

Polypodium leucotomos extract: Antioxidant activity and disposition

L. Gombau^a, F. García^c, A. Lahoz^a, Myriam Fabre^a, P. Roda-Navarro^b, P. Majano^b,
J.L. Alonso-Lebrero^{c,*}, J.P. Pivel^c, J.V. Castell^a, M.J. Gómez-Lechon^a, S. González^d

^a *Advancell, 46009 Valencia, Spain*

^b *Unidad de Biología Molecular, Hospital Universitario de la Princesa, 28006 Madrid, Spain*

^c *Industrial Farmacéutica Cantabria (IFC), C Arequipa 1 EDIF Ofic 5 planta, 28043 Madrid, Spain*

^d *Memorial Sloan-Kettering Cancer Center, NY 10022, USA*

Received 12 April 2005; accepted 19 September 2005

Available online 2 November 2005

Abstract

The extract of the fern *Polypodium leucotomos* (PL, Fernblock®) is an oral photoprotectant with strong antioxidative properties. Recent studies to determine its chemical composition have shown 4-hydroxycinnamic acid (*p*-coumaric), 3-methoxy-4-hydroxycinnamic acid (ferulic), 3,4-dihydroxycinnamic acid (caffeic), 3-methoxy-4-hydroxybenzoic acid (vanillic) and 3-caffeoylquinic acid (chlorogenic) to be among its major phenolic components. No conclusive data are available, however, on the H₂O₂-scavenging capacity of these compounds, or on their absorption and metabolism following their oral intake. In the present work, their antioxidative capacity was assessed by the luminol/H₂O₂ assay, their absorption studied using Caco-2 cells to resemble the intestinal barrier, and their metabolism investigated using cultured primary rat hepatocytes. The antioxidant capacity of PL components increased in a concentration-dependent manner, with ferulic and caffeic acids the most powerful antioxidants. The apparent permeability results correspond to a human post-oral administration absorption of 70–100% for all tested substances. Coumaric, ferulic and vanillic acids were metabolized by CYP450-dependent mono-oxygenases and partially conjugated to glucuronic acid and sulfate. These phenolic compounds may contribute to the health benefits afforded by this oral photoprotectant.

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Keywords: *Polypodium leucotomos*; Antioxidant photoprotective agent; ADME; Caco cells; Photoprotection

1. Introduction

Sunburn, immune suppression, photoaging and skin cancer may result from the overexposure of human skin to solar ultraviolet radiation (UVR). Topical and oral sun-screening agents can, however, help provide a degree of protection against the appearance of these problems. In this context, a hydrophilic extract obtained from the aerial parts of the fern *Polypodium leucotomos* (PL, Fernblock®)

is known to show strong photoprotective properties following its topical or oral administration. In vitro and in vivo studies have shown its antioxidant effects to include the scavenging of superoxide anions, hydroxyl radicals and singlet oxygen (González and Pathak, 1996; Gomes et al., 2001). It has also been shown to inhibit lipid peroxidation and increase the survival of human keratinocytes exposed to UVR (González and Pathak, 1996; Gomes et al., 2001; Alonso-Lebrero et al., 2003; Philips et al., 2003). In addition, preclinical and clinical research has shown PL to modulate the immune/inflammatory response and to prevent UVR-induced skin cancer in several animal models, perhaps by reducing DNA damage (Alcaraz et al., 1999; Gonzalez et al., 1997; Middelkamp-Hup et al., 2004a,b).

Experiments to determine the chemical composition of PL have identified the following major phenolic

Abbreviations: PL, *Polypodium leucotomos* extract; UVR, ultraviolet radiation; ECOD, 7-ethoxycoumarin de-ethylase; TEER, transepithelial electrical resistance; Papp, apparent permeability; DMSO, dimethylsulfoxide; PDTC, pyrrolidine-dithiocarbamate.

* Corresponding author. Tel.: +34 913821587; fax: +34 917640709.

E-mail address: joseluis.alonso@ifc.es (J.L. Alonso-Lebrero).

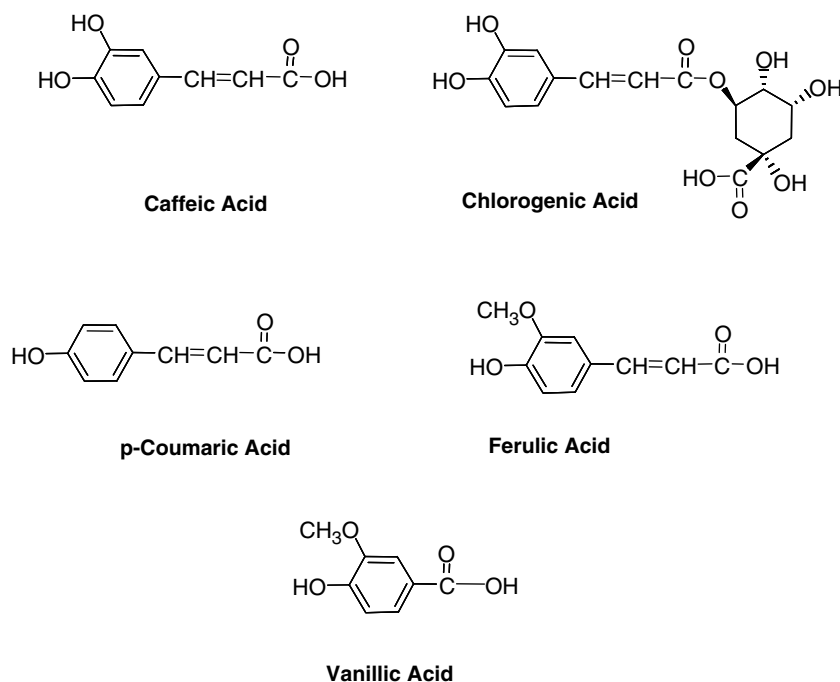


Fig. 1. Chemical structure of dietary benzoic and hydroxycinnamic acids.

compounds: *p*-coumaric, ferulic, caffeic, vanillic and chlorogenic acids (Fig. 1, unpublished). Phenolic compounds are secondary plant metabolites found in all fruits and vegetables, as well as coffee beans and tea leaves (Stavric, 1994; Williamson et al., 2000; Friedman and Jürgens, 2000). The literature refers to them as chemopreventers since they possess anticarcinogenic and other beneficial properties. Phenolic compounds in the diet are particularly bioactive and have remarkable effects in mammalian cells, including antioxidant activity, the modulation of gene expression and the inhibition of UV and carcinogen-induced tumorigenesis in different models (Stavric, 1994; Williamson et al., 2000; Lee et al., 1995; Nigdikar et al., 1998). Further knowledge on the absorption, bioavailability and metabolism of the phenolic compounds found in PL might contribute to a better understanding of the health benefits it offers. Numerous studies have highlighted the difficulty of investigating these variables in humans. It is difficult to eliminate phenolic compounds from the diet, and strict exclusion criteria are needed in the selection of volunteers (Rechner et al., 2004). To circumvent these problems, the present study employed *in vitro* cell systems to compare the antioxidant capacities, absorption and metabolism of the major phenolic components of PL.

2. Materials and methods

2.1. Reagents

Caffeic, chlorogenic, coumaric, ferulic and vanillic acids were provided by Industrial Farmacéutica Cantabria (IFC, Madrid, Spain). Dulbecco's modified Eagle's medium

(DMEM), Ham's F-12 medium, Leibovitz L15 medium, glutamine, penicillin–streptomycin, fetal bovine serum (FBS) and newborn calf serum were from Gibco (Paisley, UK). Insulin was supplied by Novo Nordisk (Bagsvaerd, Denmark). ^3H -inulin, ^{14}C -mannitol and ^3H -propranolol were from Amersham Bioscience (Freiburg, Germany), WST-1 reagent, collagenase and β -glucuronidase/arylsulfatase were purchased from Roche (Mannheim, Germany). Testosterone, 7-ethoxycoumarin, 7-hydroxycoumarin, MTT reagent, luminol, H_2O_2 , pyrrolidine-dithiocarbamate (PDTC) and DMSO were provided by Sigma (St Louis, MO, USA).

2.2. Cell culture

An human colon cancer-derived cell line (Caco-2 cells; GenBank accession number HTB37) was routinely cultured (Le Ferrec et al., 2001). Rat hepatocytes were obtained from one month-old Sprague–Dawley rats (200–300 g) by perfusion of the liver with collagenase, as described elsewhere (Gomez-Lechon et al., 1997, 2003). The viability of cells in suspension was assessed by the trypan blue dye exclusion method; results of over 90% were always obtained. Hepatocytes were seeded on fibronectin-coated plastic dishes at a density of 8×10^4 viable cells/cm 2 and cultured in Ham's F-12/Lebovitz L-15 (1:1) medium supplemented with 2% newborn calf serum, 50 mU/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 0.2% BSA and 10 nM insulin.

2.3. Transepithelial transport and uptake

Caco-2 cells were grown as previously reported (Le Ferrec et al., 2001) for 21 days on microporous polycarbonate

transwell filters. The quality of the cell barrier formed under these conditions was assessed by evaluating the paracellular transport of ^3H -inulin and ^{14}C -mannitol, and by measuring the transepithelial electrical resistance (TEER). Once the barrier status was assessed, cells were washed twice with Hank's balanced salt solution (HBSS). HBSS (pH 7.4, 37 °C) was then applied to the basolateral side (receiver side) of the cells. Caffeic, chlorogenic, coumaric, ferulic and vanillic acids were diluted in HBSS to final concentrations of 50 μM , and 200 μM and loaded onto the apical side (donor site). Cell incubations were performed at non-toxic concentrations previously determined by the WST-1 assay (Palamakula and Khan, 2004). This viability test is based on the ability of mitochondrial succinate dehydrogenase to transform the WST-1 substrate, a tetrazolium salt, into an insoluble blue formazan. The amount of colored product formed is directly related to the activity of the mitochondria, which is a clear indicator of cell viability. After 1 h of incubation at 37 °C, apical and basolateral solutions were harvested and the concentration of each test compound in each receiver compartment determined by HPLC. Basal flux (%) and the coefficient of apparent permeability (Papp [cm/min]) were calculated as follows:

$$\text{Basal flux}_{1\text{h}} = (B_{1\text{h}}/A_0) \times 100 \quad (1)$$

where A_0 is the total amount of compound applied at time 0 to the apical compartment (in nmol), and $B_{1\text{h}}$ the amount of compound present in the basal compartment after 1 h of incubation (in nmol), and

$$\text{Papp (cm/min)} = \frac{dQ}{dt \times A \times C_0} \quad (2)$$

where dQ/dt is the amount of compound present in the basolateral compartment as a function of time (in nmol/min), A is the area of the layer crossed (in cm^2), and C_0 the initial concentration of compound applied on the apical side (in nmol/ml).

^3H -propranolol was added at 0.25 nmoles/ml and used as a positive control of transcellular passive diffusion. Test compounds were applied in triplicate. The data are the means of the results of two independent experiments, except for the absorption of chlorogenic acid, for which the results correspond to single experiment.

2.4. Metabolism of hydroxycinnamic and benzoic acids in rat hepatocytes (phase I and phase II reactions)

Primary cultured hepatocytes were metabolically characterized by means of their 7-ethoxycoumarin de-ethylase (ECOD) activity and testosterone metabolism as described elsewhere (Gomez-Lechon et al., 1997, 2003).

The biotransformation of caffeic, chlorogenic, coumaric, ferulic and vanillic acids, at concentrations of 50 and 200 μM , was studied using rat cultured hepatocytes. These concentrations were shown not to cause any appreciable cytotoxic effect on these cells by the WST-1 assay. After incubation periods of 4, 8 and 24 h, aliquots of culture

medium were enzymatically deconjugated (50 mU of β -glucuronidase/ml, 30 mU of arylsulfatase/ml, acetate buffer 0.1 M, pH 4, 5) for 4 h at 37 °C. The disappearance of the test compounds in these samples was analyzed by HPLC to identify drug-metabolizing activities dependent on cytochrome P450 (phase I metabolism).

To determine the percentage of the test compounds bound to endogenous molecules (phase II reactions), aliquots of culture medium incubated for 8 h (when the metabolism of the test compounds is linear) were either left non-deconjugated or deconjugated as described above. Percentage binding was analyzed by HPLC.

2.5. HPLC analysis of hydroxycinnamic and benzoic acids

Aliquots of untreated samples were analyzed by HPLC using a C18 reversed-phase Symmetry 5 μm 4.6 \times 150 mm column at 35 °C. The mobile phase used to elute the samples consisted of three solvent systems: solvent A ($\text{H}_2\text{O}/0.2\%$ formic acid), solvent B (acetonitrile/ 0.2% formic acid) and solvent C (methanol). The HPLC pump was programmed either at a flow rate of 0.7 ml/min with a solvent ratio of 95:5:0 (0 min), 70:20:10 (40 min) and 10:75:15 (50 min), or at 1 ml/min with a solvent ratio of 0:85:15 (51 min), 0:100:0 (60 min) and 95:5:0 (65 min). Compounds were identified on the basis of their retention times and UV spectra by monitoring column effluents at 278 nm using a photodiode detector (M996 Waters).

2.6. Antioxidant capacity of hydroxycinnamic and benzoic acids

H_2O_2 -scavenging was monitored by luminol-dependent chemiluminescence (LDC), using the method described by Baker et al. (1995). In this assay, light emission occurs when the chemiluminescence substrate, luminol, is oxidized in the presence of H_2O_2 . The steady emission of light is dependent on the production of free radical intermediates within the reaction mixture. 100 μl of 5 mM hydrogen peroxide were preincubated either for 5 min at room temperature (RT) or overnight (O/N) at 37 °C with 0.5 ml of increasing concentrations of caffeic, chlorogenic, ferulic, coumaric and vanillic acids (10, 50, 250, 1250 μM) in reaction buffer (0.2 M sodium phosphate buffer pH 7.0). Once incubation was complete, 50 μl of luminol solution were added to each sample and the light emission quantified for 30 s in an automated LKB 1251 luminometer. Luminol stock solution (32.2 mM) was prepared by dissolving 5.7 mg/ml of luminol in 1 N NaOH. A working stock was prepared daily by adding 0.1 ml of luminol stock to 19.9 ml of 1 M sodium phosphate buffer, pH 7.0. The H_2O_2 used (88 μM in 0.2 M sodium phosphate, pH 7) was prepared daily from a concentrated (30%) H_2O_2 stock. Caffeic, chlorogenic, ferulic, coumaric and vanillic acids solutions were also prepared daily from DMSO-stock solutions mixed 1:4 with reaction buffer. Ascorbic acid and PDTC were used as controls.

3. Results

3.1. Transepithelial transport of hydroxycinnamic and benzoic acids across a Caco-2 cell barrier

The quantities of the test compounds found in the apical and basolateral solutions after 1 h of incubation were comparable to the initial concentrations applied to the monolayer ($t = 0$). This indicates that they had not accumulated within the cells (data not shown). Permeability was determined for all the phenolic agents listed, as well as for mannitol, inulin and propranolol. The Papp values for the tested compounds were higher when these were applied at a concentration of 50 μM than at 200 μM (Fig. 2).

Chlorogenic acid at 50 μM and all the phenolic compounds crossed the Caco-2 cell barrier more efficiently than propranolol (Fig. 2). In fact, the Papp values for these compounds were at least twice those of propranolol, in agreement with the results of other authors (Artursson and Karlsson, 1991; Yee, 1997). This suggests absorption in humans after oral administration might be as high as 100%. As indicated above, these high Papp values cannot be attributed to a toxic effect on the integrity of the monolayer. Further, inulin and mannitol (controls for paracellular transport) showed the lowest basal flux and apparent permeability values, and were within the range expected for cell barrier integrity.

3.2. Determination of phase I and phase II metabolism

To examine phase I metabolism, culture supernatants obtained by incubating rat hepatocytes with caffeic, chlorogenic, coumaric, ferulic and vanillic acids were analyzed by HPLC. Fig. 3 and Table 1 show that, when incubated

at the 200 μM concentration, coumaric, ferulic and vanillic acids were metabolized at an average rate of 40%, 75% and 95% in 4, 8 and 24 h, respectively. As expected, incubations performed with the 50 μM concentrations led to the complete metabolic breakdown of the test compounds after incubation for 8 h (data not shown). The disappearance kinetics for caffeic and chlorogenic acids were comparable, however, in the presence and absence of rat hepatocytes (Fig. 3, Panels D and E). This was probably due to the instability of these compounds under these culture conditions. Compound disappearance in samples incubated in uncoated and fibronectin/BSA/collagen-coated plastic dishes for the same time periods (4, 8 and 24 h) gave comparable results to those obtained in previous experiments (Fig. 3, Panels D and E), suggesting that the low stability of these compounds in the assay conditions was probably the cause of their disappearance in the absence of cells.

Serum esterase activity on chlorogenic acid was also examined. No significant disappearance of chlorogenic acid was seen when incubated with increasing quantities of rat serum (data not shown), indicating that serum esterases play no role in chlorogenic metabolism in vitro.

The percentage of coumaric, ferulic and vanillic acid that conjugated with glucuronic acid and sulfate (phase II metabolism) was assessed in vitro. Table 1 shows that 50.3% of the ferulic acid conjugated either to glucuronic acid or sulfate; the same was true for 38.7% of coumaric acid and 16.9% of vanillic acid.

3.3. Effect of hydroxycinnamic and benzoic acids on luminol oxidation in vitro

The incubation of increasing concentrations of caffeic, chlorogenic, ferulic, coumaric and vanillic acids in the

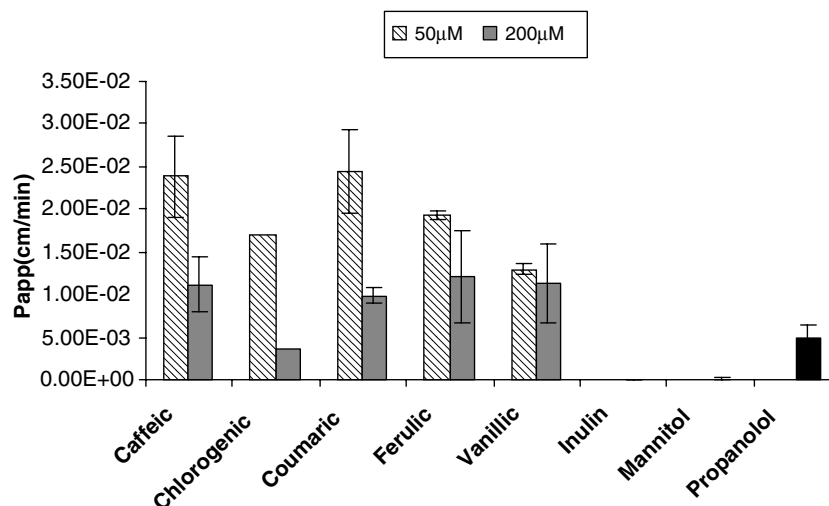


Fig. 2. Transepithelial transport of hydroxycinnamic and benzoic acids in an in vitro cell system resembling the intestinal barrier. Polarized Caco-2 cells grown on transwell filters were exposed to 50 μM and 200 μM concentrations of caffeic, chlorogenic, coumaric, ferulic and vanillic acids. After incubation for 1 h, the apparent permeability (Papp) of each compound was determined. The value for ^3H -propranolol was used as a positive control of transcellular passive diffusion. The Papp values for ^3H -inulin and ^{14}C -mannitol were representative of good Caco-2 cell barrier quality. Each bar represents the mean \pm SD of three replicates of two independent experiments, except for chlorogenic acid, for which the results come from a single experiment.

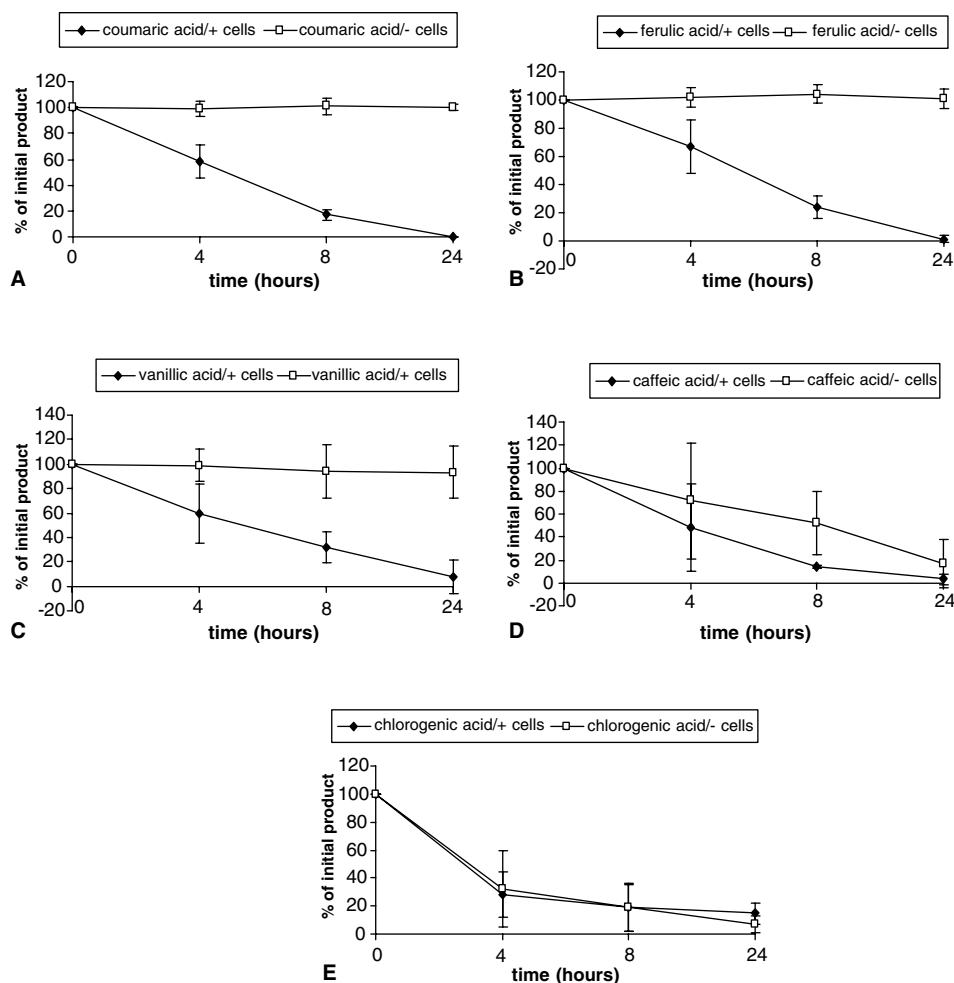


Fig. 3. Kinetics of hydroxycinnamic and benzoic acid metabolism. Coumaric, ferulic, vanillic, caffeic and chlorogenic acids at a concentration of 200 μM were incubated with rat primary hepatocytes (Panels A–E, respectively). After 4, 8 and 24 h, aliquots of media were analyzed by HPLC (method A; Section 2). The data represent the percentages left of the initial product (60 pM), and are the means obtained for three hepatocyte cultures.

Table 1
Hydroxycinnamic and benzoic acid metabolism in cultured rat hepatocytes (phase I and phase II reactions)

Metabolism	Incubation time (h)			
	4 h	8 h		24 h
	Phase I	Phase I	Phase II	Phase I
Coumaric acid	41.4 \pm 12.7	82.8 \pm 4.2	38.7 \pm 5.3	BD
Ferulic acid	32.9 \pm 18.7	76.2 \pm 7.7	50.3 \pm 0.7	98.6 \pm 2.4
Vanillic acid	40.8 \pm 24.0	67.6 \pm 12.6	16.9 \pm 7.1	92.0 \pm 13.9

Data represent the means of three independent replicates.

BD, below detection limit.

No determinations were made for caffeic and chlorogenic acids due to their instability under these culture conditions.

presence of H_2O_2 increased the resistance of luminol to oxidation (Fig. 4, Panels A and B). This resistance was comparable for all compounds at the two highest concentrations tested (250 μM and 1250 μM). In contrast, the compounds responded differently when their concentration fell below 50 μM , and more dramatically so at 10 μM . At the latter concentration, the protection of the luminol afforded by coumaric acid was almost negligible, and was comparable

only to that shown by DMSO. A reduced capacity to block luminol oxidation was also observed for vanillic acid. The IC_{50} (the concentration at which oxidation is inhibited at 50%) for ferulic and caffeic acid was 11.0 μM and 11.4 μM , respectively. Chlorogenic acid was found to be a good inhibitor of luminol oxidation with an IC_{50} value of 13.4 μM . In contrast, to obtain a 50% inhibitory effect for vanillic and coumaric acids, concentrations one order of magnitude higher were needed (109.2 μM and 121.8 μM , respectively). This antioxidative capacity was stable with time and temperature, even when incubation was at 37 $^\circ\text{C}$ overnight, and comparable to that obtained when the incubation time was shorter and at room temperature (Fig. 4, Panels A and B).

4. Discussion

Caffeic, chlorogenic and ferulic acid were the most potent inhibitors of luminol oxidation. This is in agreement with the results of other authors (Kato et al., 2003; Firuzi et al., 2003; Neudorffer et al., 2004; Kanski et al., 2002;

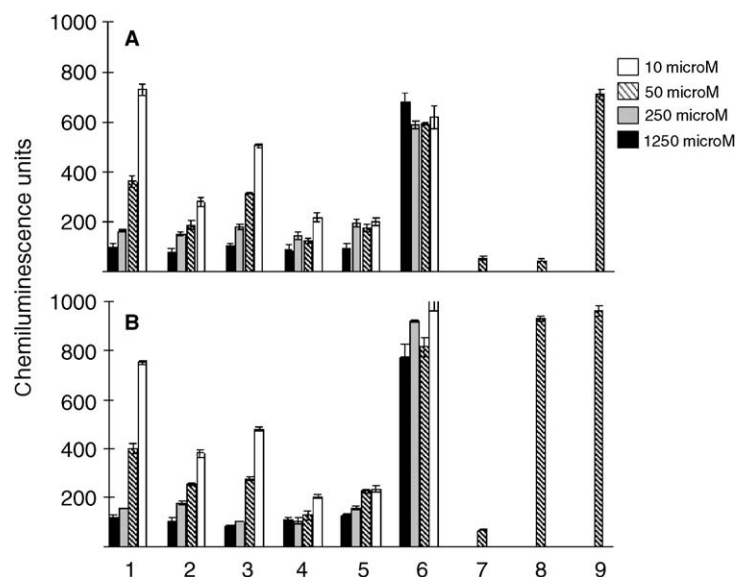


Fig. 4. Effect of hydroxycinnamic and benzoic acids on the resistance of luminol to oxidation. Increasing concentrations of coumaric (1), caffeic (2), vanillic (3), ferulic (4) and chlorogenic (5) acids, DMSO (6), PDTC (7), and ascorbic acid (8) were preincubated with 5 mM hydrogen peroxide either for 5 min at 20 °C (Panel A) or overnight at 37 °C (Panel B). Reaction buffer (0.2 M sodium phosphate buffer pH 7.0) was used as negative control (9). At the end of these periods, luminol was added to the reaction and the light emission quantified using a luminometer. Ascorbic acid was used at 50 mg/ml. Experiments were carried out in triplicate.

Bonina et al., 2002) who report the following reducing trend in oxidation potentials and IC_{50} values: caffeic acid > chlorogenic acid > ferulic acid > coumaric acid. The structural characteristics of these compounds might explain their different antioxidant capacities (Fig. 1). According to Rice-Evans et al. (1996), the incorporation of a hydroxyl group at the *para* position of a monophenol (e.g., *p*-coumaric acid) enhances its antioxidant activity. This agrees with the present data since ferulic, caffeic, chlorogenic and vanillic acids were found to be better antioxidants than *p*-coumaric acid. Among the tested polyphenols, vanillic acid was the least effective, probably due to the lack of a $-CH=CH-COOH$ chain. This chain has electron donor properties, and the stabilization of the resulting radical might be increased by electron delocalization after hydrogen donation by the hydroxyl group. As expected, glycosylation of the carboxylate group of caffeic acid (chlorogenic acid) did not significantly influence the antioxidant potential since the IC_{50} values for both compounds were comparable (11.4 μ M and 13.4 μ M, respectively).

Few studies have tried to estimate the bioavailability of phenolic compounds in the diet. In the present study, the transcellular transport of PL's major phenolic compounds was studied by applying them to the apical site of a Caco-2 cell monolayer. The concentrations tested were selected according to their content in fruits and beverages described in the literature (DuPont et al., 2002; Simonetti et al., 2001; Clifford, 2001). Compound uptake was not a linear function of substrate concentration. In fact, compounds applied at 50 μ M on the apical donor compartment were transported more efficiently than when applied at 200 μ M. Paracellular diffusion was not involved, as shown

by the low Papp values for inulin and mannitol compared to the test compounds. These results suggest a concentration-dependent saturation of an active transport system for these compounds. Indeed, the literature refers to the absorption of chlorogenic acid, ferulic acid and other hydroxycinnamates as a Na^+ /dependent carrier-mediated transport process that is not saturated at 50 μ M (Williamson et al., 2000; Adam et al., 2002; Konishi et al., 2002). This might explain the differences found between the Papp values. Further experiments including the examination of basolateral-apical transport and the use of inhibitors are required to elucidate the absorption mechanism. Independently, the Papp values determined for caffeic, coumaric, ferulic and vanillic acids were comparable or even higher than those obtained for propranolol, predicting that their absorption in humans after oral administration might be as high as 100% (Artursson and Karlsson, 1991; Yee, 1997). Finally, chlorogenic acid, at least at 200 μ M and under the experimental conditions of the assay, less easily penetrated the cells than did the other compounds. This has been reported by other authors (Olthof et al., 2001; Azuma et al., 2000).

Although little is known about the metabolism of hydroxycinnamic and benzoic acids in the diet, their biotransformation by different metabolizing systems (i.e. P450/NADPH) has been reported (Williamson et al., 2000; Moridani et al., 2001), usually generating more polar and reactive metabolites. In the present study, the metabolism of these phenolic components was studied in vitro using rat isolated primary cultured hepatocytes. Hepatocytes have been widely used in drug metabolism studies as a model of in vivo metabolism since these cells express

phase I and phase II biotransformation enzymes in culture (Donato et al., 2002). The present results show comparable metabolic breakdown rates for coumaric, ferulic and vanillic acids. The metabolic kinetics of caffeic and chlorogenic acid could not be determined since both disappeared in the absence and presence of these cells. This same behavior has been previously reported (Friedman and Jürgens, 2000), in which caffeic and chlorogenic acids were found to be unstable at pH 7–11.

Several papers report that the metabolites of most consumed phenols are found in plasma and urine in both rats and humans, usually in the form of glucuronide sulfate and sulfate/glucuronide conjugates (Williamson et al., 2000; Lee et al., 1995; Clifford, 2001; Adam et al., 2002; Azuma et al., 2000; Cremin et al., 2001). In agreement with these studies, the present data indicate that ferulic and coumaric acids also suffer extensive phase II metabolism in hepatocytes, with 50% and 40%, respectively of these molecules binding to glucuronic acid and sulfate. A smaller binding percentage was found for vanillic acid, although no data are available on its phase II metabolism.

The analysis of serum esterase activity on chlorogenic acid was performed since the literature reports its cleavage into caffeic and quinic acids, probably by the action of the esterases of the colonic microflora (Azuma et al., 2000; Plumb et al., 1999). Incubating chlorogenic acid with increasing concentrations of human serum did not result in the disappearance of the compound (as determined by HPLC).

In conclusion, these major phenolic components of PL showed significant antioxidant activity. These molecules are absorbed very efficiently in a cellular model resembling the intestinal barrier and are metabolized slowly. The data obtained suggest that almost complete absorption of these compounds occurs in humans after the oral administration of PL.

Acknowledgment

This work was partially supported by an IFC research grant. Dr. S. González is an IFC consultant.

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