filip et al., 1998, 2001). Pure standards were obtained from Carl Roth. A reverse phase IB-SIL RP 18 (5 μm, 250 × 4.6 mm I.D.) Phenomenex column and a gradient consisting of Solvent A: water:acetic acid (98:2); Solvent B: methanol:acetic acid (98:2). was used. For caffeoyl derivatives the gradient was: from 15% B to 40% B, 30 min; 40% B to 75% B, 10 min; 75% B to 85% B, 5 min, with a flow rate of 1.2 ml/min. For caffeine the gradient was: from 17% B to 20% B, 10 min; 20% B (isocratic), 5 min; 20% B to 23% B, 10 min, 23% B to 100% B, 5 min with a flow rate of 1.0 ml/min. Identification and quantitation was carried out by simultaneous detection with a UV Varian 9050 UV detector and Varian 9065 Photodiode-Array Detector at 325 nm for caffeoyl derivatives and 273 nm for caffeine. Samples were injected with a Rheodyne injector fitted with a 100 μl loop.

2.5. Spectrophotometric determination of total polyphenol content

Quantification was performed using a of a Shimadzu U.V.2101-3101 PC system and chlorogenic acid (Carl Roth) as a standard. A linear relationship (λ_{max} 330 nm) between area and concentration was observed in the range of 0.12–2.5 g% using six different concentrations. The regression equation of the curve was: $y = 0.4274x - 0.0107$ with a regression coefficient of determination $r^2 = 0.9992$.

Each concentration was measured three times and the intraassay variation coefficient was below 2%. Suitable amounts of standard chlorogenic acid were added to a

sample of *I. paraguarensis* and then analysed by the procedure stated above. The results showed that the recoveries were: 96.7–102.0% (Filip, Lotito, Ferraro, & Fraga, 2000).

2.6. Determination of peroxidase-like activity

Peroxidase activity was determined by the method described by Herzog and Fahimi (1973). Different concentrations of each extract (from 10 to 10.000 μg/ml), chlorogenic acid, caffeic acid and caffeine (from 1 to 20.000 μg/ml) were analysed. To do this, 200 μl of each sample was incubated with 775 μl of DAB (5×10^{-4} M) and 25 μl of H_2O_2 (solution of H_2O_2 Parafarm R, 30% v/v diluted 1/86 in distilled water). The reaction started by the addition of H_2O_2. DAB without H_2O_2 was used as blank. Final volume in reaction tube: 1000 μl. Then, change in absorbance readings were recorded at 30 s intervals for 5 min using a Shimadzu recording spectrophotometer UV-240 (graphic printer PR-1) set at 465 nm, and Δ absorbance/min was calculated. A standard curve with a linear relationship between activity concentration and Δ absorbance/min of radishhorse peroxidase, in the range of 1.95×10^{-3} to 2.5×10^{-5} U/ml, was obtained. The activity of samples was calculated by interpolation from the standard curve.

Results were expressed as peroxidase activity $\times 10^{-4}$ (U/ml) or as hydrogen peroxide removal (nM)/min (calculated taken into account international units definition: one units of peroxidase catalysed the descomposition of 1 μM of H_2O_2/min at 25 °C). Results represent the Media ± SEM of three or more experiments performed by triplicate. EC_{50} represented the concentration of extracts or isolated compounds, which produced a removal of hydrogen peroxide of 50%. EC_{50} values were calculated by the method of Alexander (Alexander, Browse, Reading, & Benjamin, 1999).

2.7. Statistical analysis

Student's "*t*" test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary, the Dunnett's test (Dunnett, 1964) was applied after ANOVA. Differences between means were considered significant if $P < 0.05$.

3. Results and discussion

The extracts assayed (*I. paraguariensis* and a commercial sample of *I. paraguariensis* "*Yerba Mate*") exhibited peroxidase-like activity. Fig. 1 shows that the

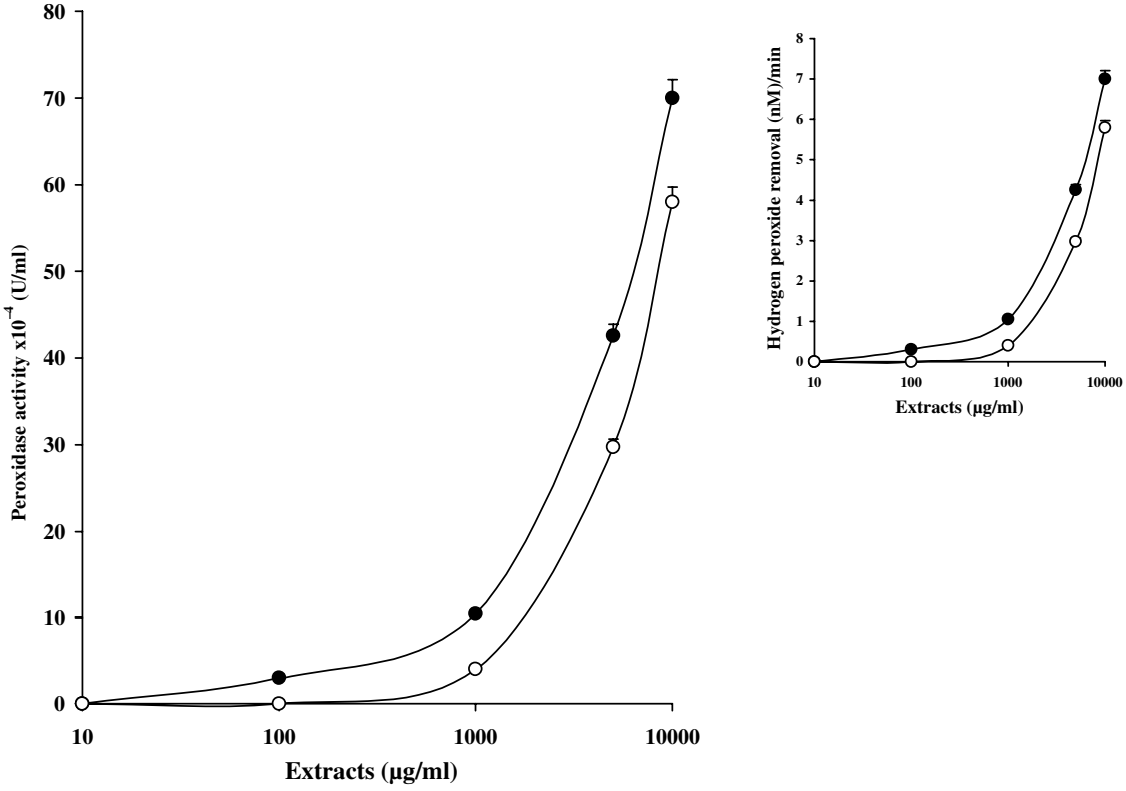

Fig. 1. Effect of aqueous extracts from *I. paraguariensis* and "*Yerba Mate*" aqueous extract on hydrogen peroxide removal. Different concentrations of the extracts were incubated in presence of reactives (DAB and H_2O_2) to determine peroxidase-like activity. Results are expressed as peroxidase activity $\times 10^{-4}$ (U/ml) and as hydrogen peroxide removal (nM)/min and represent the media ± SEM of three or more experiments performed in triplicate. (•) *I. paraguariensis* and (○) "*Yerba Mate*".

aqueous extract of *I. paraguariensis* was significantly more active than "*Yerba Mate*", as indicated in Table 1 when comparing the EC_{50} values.

Different preparations from plant material exert peroxidase activity because the enzyme is present as was observed in *Latuca sativa* L. (Bestwick, Brown, & Mansfield, 1998). In our study, the extracts were prepared in boiling water, which necessarily denaturalized any enzymes present in them. It was observed that temperatures of 60 °C or less induce the protein globule to unfold resulting in the loss of the peroxidase activity of peroxidase present in horseradish (Artyukhov, Basharina, & Iskusnykh, 2003). Peroxidase activity found in the African oil palm tree *Elaeis guineensis* is lost at 72 °C (Rodríguez et al., 2002). Therefore, the peroxidase-like activity found in our aqueous extracts could only be due to the compounds present in them.

Antioxidant activity of *Ilex* species was previously demonstrated in others systems (Filip et al., 2000). Peroxynitrite and lipoxygenase-induced human LDL oxidation are inhibited by *I. paraguariensis* extracts in a potent, dose-dependent fashion (Bracesco et al., 2003).

It has been proved that polyphenols exert antioxidant activity (Lodovici et al., 2001). In order to establish the relationship between the total phenolics content and the antioxidant activity, total polyphenols were quantified in the crude extracts. The extract of *I. paraguariensis* contained a significantly higher polyphenol content than commercial "*Yerba Mate*" (Table 2). The higher enzymatic activity found in *I. paraguariensis* extract could be related to the higher polyphenols content. Hence, there was a positive correlation between the polyphenolic compounds content in the aqueous extracts and peroxidase-like activity. Among the polyphenols investigated, chlorogenic and caffeic acids were identified and quantified by HPLC in the aqueous extracts. The retention times of the identified compounds were: chlorogenic acid: 10.9 min; caffeic acid: 13.0 min.

Table 2 shows that the content of chlorogenic acid was higher than caffeic acid in both *I. paraguariensis* and "*Yerba Mate*". Furthermore, the content of chlorogenic acid was higher in *I. paraguariensis* than "*Yerba Mate*". The capacity of chlorogenic acid and caffeic acid to eliminate H_2O_2 was analysed and the results show that both compounds presented peroxidase-like activity at these levels of concentration (Fig. 2). The range of concentrations tested were chosen according the content of chlorogenic and caffeic acid present in the crude extracts, as shown in Table 2. Caffeic acid exhibited higher activity than chlorogenic acid, as was shown by EC_{50} values, reported in Table 3.

Table 1
EC_{50} of *Ilex paraguariensis* extract and *Yerba Mate* extracts for hydrogen peroxide removal

Extracts	EC_{50} (μg/ml)
Ilex paraguariensis	3364 ± 100
Yerba Mate	4774 ± 150*

The EC_{50} values represented the concentration of extracts, which decreased the hydrogen peroxide concentration in about 50%.
* $P < 0.05$ significant differences of EC_{50} of Yerba Mate with respect to EC_{50} of *Ilex paraguariensis*, according to Student's *t* test.

Table 2
Polyphenols, chlorogenic and caffeic acids content in *I. paraguariensis* and *Yerba Mate* extracts

Compounds	*Ilex paraguariensis*	Yerba Mate
Total polyphenol contents	10.71 ± 0.40	6.89 ± 0.29*
Chlorogenic acid	2.80 ± 0.30	1.98 ± 0.37**
Caffeic acid	0.023 ± 0.004[a]	0.020 ± 0.003***

Total polyphenol content in *I. paraguariensis* and commercial "*Yerba Mate*" was determined by spectrophometry. The results are shown as mean ± SD and expressed as equivalents of chlorogenic acid (g/100 g dried plant material).
[a] Significantly differences of caffeic acid contents respect to chlorogenic acid of *I. paraguariensis*. Significantly differences accordingly to Dunnett's test.
* $P < 0.05$ significantly differences of the polyphenol contents of *Yerba Mate* with respect to polyphenol contents of *I. paraguariensis*, according with Student's *t* test. The identification and quantification of chlorogenic and caffeic acids, were determinated by HPLC. The values are expressed in (g/100 g dried plant material).
** Significantly differences of chlorogenic acid contents of "*Yerba Mate*" respect to chlorogenic acid content of *I. paraguariensis* ($p < 0.05$).
*** Significantly differences of caffeic acid content respect to chlorogenic acid of "*Yerba Mate*" ($p < 0.05$).

The antioxidant activity of chlorogenic and caffeic acids has also been determined in others systems. For example, their capacity to enhance the ability of plasma to reduce the ferric ion by about 10% was reported. (Lekse, Xia, Stark, Morrow, & May, 2001). Moreover, chlorogenic acid and its isomers neochlorogenic acid and cryptochlorogenic acid isolated from *Prunus domestica* possess scavenging activity against the superoxide anion radical and an inhibitory effect against oxidation of methyl linoleate (Nakatani et al., 2000). Chlorogenic acid and caffeic acid also, possess aminooxidase-like activity, as reported by Akagawa and Suyama (2001).

Taking into account that there was no significant difference ($p > 0.05$) between the caffeic acid content in the extracts of *I. paraguariensis* and "*Yerba Mate*", it seemed highly unlikely that the caffeic acid content could be responsible for the the significant differences in peroxidase-like activity exerted by the *I. paraguariensis* extract. On the other hand, it could possible be that chlorogenic acid participated in the higher activity exerted by *I. paraguariensis* apart from others polyphenolic compounds such as the flavonoids quercetin and rutin present in it (Filip et al., 2001). Also, the presence of others polyphenolic compounds coud be related with the higher activity exerted by the crude extracts in comparison with pure compounds.

Caffeine is a well known component of *I. paraguariensis* (Filip et al., 1998). Its peroxidase activity was as-

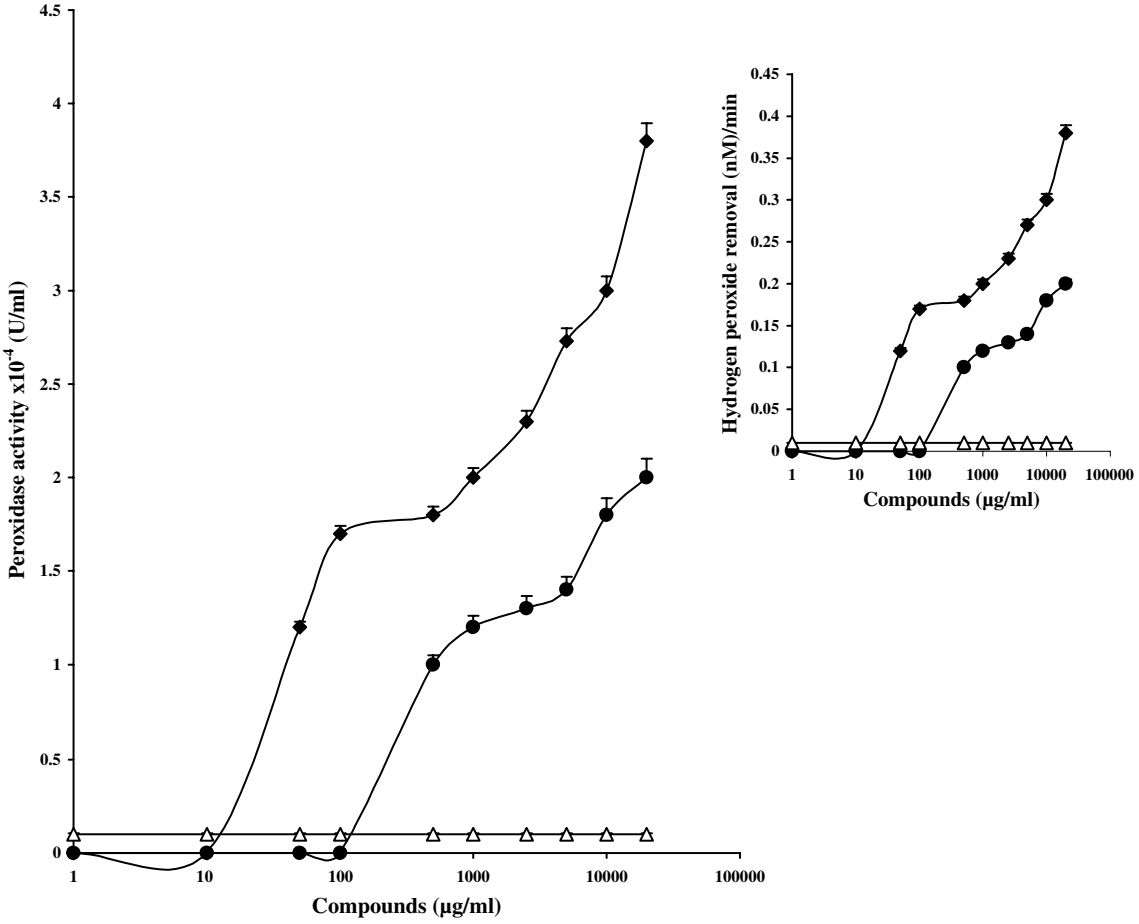

Fig. 2. Effect of Chlorogenic acid, caffeic acid and caffeine on hydrogen peroxide removal. Different concentrations of the compounds were incubated in presence of reactives (DAB and H_2O_2) to determine peroxidase-like activity. Results are expressed as peroxidase activity $\times 10^{-4}$ (U/ml) and as hydrogen peroxide removal (nM)/min and represent the media ± SEM of three or more experiments performed in triplicate. (◆) Caffeic acid, (●) chlorogenic acid and (△) caffeine.

Table 3
EC_{50} of chlorogenic and caffeic acid on hydrogen peroxide removal

Compounds	EC_{50} (µg/ml)
Chlorogenic acid	676 ± 60
Caffeic acid	98.62 ± 5.0*

The EC_{50} values represented the concentration of extracts, which diminished the hydrogen peroxide concentration in about 50%.
* $P < 0.05$ significantly differences of EC_{50} of caffeic acid with respect to EC_{50} of chlorogenic acid, according with Student's t test.

sayed in order to evaluate its possible contribution to the activity observed in the extract. The results showed no peroxidase activity at the concentrations analysed (Fig. 2)

4. Conclusions

The results obtained in this work proved the peroxidase-like activity of *I. paraguariensis* and "*Yerba Mate*" aqueous extracts, commonly used by people, and show their potential as antioxidants and therefore chemoprotective agents.

Acknowledgement

This work was carried out with the aid of grant from University of Buenos Aires UBACYT: B090.

References

Akagawa, M., & Suyama, K. (2001). Aminooxidase-like activity of polyphenols. Mechanism and properties. *European Journal of Biochemistry, 268*, 1953–1963.

Alexander, B., Browse, D. J., Reading, S. J., & Benjamin, I. S. (1999). A simple and accurate mathematical method for calculation of the EC_{50}. *Journal of Pharmacological and Toxicological Methods, 41*, 55–58.

Alonso Paz, E., Bassagoda, M., & Ferreira, F. (1992). Yuyos. In *Uso racional de Plantas Medicinales* (pp. 114–115). Uruguay: Ed. Fin de Siglo.

Artyukhov, V. G., Basharina, O. V., & Iskusnykh, A. Yu. (2003). Peculiarities of structural and functional properties of thermomodified horseradish peroxidase. *Ukrains'kii Biokhimichnii Zhurnal, 75*, 45–49.

Bestwick, C. S., Brown, I. R., & Mansfield, J. W. (1998). Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. *Plant Physiology, 118*, 1067–1078.

Bracesco, N., Dell, M., Rocha, A., Behtash, S., Menini, T., Gugliucci, A., et al. (2003). Antioxidant activity of a botanical extract preparation of *Ilex paraguariensis*: prevention of DNA double-strand breaks in *Saccharomyces cerevisiae* and human low-density lipoprotein oxidation. *Journal of Alternative and Complementary Medicine, 9*, 379–387.

Carbonaro, M., Grant, G., & Pusztai, A. (2001). Evaluation of polyphenol bioavailability in rat small intestine. *European Journal of Nutrition, 40*, 84–90.

Clifford, M. N., & Ramirez-Martinez, J. R. (1990). Chlorogenic acids and purine alkaloids contents of Maté (*Ilex paraguariensis*) leaf beverage. *Food Chemistry, 35*, 13–21.

Dunnett, C. W. (1964). New tables of multiple comparisons with a control. *Biometrics, 20*, 482–491.

Filip, R., & Ferraro, G. E. (2003). Researching of new species of "Mate": *Ilex brevicuspis*. Phytochemical and pharmacology study. *European Journal of Nutrition, 42*, 50–54.

Filip, R., López, P., Coussio, J., & Ferraro, G. E. (1998). "Mate substitutes or adulterants": study of xanthine content. *Phytotherapy Research, 12*, 129–131.

Filip, R., López, P., Giberti, G., Coussio, J., & Ferraro, G. (2001). Phenolic compounds in seven South American *Ilex* species. *Fitoterapia, 72*, 774–778.

Filip, R., Lotito, S. B., Ferraro, G. E., & Fraga, C. G. (2000). Antioxidant activity of *Ilex paraguariensis* and related species. *Nutrition Research, 20*, 1437–1446.

Giberti, G. (1979). Las especies argentinas del género *Ilex* (Aquifoliaceae). *Darwiniana, 22*, 217–240.

Gorzalczany, S., Filip, R., Alonso, M. R., Miño, J., Ferraro, G. E., & Acevedo, C. (2001). Choleretic effect and intestinal propulsion of "Mate" (*Ilex paraguariensis*) and its substitutes or adulterants. *Journal of Ethnopharmacology, 75*, 291–294.

Halliwell, B., Gutterdge, J. M., & Cross, C. E. (1992). Free radicals. Antioxidants and human disease: where are we now. *The Journal of Laboratory and Clinical Medicine, 119*, 598–620.

Herzog, V., & Fahimi, H. D. (1973). A new sensitive colorimetric assay for peroxidase using 3,3′-diaminobenzidine as hydrogen donor. *Analytical Biochemistry, 55*, 554–562.

Lekse, J. M., Xia, L., Stark, J., Morrow, J. D., & May, J. M. (2001). Plant catechols prevent lipid peroxidation in human plasma and erythrocytes. *Mollecular and cellular Biochemistry, 226*, 89–95.

Lodovici, M., Guglielmi, F., Casalini, C., Meoni, M., Cheynier, V., & Dolara, P. (2001). Antioxidant and radical scavenging properties in vitro of polyphenolic extracts from red wine. *European Journal of Nutrition, 40*, 74–77.

McCord, J. M. (1974). Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. *Science, 185*, 529–531.

Nakatani, N., Kayano, S., Kikuzaki, H., Sumino, K., Katagiri, K., & Mitani, T. (2000). Identification, quantitative determination and antioxidative activities of chlorogenic acid isomers in prune (*Prunus domestica* L.). *Journal of Agricultural and Food Chemistry, 48*, 5512–5516.

Rodríguez, A., Pina, D. G., Yélamos, B., Castillo León, J. J., Zhadan, G. G., Villar, E., et al. (2002). Thermal stability of peroxidase from the african oil palm tree *Elaeis guineensis*. *European Journal of Biochemistry, 269*, 2584–2590.

Shapira, L., Borinski, R., Sela, M. N., & Soskolne, A. (1991). Superoxide formation and chemiluminescence of peripheral polymorphonuclear leukocytes in rapidly progressive periodontitis patients. *Journal of Clinical Periodontology, 18*, 44–48.

Wang, H., Nair, M. G., Strasburg, G. M., Booren, A. M., & Gray, J. I. (1999). Novel antioxidant compounds from tart cherries (*Prunus cerasus*). *Journal of Natural Products, 62*, 86–88.