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DNA Repair Activity of *Ilex paraguariensis* in Human Cells *In Vitro*

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***SUMMARY*. Yerba mate *(Ilex paraguariensis)* has been consumed as popular infusions in South America for centuries, with potential beneficial effects on health. The aim of this study was to evaluate yerba mate antioxidant properties in human leucocytes exposed *in vitro* to oxidative damage, using the Comet assay. Human cells were exposed to 10 μM H_2O_2 and then allowed to repair with increasing concentrations of two different yerba mate infusions: 10 μl/mL, 100 μl/mL and 1000 μl/mL. Damage Index (DI) and the percentage of damage reduction (%DR) were calculated. DI showed that both infusions produced similar protective effects ($p > 0.05$) and the %DR indicated a decreased in oxidative damage as infusion concentration increases ($p < 0.05$). The greatest protective effects were found using the highest concentration of mate (1000 μl/mL). Thus, we conclude that the regular ingestion of *Ilex paraguariensis* infusions could contribute to antioxidant defense on humans.**

RESUMEN. La yerba mate *(Ilex paraguariensis)* se consume como infusión popular en América del Sur desde hace siglos, con potenciales efectos beneficiosos sobre la salud. El objetivo de este estudio fue evaluar las propiedades antioxidantes de la yerba mate en leucocitos humanos expuestos *in vitro* a un daño oxidativo, utilizando el ensayo cometa. Células humanas fueron expuestas a 10 µM de H_2O_2 y luego reparadas con concentraciones crecientes de dos diferentes infusiones de yerba mate: 10, 100 y 1.000 µL/mL. Se calculó el índice de daño (DI) y el porcentaje de reducción de daño (% DR). El DI mostro que ambas infusiones producen efectos protectores similares ($p > 0,05$) y el % DR indicó una disminución en el daño oxidativo al aumentar la concentración de la infusión ($p < 0,05$). Los mayores efectos protectores fueron encontrados con la concentración más alta de mate (1.000 µl/mL). Por lo tanto, concluimos que la ingestión regular de infusiones de *Ilex paraguariensis* podría contribuir a la defensa antioxidante en seres humanos.

INTRODUCTION

Ilex paraguariensis St. Hilaire (Aquifoliaceae), common name 'mate' or 'yerba mate', is an economically very important plant in South America, which grows naturally but is also widely cultivated in Argentina, Brazil and Paraguay. The leaves are used to prepare different beverages, such as mate, tereré and the mate tea. The *per capita* consumption of mate in Brazil is estimated in 1.2 kg per year, while in Argentina and Uruguay people use around 5-7 kg of dried mate per year for different preparations. More recently, mate-based products have no longer been limited to their producing countries; they have reached external markets such as the USA and Europe [1].

I. paraguariensis is also included in the British Herbal Pharmacopoeia [2] and in the Martindale [3] due to the fact that yerba mate is rich in several bioactive compounds such as caffeine, phenolic acids and saponins; these compounds are absorbed by the body and may act as antioxidants or as free radical scavengers [4]. Clinicians and biomedical scientists are interested in antioxidants because they can retard the oxidative damage by increasing those natural defenses. There is an increasing interest in the antioxidant properties of compounds derived

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from herbs which could be relevant in relation to their nutritional incidence and role in health and disease [5]. In particular, *I. paraguariensis* is reputed to have a characteristic bitter taste and hepatoprotective, choleretic, hypocholesterolemic, antioxidant, antirheumatic, diuretic, glycogenolytic and lipolytic properties. In fact, it is employed in commercial herbal preparations as tonic, anticellulitic and antiaging. Some of these pharmacological activities are atributed to the high content of caffeoyl derivatives and flavonoids [6] as well as to the presence of methylxanthines (caffeine, theobromine, and theophylline) and phenolic acids. In effect, several studies with plants have shown a relationship between the polyphenolic content and the free radical-scavenging ability, including studies with mate [1]. Currently, it is well established that oxidative stress is involved in various pathological states such as cancer, cardiovascular disorders, diabetes, arthritis, inflammation and liver diseases. The eukaryotic cells are continuously attacked by reactive oxygen species (ROS), which arise as natural by-products of normal cellular energy production or are generated in large amounts by exhaustive exercise or by chemical agents in the environment. The oxidative damage induced to DNA results in base modifications, single and double strand breaks and the formation of apurinic/apyrimidinic lesions. Since the oxidized adducts of DNA are pro-mutagenic lesions, if they are not repaired, they can result in mutations. In animal models, it was demonstrated that mate infusion is not genotoxic in liver, kidney and bladder cells, and the regular ingestion of mate tea increases the resistance of DNA to H_2O_2-induced DNA strand breaks and improves the DNA repair after H_2O_2 challenge in liver cells, regardless of the dose ingested. These results suggested that mate tea could protect against DNA damage and enhance the DNA repair activity [4].

The comet assay, or single cell gel electrophoresis, is a simple, rapid and sensitive method of detecting DNA single and double strand breaks, alkali sites and cross-linking at the level of the individual cell [7]. It is a useful tool which has been used to evaluate the protective effects and DNA repair of various dietary factors, both *in vitro* and *in vivo* [8].

The aim of this work was to evaluate yerba mate antioxidant properties in human leucocytes exposed *in vitro* to oxidative damage, using the Comet assay.

MATERIALS AND METHODS

Mate samples

Two different samples of yerba mate from commercial brands (infusion 1 and 2) were analyzed. All samples are produced by Argentinean companies. Infusion 1: from Misiones (Argentina state), Rosamonte®, Hreñuk S.A Company. Infusion 2: from Corrientes (Argentina state), Playadito®, Colonia Liebig Ltda. S.A. Company.

Preparation of the extracts

The extracts of yerba mate were prepared as infusions. Mate samples (5 g) were weighed into 125 mL Erlenmeyer flasks and 100 mL of boiling distilled water were added and left to cool down to 40 °C. Both preparations were sterilized through a 0.22 µm filter, and stored at -20 °C.

Blood samples

Blood samples were obtained from fourteen healthy subjects (males and females) by venipuncture, using a heparinized sterile syringe. Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral Ethical Committee established the regulations for the development of the study and informed consent was given by each individual prior to the beginning of the evaluation.

Evaluation of protective capacity against DNA damage: comet assay

The procedure described by Singh *et al.* [7] was used with modifications. Briefly, 50 µL of fresh blood was added to 940 µL of RPMI 1640. Then, human leucocytes were exposed *in vitro* to H_2O_2, by adding 10 µL H_2O_2 1 mM for 10 min at 37 °C. The reaction was quenched using 0.5 mL of 2 % DMSO solution in PBS. Each cell sample was centrifuged at 1000 g, washed again with RPMI 1640, and resuspended in RPMI 1640 medium supplemented with 10 % fetal bovine serum and increasing concentrations of yerba mate infusion (YMI) for 30 min at 37 °C: repair I (RI: 10 µL/mL YMI), repair II (RII: 100 µL/mL YMI) and repair III (RIII: 1000 µL/mL YMI); while another one remained as a control without repair (CWR), with no addition of the infusion. As a positive control (PC), cells in RPMI 1640 were exposed to 50 µM H_2O_2 while the negative control (NC) remained without H_2O_2 [9].

Viability of each cell suspension was evaluated by mean of fluorescent DNA binding dyes [10]. After incubation, samples were centrifuged and

pellets obtained were resuspended in 200 µL of 1 % low-melting point agarose solution (37 °C) and spread onto slides precoated with 1 % normal melting point agarose. The slides were submerged in cold, freshly prepared lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Trizma, 1 % Triton X-100 and DMSO 10 %, pH 10) and left overnight at 4 °C. Then, slides were placed in cold electrophoresis alkaline buffer (10 N NaOH, 200 mM Na_2EDTA, pH > 13) and left for 20 min to allow DNA unwind, before electrophoresis at 25 V and 300 mA (0.75 V/cm) for 20 min too. The slides were then washed with neutralization buffer (Tris 0.4 M, pH 7.5) and the DNA stained with ethidium bromide (0.02 mg/mL) and observed using a fluorescent microscope at 400 X. All procedures were carried out in darkness to avoid additional DNA damage. A total of 100 randomly selected cells were visually analyzed on a scale of 1-4, depending on the grade of damage, and Damage Index (DI) calculated as follows: $DI = (1 \times n_1)+(2 \times n_2)+(3 \times n_3)+(4 \times n_4)$, where n is the number of cells in each damage category.

The percentage of damage reduction (%DR) was calculated according to Delmanto *et al.* [11] and Serpeloni *et al.* [12] by Eq. [**1**]:

$$\%DR = \frac{mean\ score\ in\ A - mean\ score\ in\ B}{mean\ score\ in\ A - mean\ score\ in\ C} \times 100 \quad [\mathbf{1}]$$

where A is the DI of cells treated with H_2O_2 50 µM (PC); B is the DI of cells treated with different infusion concentrations (RI, RII, RIII) and C is the DI of the negative control (NC).

Statistical Analysis

The results are presented as means ± standard deviation. Statistical analysis was performed with the software SPSS 14.0 for Windows. Differences between controls and treatments were analyzed by one-way ANOVA followed by Tukey test; Student's t-test was used for the comparison between protective effects of the two yerba mate brands. A value of $p < 0.05$ was considered statistically significant for all the endpoints evaluated.

RESULTS

All cells exhibited more than 95 % viability before the comet assay. NC showed a high proportion of type I comets, indicating a low level of DNA damage (DI: 132.5 ± 36.7). As expected, cells treated with H_2O_2 50 µM (PC) showed most of the comets of type II or III (DI: 250.5 ± 46.4).

Figs. 1 and 2 show the effect of *I. paraguariensis* in the repair process (DI) of human cells previously exposed *in vitro* to 10 µM H_2O_2 for infusion 1 and 2, respectively. Differences in the DI were found among treatments of yerba mate (ANOVA test $p < 0.05$ in both cases). The comparison between the two different yerba mate brands showed no difference for any of the repair groups (RI, RII, RIII; T-test $p > 0.05$), indicating similar protective effects of both infusions.

The antigenotoxic effects of *I. paraguariensis,* expressed as percentage of DNA damage re-

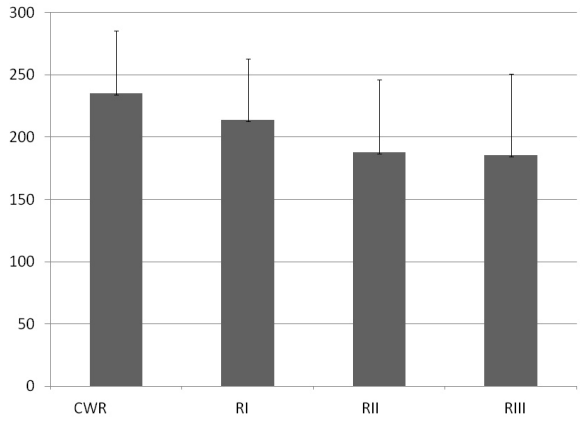

Figure 1. DI in CWR, RI, RII and RIII using Infusion 1 in human cells. Boxes are limited by first and third quartiles divided by median; thin vertical lines represent minimum and maximum values except when outliers are present. P < 0.05 ANOVA test.

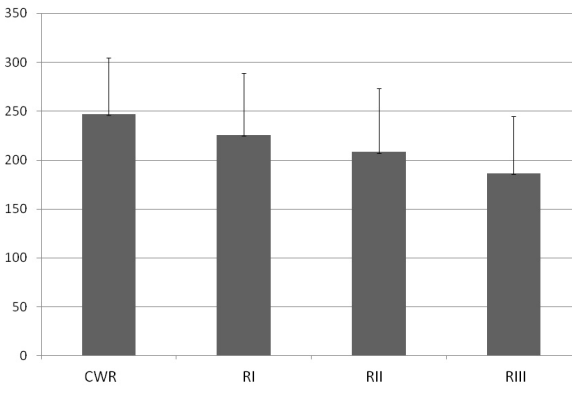

Figure 2. DI in CWR, RI, RII and RIII using Infusion 2 in human cells. Boxes are limited by first and third quartiles divided by median; thin vertical lines represent minimum and maximum values except when outliers are present. P < 0.05 ANOVA test.

Infusions	% CWR	% R I (10 µL/mL)	% R II (100 µL/mL)	% R III (1000 µL/mL)
1	34.05 ± 12.12	52.07 ± 13.98	122.34 ± 16.53	124.34 ± 14.98
2	31.21 ± 11.08	44.52 ± 12.84	69.00 ± 14.62	106.13 ± 15,07

Table 1. Reduction % for both infusions.

duction are shown in Table 1. The %DR indicated a decrease in oxidative damage as infusion concentration increases ($p < 0.05$). A significant increase in %DR was observed at RIII (1000 µL/mL) for infusions 1 and 2 (124.34 % and 106.13 %, respectively; $p < 0.05$), while at RII (100 µL/mL) only the Infusion 1 showed increased %DR (122.34 %) compared to the CWR (34.05%; $p < 0.05$), while the infusion 2 at the same concentration no difference was observed. No protective effects were observed at the lowest concentration for any of the Infusions compared to the CWR (Table 1).

DISCUSSION

Herbal extracts have been used for centuries around the world to treat different diseases and there is a long history of the use of plants of all kinds by herbalists in many cultures. Phytochemical investigations of *I. paraguariensis* found many classes of chemical constituents as purine alkaloids (caffeine), amino acids, polyphenols (chlorogenic acids) and flavonoids (quercetin, rutin and kaempferol). Polyphenols and flavonoids have already been implicated in the protective role extracts play against several diseases [5].

There is evidence that polyphenol compounds may have beneficial effects on human health and that some may exert antioxidant activity. Thus, it is expected that individuals who consume diets with a high content of phenolic antioxidant should be better protected against oxidative cellular damage than individuals who do not [4]. In the past 15 years, there was a several-fold increase in the literature studying yerba mate properties, showing effects such as antioxidant properties in chemical models and *ex vivo* lipoprotein studies, vaso-dilating and lipid reduction properties, antimutagenic effects, antiglycation effects and weight reduction properties. Lately, promising results from human studies have surfaced and the literature offers several developments on this area [13]. On the other hand, there is some controversial data about yerba mate may produce cito- and genotoxic effects on human cells [14].

In the present study, the potential antioxidant activity of the very-popular South American beverage mate was studied. Our results are in agreement with numerous investigations that reported antioxidant effects of *I. paraguariensis* using different methodologies [15-19].

Gugliucci [20] showed that an *I. paraguariensis* extract was able to inhibit the *in vivo* copper-induced low density lipoprotein oxidation in human plasma. Such effect was attributable to polyphenols and flavonoids present in the extract. Although the extent to which these potentially important antioxidants can be absorbed is not clear, evidence indicates that substantial quantities of the compounds are absorbed and reach levels in plasma high enough to inhibit the lipoprotein oxidation. Nevertheless, the amount of the extract ingested to obtain that effect was not indicated.

Other authors demonstrated that regular ingestion of mate tea increased the resistance of DNA to induced strand breaks and improved repair after H_2O_2 challenge in liver cells, regardless of the dose ingested. These results suggest that mate tea could protect against DNA damage and enhance DNA repair activity [4]. In the same way, Leonard *et al.* [21] showed that yerba mate possesses potent antioxidant effects against superoxide and hydroxyl radicals, concluding DNA-protective properties of this infusion.

Our results allow us to suggest that ingestion of mate might be a very effective and economic way to provide an important amount of compounds that increase the antioxidant defense system of an organism. Anyway, it is necessary to continue studying this to discard any negative effect of yerba mate.

CONCLUSIONS

This work contributes to support the idea of mate as a potential source of antioxidants. The greatest protective effects were found using the highest concentration of *I. paraguariensis* (1000 µL/mL) in an *in vitro* human system using the Comet assay. It is possible to speculate that the regular consumption of this beverage may significantly contribute to improve human antioxi-

dant defenses. However, future studies are necessary to examine the biological activities of *I. paraguariensis* extracts.

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