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Ilex paraguariensis extracts are potent inhibitors of nitrosative stress: A comparative study with green tea and wines using a protein nitration model and mammalian cell cytotoxicity

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Abstract

Due to the increasing importance of nitrosative stress in pathology and the efficacy displayed by flavonoids in canceling the effects of peroxynitrite, we decided to conduct a comparative study of three commonly used beverages with the highest polyphenol contents and proven antioxidant properties: mate (Ilex paraguariensis); green tea (Camelia sinensis) extracts and white and red wines of the main varietals. We directly evaluated and compared the extracts and wines as protein nitration inhibitors using 3-nitrotyrosine as a biomarker, we studied the extracts as protectors from OONO-induced cytotoxicity in two mammalian cell lines. Both green tea and mate extracts have a high polyphenol content, in the case of Ip, its higher concentration and higher free radical quenching activity on the DPPH assay may be mainly due to the sui generis extraction procedure. When BSA was incubated in the presence of SIN-1, a time and dose dependent nitration of the protein is clearly shown. Co-incubation of BSA with Ip, green tea or red wines led to a dose dependent inhibition of the effect. Ip displayed the highest inhibitory activity, followed by red wines and the green tea. Dilutions as low as 1/1500 produced more than 80% inhibition of albumin nitration. When we studied peroxynitrite-induced cytotoxicity in murine RAW 264.7 macrophages and 31EG4 mammary cells., we found a potent, dose-dependent protective effect that was Ilex paraguariensis > red wines > green tea. Taken together, our results indicate that when the herbal preparations studied here are prepared the way they are usually drunk, Ip displays the highest inhibition of protein nitration, and the highest promotion of cell survival, whereas green tea or red wines display significant but lesser effects at the

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same concentrations. Further studies aiming at isolation of the active principles and assessment of their bioavailability are warranted.

Keywords: Peroxynitrite; Nitrotyrosine; Oxidation; Free radicals; Diabetes; Flavonoid

Introduction

Evidence for nitration of biomolecules and their role in pathology is increasing (Hoeldtke, 2003; Rose et al., 2003; Radi, 2004). Atherosclerosis, diabetic complications and diseases of neuronal degeneration are among the main entities for which an important role of nitrosative stress has been proposed (Cowell and Russell, 2004; Kuhn et al., 2004). Nitrosative stress is induced whenever conditions are favorable for increased superoxide formation and NO is locally available. Superoxides can react with NO, forming peroxynitrite ($ONOO^-$), which rapidly causes protein nitration or nitrosylation, lipid peroxidation, DNA damage, and cell death (Hoeldtke, 2003; Radi, 2004). $ONOO^-$, formation is dependent on both superoxide and NO concentrations; consequently, cells that constitutively express NO synthase, such as endothelial cells and neurons, may be more vulnerable to $ONOO^-$ induced cell death in conditions favoring the production of superoxides (Hoeldtke, 2003). The cytotoxic effect of peroxynitrite are mediated by initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate dehydrogenase, or inactivation of membrane sodium channels (Hoeldtke, 2003; Rose et al., 2003). In addition, peroxynitrite is a potent trigger of DNA strand breakage, with subsequent activation of the nuclear enzyme poly-ADP ribosyl synthetase or polymerase, with eventual severe energy depletion and necrosis of the cells (Szabo, 2003). The need for defense against peroxynitrite seems to be a defensible hypothesis. Prevention, interception and repair are three physiological and pharmacological strategies for protection against peroxynitrite (Cowell and Russell, 2004).

Ideal therapeutic approaches for nitrosative stress in atherosclerosis and diabetes should limit the formation of superoxides and $ONOO^-$, while preventing reductions in vascular NO that would cause vasoconstriction (Hoeldtke, 2003). On more realistic grounds, substances capable of quenching $ONOO^-$, while not affecting NO mechanisms could prove beneficial. Precisely, cardioprotective effects including antioxidant properties and activation of endothelial nitric oxide synthase, thus increasing NO concentrations have been ascribed to flavonoids (Ader et al., 2000; Duthie and Crozier, 2000; Aviram and Fuhrman, 2002). With regards to interception of peroxynitrite or its derivatives, flavonoids are good exogenous candidates to perform that function. Flavonoids are general free radical scavengers and chelate transition metals (Aruoma, 1999; Hollman and Katan, 1999a,b; Terao, 1999; Radi, 2004).

They react with nitric oxide and superoxide and protect against peroxynitrite oxidation and nitration reactions. It is likely that these compounds react with nitrating and oxidizing intermediate species formed during peroxynitrite decay and before the species attack their protein or DNA target and not with peroxynitrite itself (Radi, 2004).

Apples, onions, chocolate, red wines, green tea and other plant extracts are good sources of flavonoids (Hollman and Katan, 1997; Hollman et al., 1997). One such plant, “mate” [mä’tā], is an infusion popular in Argentina, Uruguay, Paraguay, and southern Brazil, where it is a stimulating tea

drank in many cases instead of coffee (Gugliucci and Stahl, 1995; Filip et al., 2001). Its popularity is increasing in the USA, Canada and Europe. Mate is brewed from the dried leaves and stemlets of the perennial tree *Ilex paraguariensis* (Ip). The tree grows between the parallels 10° and 30° (South) in the Paraguay and Paraná river basins (Gugliucci and Stahl, 1995; Filip et al., 2001). It is a stimulating drink, greenish in color, containing 1–1.5% w/w caffeine, 7–11% w/w tannins and several vitamins such as B_1, B_2 riboflavin, pantotenic acid, C, E and β-carotene. We have previously shown that Ilex paraguariensis (Ip) extracts are very potent inhibitors of LDL oxidation by peroxynitrite and described an antimutagenic effect of mate infusions in yeast cell populations (Gugliucci and Stahl, 1995; Gugliucci, 1996; Bracesco et al., 2003). In this work we set out to directly evaluated Ip extracts as protein nitration inhibitors using 3-nitrotyrosine as a biomarker, we studied Ip extracts as protectors from OONO-induced cytotoxicity in two mammalian cell lines and we compared their activity with other well studied antioxidant beverages.

Material and methods

Chemicals

All chemicals are analytical grade and purchased from Sigma (Missouri, USA).

Apparatus

Spectrophotometric measurements were made in a Beckman DU 640 Spectrophotometer (Beckman Coulter Inc, Fullerton CA). Protein concentrations were measured by the Bradford (Bradford, 1976) method (BioRad, Hercules, CA).

Preparation of Ilex paraguariensis, Camelia sinensis and Achyrocline satureoides (marcela) extracts

The yerba mate from commercial sources (Canarias, Pando Uruguay) was freshly prepared into a mate infusion (50 g of herb per liter of water at 90 °C) in a gourd. The extracts were filtered through a 0.45 mm Millipore filter and used the same day. The inter-assay CV of the total polyphenol content of the different preparations was less than 10%. Green and black tea (in tea bags) were obtained from commercial sources and infusions were prepared (5 g/200 ml water). All comparative studies between the herbal preparations and wines were done extemporaneously. *Achyrocline satureoides (marcela)* is another popular herbal preparation used in alternative medicine in South America and studied in our laboratory (Gugliucci and Menini, 2002a,b).

Wine samples were obtained from commercial sources, bottles were opened and wines were used in experiments immediately or kept under Argon at −80 °C for less than one week.

Determination of polyphenol concentration

Total polyphenol concentrations in Ip samples was determined spectrophotometrically with the Folin-Ciocalteau phosphomolybdic-phosphotungstic acid reagents as modified by (Vinson et al., 2001).

Free radical scavenging capacity

The free radical scavenging capacity of Ip, green and black tea samples was analyzed by using the 1,1-diphenyl-2-picrylhydrazyl assay (DPPH) as previously described (Malterud et al., 1993). Aliquots of the herbal extracts samples at the given concentrations were mixed with 1 ml of 0.1 mmol/l DPPH (in ethanol) in a cuvette. The time course of the change in absorbance at 517 nm was then kinetically monitored.

Nitration of bovine serum albumin

BSA (1 mg/ml in 140 mmol/l NaCl, 20 mmol/l sodium phosphate, pH 7.4) was incubated in the absence (control) or the presence of 1 mmol/l SIN-1 with or without the addition of the aforementioned substances. After incubation at 37 °C for 180 min, samples were dialysed and 3-nitrotyrosine in the samples was determined by Western blot as described below.

SDS-PAGE

Electrophoresis was run according to Laemmli on 10% gels (reducing conditions). Each lane was loaded with 10 μg protein. Equipment employed was Mini Gel III from BioRad (BioRad, Hercules, CA). Gels were stained with Coomassie Brilliant Blue.

Immunoblotting

Immunochemical detection of 3-nitrotyrosine was performed after electrophoresis and Western blotting. The antibody employed was using a murine monoclonal anti-nitrotyrosine antibody HM11 (Abcam, Cambridge, MA). Immunoblots were revealed by an anti-rabbit peroxidase-labelled second antibody and enhanced chemiluminiscence using Immun-Star detection system from BioRad (BioRad, Hercules, CA).

Cytotoxicity assays in 2 mammalian cell lines

Murine RAW264.7 macrophages were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were routinely cultured in RPMI/1640 medium Sigma supplemented with 10% fetal bovine serum with 1% of 10,000 units/ml penicillin and 10 mg/ml streptomycin solution in 95% humidified air and 5% CO_2 at 37 °C. Cells were seeded at a density of 200,000 cells per well and plated on 12 well plates. Cells were allowed to grow to confluency for 24 h approximately and "starved" in serum free RPMI/1640 overnight with the addition of SIN-1 at different time periods in the presence or absence of inhibitors mate, green tea, and Tannat. They were incubated for different times up to 24 h. At the indicated time periods cell integrity and viability were determined through cell morphology, cell count and cytoxicity LDH assessment. Cells were washed 3 times in PBS, then with trypsin (Cell Gro,), stopped with added media not containing serum, centrifuged, and brought back up in 2 ml PBS. Viability was determined through a cell count where a 50 μl cell sample was combined with 50 μl trypan blue. Cells were lysed by freeze thawing. Cell lysates (2 μg protein) were loaded onto 10% SDS-PAGE and developed by silver stain. Protein bands were analyzed by densitometry (NIH Image software).

Murine mammary epithelial cells 31EG4 were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were routinely cultured in DMEM medium Sigma supplemented with 5% fetal bovine serum, 2 mmol/l l-glutamine and 5 μg/ml insulin with 1% of 10,000 units/ml penicillin and 10 mg/ml streptomycin solution in 95% humidified air and 5% CO_2 at 37 °C. Cells were seeded at a density of 200,000 cells per well and plated on 12 well plates. Cells were allowed to grow to confluency for 24 h approximately and "starved" in serum free DMEM overnight with the addition of SIN-1 at different time periods in the presence or absence of inhibitors mate, green tea, and Tannat. They were incubated for different times up to 24 h and processed using the same procedures described above for macrophages.

Cytotoxicity was determined using the LDH cytotoxicity assay from Roche.

Statistical analysis

Unless otherwise stated, data are expressed as mean ± SD. Comparisons between data were performed by the Student's t test (two-tailed) for unpaired samples. Data was processed on SPSS 12.0.

Results

Polyphenol concentrations

The polyphenol content in the preparations used in these studies are depicted in Fig. 1A. Ilex paraguariensis extracts as they are usually brewed and drunk in South America have the highest concentration of polyphenols, followed by red wines and green tea.

Free radical scavenging capacity

Quenching of the DPPH free radical by the preparations used in this study is shown in Fig. 1B. When 1/10 dilutions of Ip, green and black tea and several white and red wines are prepared extemporaneously, Ilex paraguariensis extracts display the highest activity. When this activity is corrected for the amount of polyphenols in each preparation, Ilex paraguariensis extracts have 70 ± 10% of the activity of green tea.

Inhibition of BSA nitration by peroxynitrite

Sydnonimine (SIN-1) is a generator of nitric oxide and superoxide which, in turn, combine to form peroxynitrite. Fig. 2A depicts the dose dependency of the inhibition of peroxynitrite-induced nitration of albumin by Ip and green tea. Control BSA does not have 3-nitrotyrosine immunoreactivity, which becomes manifest after 3 h of incubation with SIN-1. We extended our incubations up to 16 h. The reaction reaches a plateau after 3 h, therefore we chose this time. As shown in the figure, dilutions of Ip as low as 1/750 produce 95% of inhibition of this reaction as quantified by densitometry.

Green tea also inhibits protein nitration, however the effect is lower: at 1/750 dilutions the reduction is only 30%. ($p < 0.05$). Fig. 2B shows the effects of red wines from the Tannat and Merlot varietals. A

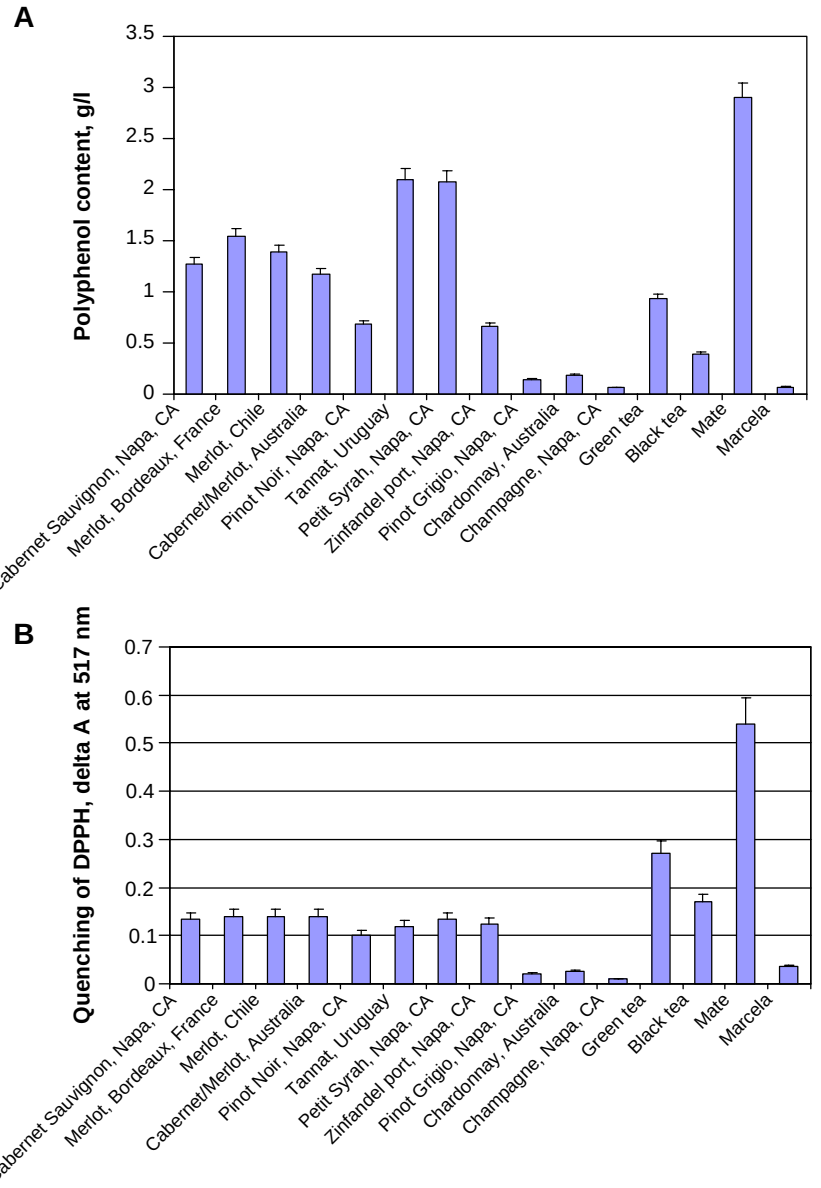

Fig. 1. Polyphenol content (A) and free radical quenching activity (B) of several wines, teas and Ilex paraguariensis. Polyphenols were determined by the modified Folin-Ciocalteu method as described in Materials and methods. Quenching of the radical DPPH was measured as described in Materials and methods and expressed as absorbance change at 517 nm after 5 minutes of incubation. Data represent mean ± SD of 3 independent experiments in which each sample was analyzed in duplicates.

dose dependent inhibitory effect is also apparent for these samples. No significant differences were observed between the two varietals. At 1/1500 dilutions, the inhibitory activity of Ip extracts is significantly higher than that of either red wine: 80 ± 10% vs 20 ± 6% ($p < 0.05$).

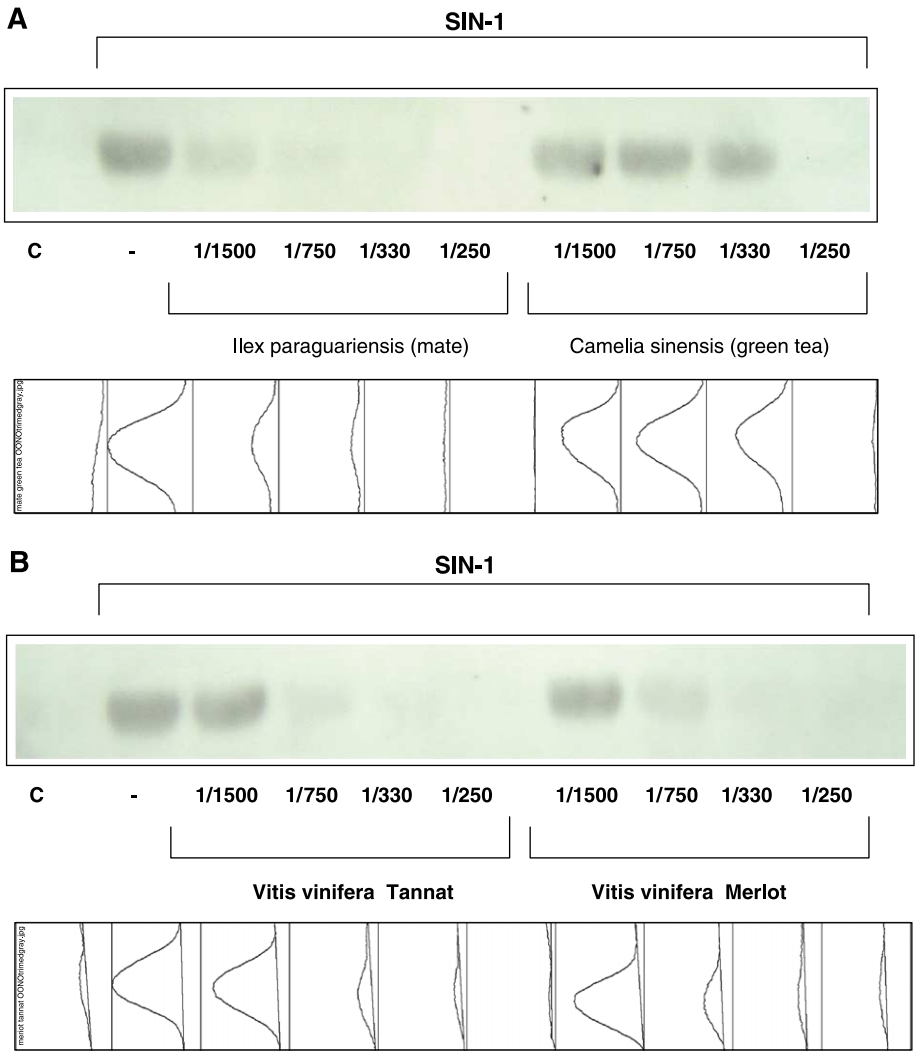

Fig. 2. Nitration of bovine serum albumin. 2A: inhibition by Ilex paraguariensis and green tea extracts. 2B: inhibition by red wines. BSA (1 mg/ml in 10 mmol/l PBS) was incubated for 3 hours at 37 °C in the presence of 1 mmol/l SIN-1 as a peroxynitrite generator with or without the addition of the dilutions of Ip or green tea extracts depicted in 2A or with or without the addition of the dilutions of Vitis vinifera Merlot or Tannat aliquots depicted in 2B. After SDS-PAGE (10% acrylamide) and blotting, 3-nitrotyrosine was immunologically detected by ECL. Western blot (top) and densitogram (bottom) of a typical experiment out of 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Protective effect of Ilex paraguariensis against peroxynitrite-induced cytotoxicity in murine macrophages

We next tested the effects of peroxynitrite on murine RAW264.7 macrophages by monitoring cell survival, cytotoxicity as LDH release, morphological changes and changes in protein profiles.

Cell survival and morphological changes

When cells were incubated in the presence of 100 μmol/l SIN-1, significant morphological changes were apparent after 5 h as depicted in Fig. 3A (control) and B (SIN-1), where clear signs of cell injury and nuclear changes are observed. Further incubation intensified these changes and led to more cell death (data not shown). We chose 5 h as our time frame for our following experiments. Co-incubation of cells with SIN-1 and Ip (1/500) led to protection from the oxidative stress, cells are morphologically indistinguishable from controls as shown in Fig. 3C. Fig. 3D depicts cells after 5 h co-incubation with SIN-1 and Tannat red wine at 1/500 dilution. Again, this led to protection from the oxidative stress, cells are morphologically indistinguishable from controls. When survival rates were assessed by Trypan blue exclusion, Ip afforded 90% protection and red wine 85%.

Changes in protein profiles

Cell lysates from the experiments above were analyzed by SDS-PAGE, to test for changes in protein patterns induced by the nitrosative stress. Fig. 4A shows the silver stained gel and Fig. 4B the corresponding densitograms. Cells incubated in the presence of 100 μmol/l SIN-1 display

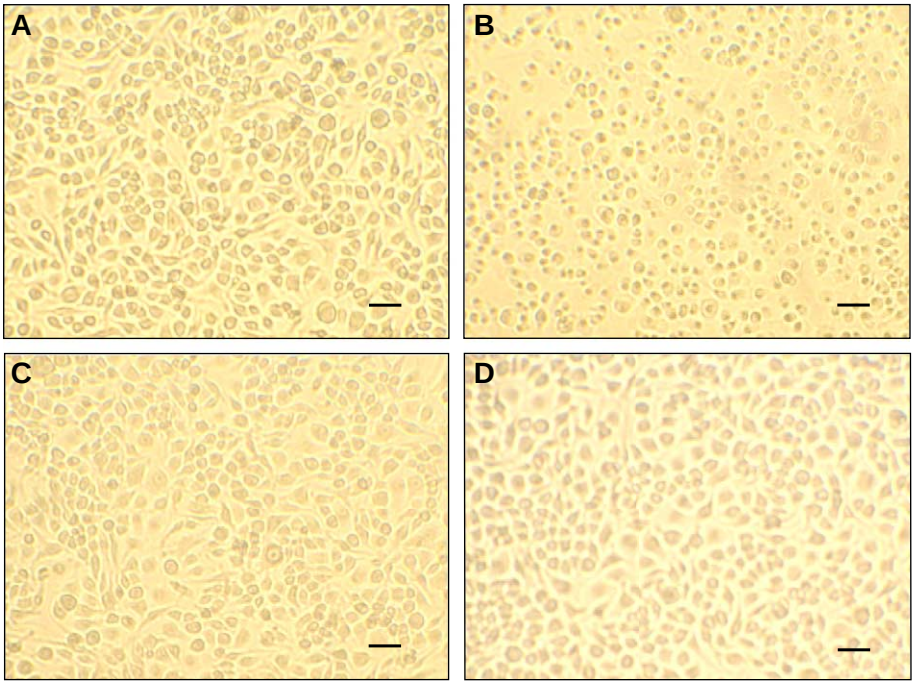

Fig. 3. Cytoxicity of peroxynitrite vis-à-vis murine RAW264.7 macrophages. Cells at confluence were incubated for 5 h in the absence (A) or the presence (B) of 100 μmol/l SIN-1. In (C) cells were incubated in the presence of 100 μmol/l SIN-1 and 1/500 dilution of Ilex paraguariensis extracts and in (D) 1/500 dilution of Tannat red wine. Bar is 20 μm. Both Ilex paraguariensis and Tannat red wine afford a protective effect. See also Fig. 6A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

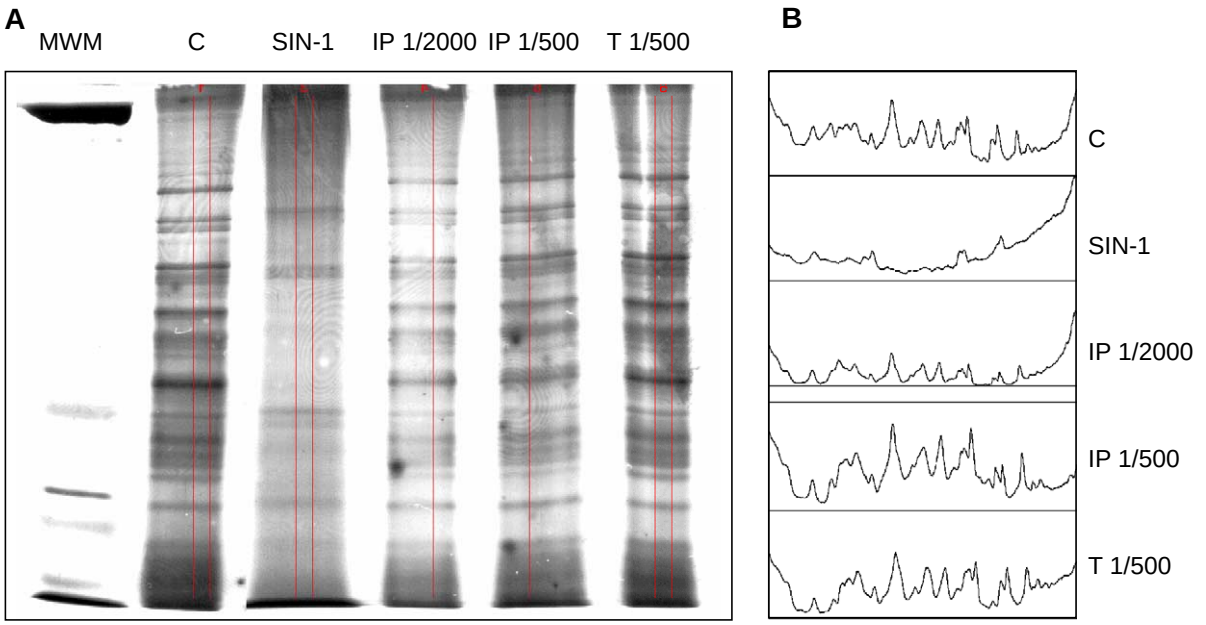

Fig. 4. SDS-PAGE protein profiles of RAW264.7 cell lysates. A: Silver stain; B: densitogram. Cells were incubated for 5 h in the absence (C) or the presence (SIN-1) of 100 μmol/l SIN-1 with the addition of 1/2000 (IP 1/2000) or 1/500 (IP 1/500) dilutions of Ilex paraguariensis extracts 1/500 (T 1/500) dilution of Tannat red wine. After incubation cells were scraped and lysed and 2 μg protein were loaded onto 10% acrylamide gels, run and Silver stained.

significant changes in protein patterns, notably many species in the middle range of molecular weights are attenuated while high molecular weight aggregates become apparent. Co-incubation with Ip extracts at dilutions as low as 1/2000 revert these changes significantly and restore the profile to control levels at 1/500 dilutions. A similar effect is afforded by red wine of the Tannat variety. Effect of green tea at the same dilutions was noticeable but far less significant (data not shown).

Protective effect of Ilex paraguariensis against peroxynitrite-induced cytotoxicity in murine mammary epithelial cells

In order to extend our observation to another cell line we performed some of the experiments above using 31EG4 murine mammary epithelial cells. When these cells were incubated in the presence of 100 μmol/l SIN-1, significant morphological changes were apparent after 5 h and became striking at 24 h as depicted in Fig. 5A (control) and B (SIN-1), where signs of cell injury and nuclear changes are observed. Co-incubation of cells with SIN-1 and Ip (1/500) led to protection from the oxidative stress, cells are morphologically indistinguishable from controls, as shown in Fig. 5C.

Cytotoxicity assays

Fig. 6A depicts the results obtained when cell LDH release to media was monitored in experiments with murine macrophages. SIN-1 promoted LDH release over control values and 1/500 dilutions of Ip

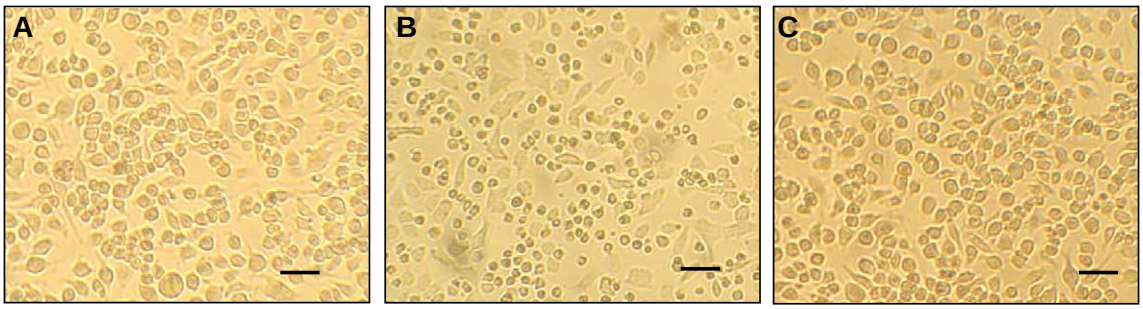

Fig. 5. Cytoxicity of peroxynitrite vis-à-vis murine mammary epithelial cells. Cells at confluence were incubated for 24 h in the absence (A) or the presence (B) of 100 μmol/l SIN-1. In (C) cells were incubated in the presence of 100 μmol/l SIN-1 and 1/500 dilution of Ilex paraguariensis extracts. Bar is 20 μm. See also Fig. 6A.

abolished this release and even conferred protection beyond control values. In these experiments, cells were incubated for 24 h without any supplement which leads to LDH release in control conditions. At the same dilutions, neither green tea nor red wines displayed a significant protective effect. Similar experiments were also conducted with the murine mammary cell line and the results are shown in Fig. 6B. At 1/500 dilutions, only Ip showed a significant protective effect against cytoxicity induced by nitrosative stress.

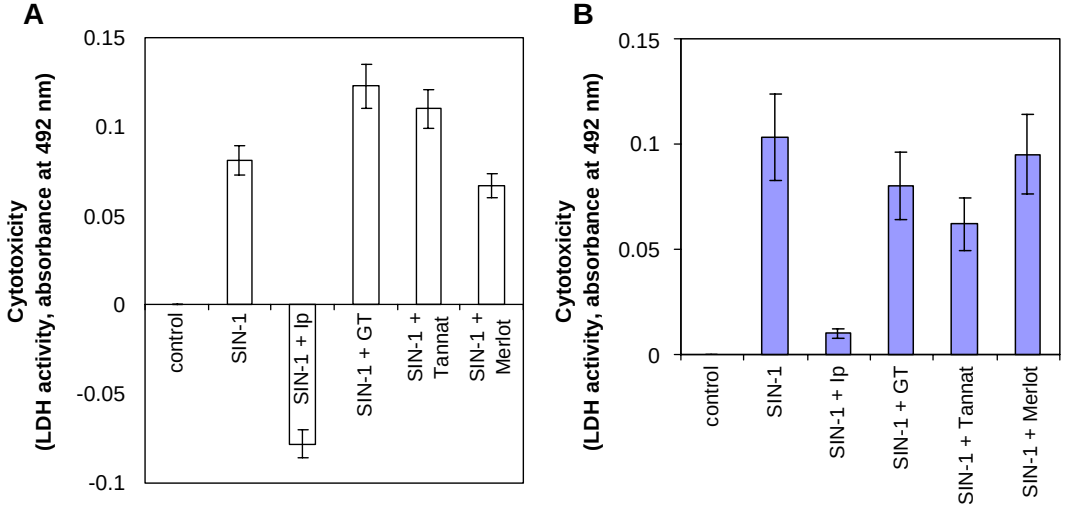

Fig. 6. Cytoxicity of peroxynitrite vis-à-vis murine RAW264.7 (A) and mammary epithelial cells (B). Cells at confluence were incubated for 24 h in the absence (control) or the presence of 100 μmol/l SIN-1 (SIN-1), with the addition of 1/500 dilution of Ilex paraguariensis extracts (SIN-1 + Ip); green tea (SIN-1 + GT); red wine Vitis vinifera Tannat (SIN-1 + T) or red wine Vitis vinifera Merlot (SIN-1 + Merlot). LDH activity on cell media was determined colorimetrically to determine cytotoxicity. Data represent mean ± SD of 2 independent experiments in which each condition was analyzed in triplicates. In A, the difference between Ip and SIN-1 is significant with a $p < 0.005$, the difference between SIN-1 and green tea is significant with a $p < 0.05$. In B, the difference between Ip and SIN-1 is significant with a $p < 0.01$, the difference between SIN-1 and Tannat is significant with a $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Discussion

Due to the increasing importance of nitrosative stress in pathology and the efficacy displayed by flavonoids in canceling the effects of peroxynitrite, we decided to conduct a comparative study of three commonly used beverages with the highest polyphenol contents and proven antioxidant properties: Ilex paraguariensis; Camelia sinensis extracts and white and red wines of the main varietals.

In the case of mate and teas, we conducted all the comparison experiments with extracts prepared in the way they are usually brewed and drunk. We believe this is very important if one wants to obtain nutritionally or physiologically relevant conclusions. We first determined the polyphenol content of these beverages. Data in Fig. 1A demonstrate that Ip extracts contain the highest concentration of polyphenols of all the beverages studied. A very recent report (Chandra and De Mejia Gonzalez, 2004) shows somewhat lower concentrations; we believe this is due to the different extraction procedure employed by the authors, as well as the ratio of herb and water, which is not the way Ip is usually drunk. If this is taken into account, the data are comparable. Both green tea and mate extracts have a high polyphenol content, in the case of Ip, its higher concentration may be mainly due to the sui generis extraction procedure. The concentration ranges for green tea, red and white wines are in agreement with data on the literature (Actis-Goretta et al., 2002; Aviram and Fuhrman, 2002).

Next, we screened all the beverages for their free radical quenching activity. Fig. 1B displays the quenching of the radical DPPH and demonstrates a higher scavenging activity for Ip as compared to the rest. When free radical quenching activity is corrected for polyphenol content, green tea extracts display the highest levels in this assay. Again, this is in agreement with recent data from other investigators (Chandra and De Mejia Gonzalez, 2004). We employed these assays to select the extracts with the highest polyphenol concentration and the highest free radical scavenging properties as candidates for our nitrosative stress studies.

We next set out to demonstrate the relative anti-nitration activities of the extracts chosen from the experiments above, in a protein nitration model and in mammalian cell culture. For this purpose we employed a peroxynitrite-generating substance, SIN-1, and monitored its effects on bovine serum albumin by measuring the appearance of 3-nitrotyrosine as a biomarker. During inflammation, activated macrophages and neutrophils can produce great amounts of peroxynitrite. The determination of nitrotyrosine, which is a stable final metabolite of peroxynitrite-modified proteins, provides an important indicator of tissue disorders caused by peroxynitrite (Kuhn et al., 2004). For instance, nitrotyrosine levels are elevated in diabetic patients, in whom nitrosative stress may enhance the development of complications (Hoeldtke, 2003). Nitrated HDL is present in atheroclerotic lesions (Pennathur et al., 2004).

When BSA was incubated in the presence of SIN-1, a time and dose dependent nitration of the protein is clearly shown. This is in agreement with previous studies (McConnell et al., 2003), and the range of concentrations of SIN-1 employed is within those currently employed by other authors in other in vitro and in vivo studies (Rose et al., 2003; Szabo, 2003; Kuhn et al., 2004). Co-incubation of BSA with Ip, green tea or red wines led to a dose dependent inhibition of the effect. In previous studies, the epicatechin/gallate family of flavonols, constituents of green tea, red wine, etc., was shown to afford the most extensive inhibitory properties against both tyrosine nitration (Oldreive et al., 1996). Our data on green tea are in agreement with this previous work, and show that Ip displayed the highest inhibitory activity, followed by red wines and the green tea. Dilutions as low as 1/1500 produced more than 80% inhibition of albumin nitration. A 95% inhibition of protein nitration was achieved with a third of the Ip

extract as compared to green tea. The difference may reside in the higher polyphenol concentrations in Ip extracts or in the different composition. It is interesting to note that it has been reported that 196 volatile chemical compounds are found in Ip, of which 144 are also found in green tea (Kawakami and Kobayashi, 1991). Mate's processing method, like that of green tea, but unlike that of black tea to a certain degree, preserves antioxidants and nutritive values. On the contrary, with regard to phenolics, a recent study showed that HPLC polyphenol fractions differ significantly between green tea and Ip extracts (Chandra and De Mejia Gonzalez, 2004). Ilex paraguariensis contains high levels of chlorogenic acid which are low or absent in green tea, and several polyphenol fractions still not identified and not present in green tea.

We show that Ip extracts not only display a strong, dose dependent free radical scavenging activity, but are also very potent inhibitors of peroxynitrite damage to proteins.

We then explored the protective effects of Ip, green tea and red wines extracts against peroxynitrite-induced cytotoxicity. This cytotoxicity has been implicated in the pathogenic mechanisms of stroke, myocardial ischemia, diabetes and diabetes-associated cardiovascular dysfunction (Szabo, 2003). When we studied peroxynitrite-induced cytotoxicity, we found a potent, dose- dependent protective effect for green tea, red wines and Ilex paraguariensis. This effect was evidenced in the two mammalian cell lines studied. Ip extracts proved to be more potent than green tea and red wines as demonstrated by the cytotoxicity assays depicted in Fig. 6, conferring protection at 1/500 dilutions while either green tea or red wines did not. In this experimental work we did not explore whether the main mechanism for toxicity was apoptosis or necrosis, but both might be involved according to the available evidence from the literature (Szabo, 2003). Indeed, low concentrations of peroxynitrite have been reported to trigger apoptotic death, while higher concentrations induce necrosis. Cellular energy balance serves as switch between the two modes of cell death. Peroxynitrite damages DNA and triggers the activation of DNA repair systems. A DNA nick sensor enzyme, poly(ADP-ribose) polymerase-1 (PARP-1) also becomes activated upon sensing DNA breakage (Cowell and Russell, 2004). Activated PARP-1 cleaves NAD(+) into nicotinamide and ADP-ribose and polymerizes the latter on nuclear acceptor proteins. Peroxynitrite-induced overactivation of PARP consumes NAD(+) and consequently ATP culminating in cell dysfunction, apoptosis or necrosis (Szabo, 2003).

We provide evidence for a dramatic change in the patterns of proteins in the cells under nitrosative stress, where middle-sized peptides disappear while high molecular weight peptides increase. These changes are reverted in a dose dependent fashion by Ip and red wines and are cancelled at 1/500 dilutions of either one, whereas almost no effect is seen for green tea at the same dilutions.

Taken together, our results indicate that when the products studied here are prepared as they are usually drunk, Ip displays the highest inhibition of protein nitration, and the highest promotion of cell survival, being over 60% at dilutions of 1/1200, whereas green tea or red wines displayed significant but modest effects at the same concentrations.

As is the case for all studies on flavonoid containing beverages, extrapolation of in vitro and cell culture, results to humans is very difficult. However, based on the high potency shown here for Ilex paraguariensis, its polyphenol content and the potential interest in this herb against nitrosative stress, we can compare the potency demonstrated in our studies with in vivo data from the literature. For instance, work on the pharmacokinetic properties of green tea flavonoids, have provided indications of the plasma levels and circulating molecular forms that may be expected in humans following tea consumption. Measurement of the total antioxidant capacity of plasma after the consumption of polyphenol-rich food allows a comparison of their contribution to the total antioxidant capacity to that of ascorbic acid, the

other main water soluble antioxidant found in our diets. Data from Whitehead (Whitehead et al., 1995) and Duthie (Duthie et al., 1998; Duthie and Crozier, 2000) show that after ingestion of 300 ml red wine (500 mg polyphenols) the total effective concentration of polyphenols and their metabolites in plasma is about 50 μmol/l. If polyphenols in mate have similar bioavailability to those of red wines, this concentration corresponds to 1/200 dilutions of the extracts. Throughout this study we have shown dramatic inhibition of protein nitration or cell toxicity with at least half of that concentration of our extracts. Most of the flavonoids absorbed from the small intestine have a short half-life of 1–2 h (Bourne and Rice-Evans, 1999; Terao, 1999; Ader et al., 2000). The particular way in which mate is drunk, which usually implies repeated extraction from a large (50–100 g) quantity of dry product in a gourd, and intermittent consumption, many times for hours in a row, would allow for maintenance plasma levels of polyphenols which are usually not attained with green tea or moderate wine drinking.

In view of the critical involvement of peroxynitrite in cardiovascular disorders, the results of this study might have implications for the cardiovascular protection associated with some nutrients.

Classic antioxidants, such as vitamin E, which work by scavenging already-formed toxic oxidation products, have failed to show beneficial effects on diabetic complications. New and "causal" antioxidant therapy is warranted. Anti-nitration drugs (to be designed) might be additional therapeutic goals to slow the course of diabetes macrovascular complications. While waiting for these focused tools, flavonoid-rich beverages could represent an alternative coadjuvant in treatment. In this regard, our studies suggest that Ilex paraguariensis extracts are more effective than either green tea or red wines. Further studies aiming at isolation of the active principles and assessment of their bioavailability are warranted.

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