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Influence of anthocyanins and derivative pigments from blueberry (*Vaccinium myrtillus*) extracts on MPP^+ intestinal uptake: A structure–activity approach

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Abstract

The aim of the present study was to investigate the influence of an anthocyanin extract (extract I), and two other derivative extracts (extracts II and III), which are being developed aiming to be further applied in the food industry, on intestinal uptake of organic cations. For this purpose, the effect of these compounds on ^{3}H-MPP^+ uptake was evaluated in Caco-2 cells (an enterocyte-like cell line derived from a human colonic adenocarcinoma).

Extracts I and III did not present any effect on ^{3}H-MPP^+ uptake. Extract II, an anthocyanin–pyruvic acid adduct extract, decreased organic cation uptake. Some phenolic molecules were also tested, aiming to study possible structural features responsible for these effects. The results are compatible with the hypothesis of phenolic molecules bearing a carboxylic group with extended electronic conjugation being the responsible feature for the inhibitory effect on ^{3}H-MPP^+ uptake.

The use of these compounds in foodstuffs must be carefully assessed since interactions with organic cations present in the diet, as nutrients or xenobiotics, may affect their absorption and bioavailability. Type and complexity of the ingested compounds are important factors in this regard, and should be taken into consideration.

Keywords: Absorption; Anthocyanin; Bioavailability; Caco-2 cells; Diet; Intestinal epithelia; MPP^+; Organic cation

1. Introduction

Anthocyanins are a group of naturally occurring phenolic compounds responsible for the colour of many flowers, fruits (particularly berries) and vegetables. Many of the health-promoting properties of berries are thought to be attributable to their bioactive compounds (namely proanthocyanidins and anthocyanins) (Sanchez-Moreno, Cao, Ou, & Prior, 2003; Santos-Buelga & Scalbert, 2000). Anthocyanins are present in blueberries at high concentrations (Kähkönen, Heinämäki, Ollilainen, & Heinonen, 2003) and these are among the fruits that are best recognized for their potential health benefits.

More recently, new stable anthocyanin-derived pigments, namely anthocyanin–pyruvic acid adducts and vinylpyranoanthocyanin–catechins (portisins) have been detected in wine (Bakker & Timberlake, 1997; Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998; Mateus, Silva, Rivas-Gonzalo, Santos-Buelga, & de Freitas, 2003). These compounds exhibit unusual colours (orange and blue) and, because of their structural properties, they are very stable, display great resistance to colour bleaching by sulfur dioxide, and express more colour at

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higher pH values than do their anthocyanin precursors (Oliveira et al., 2006; Oliveira, Santos-Buelga, Silva, de Freitas, & Mateus, 2006; Sarni-Manchado, Fulcrand, Souquet, Cheynier, & Moutounet, 1996; Vivar-Quintana, Santos-Buelga, & Rivas-Gonzalo, 2002). Little is known about the absorption and uptake of anthocyanins and respective derivative pigments and the interaction of these compounds with the intestinal absorption of dietary compounds, despite their common occurrence in diet (Hillebrand, Schwarz, & Winterhalter, 2004; Koponen, Happonen, Mattila, & Torronen, 2007; Macz-Pop, Gonzalez-Paramas, Perez-Alonso, & Rivas-Gonzalo, 2006; Mateus, Silva, Vercauteren, & de Freitas, 2001; Wu et al., 2006). It is becoming increasingly evident that the absorption of certain nutrients and drugs may be influenced by the concomitant ingestion of other substances. For example, polyphenols are naturally occurring compounds in foodstuffs of vegetal origin and are widely present in human diet. In fact, it has been recently reported (Faria, Mateus, de Freitas, & Calhau, 2006; Monteiro et al., 2005; Monteiro, Calhau, Martel, Guedes de Pinho, & Azevedo, 2005) that beverages, such as red wine and tea, and procyanidins modulate the intestinal uptake of organic cations, and most vitamins, nutrients and xenobiotics pass the intestinal barrier as organic cations.

Intestinal transporters may play a crucial role in limiting and/or promoting the absorption or secretion of organic cations. Indeed, many organic cations are incompletely absorbed after oral administration and may also be actively secreted in the intestine (Hardman, Limbird, Molinoff, Ruddon, & Gilman, 2002). Drugs from a wide array of therapeutic groups, including antihistamines, skeletal muscle relaxants, calcium channel blockers and ß-adrenoceptor blocking agents, as well as various xeno- and endobiotics, are organic cations. Several endogenous bioactive amines (such as catecholamines, 5-hydroxytryptamine and histamine) and some vitamins (such as thiamine and riboflavin) are also organic cations.

The aim of this work was to characterize the modulation of the intestinal apical uptake of organic cations by a blueberry (*Vaccinium myrtillus*) extract and two anthocyanin-derived extracts which are being developed to be used as food colorants. Caco-2 cells, an enterocyte-like cell line derived from a human colonic adenocarcinoma, were used as an intestinal model. This human intestinal epithelial cell line forms confluent monolayers of well-differentiated enterocyte-like cells with the functional properties of transporting epithelia (Artursson, 1991; Artursson & Karlsson, 1991; Hidalgo, Raub, & Borchardt, 1989; Yee, 1997).

2. Materials and methods

2.1. Reagents

^{3}H-MPP^{+} (*N*-[methyl-^{3}H]-4-phenylpyridinium acetate; specific activity 82 Ci mmol^{-1}) (New England Nuclear Chemicals, Dreieich, Germany) and MPP^{+} (1-methyl-4-phenylpyridinium iodide) (Research Biochemicals International, Natick, MA, USA) were used. Toyopearl gel was purchased from Tosoh® (Tokyo, Japan); caffeic acid, catechol, gallic acid, Hepes, NaCl, phloroglucinol, pyruvic acid and vinylphenol were purchased from Sigma-Aldrich® (Madrid, Spain), ferulic acid, *para*-coumaric acid and tannic acid were purchased from Fluka® (Madrid, Spain) and Triton X-100 from Merck® (Darmstadt, Germany).

2.2. Extract preparation

Two hundred grams of blueberries (*V. myrtillus*) picked in field were subjected to extraction with 500 ml of 50% aqueous ethanol (pH 1.5) for 30 min at room temperature. The blueberry anthocyanin extract (I) was filtered in a 50 μm nylon membrane and then purified by Toyopearl gel column chromatography according to the procedure described previously (Pissarra, Mateus, Rivas-Gonzalo, Santos Buelga, & Freitas, 2003).

Anthocyanin–pyruvic acid adducts (extract II) were prepared through reaction of the genuine anthocyanin extract with pyruvic acid in water (pH 2.6, 35 °C) at an approximate molar ratio, pyruvic acid/anthocyanin, of 50:1 during 5 days. The resulting extract was purified by Toyopearl gel column chromatography with the anthocyanin–pyruvic acid adducts fraction eluted with water/ethanol 20% (v/v).

Vinylpyranoanthocyanin–catechins (portisins) (extract III) were prepared through reaction of anthocyanin–pyruvic acid adducts (extract II) with catechin and acetaldehyde at 35 °C in 20% aqueous ethanol (pH 1.5) at an approximate molar ratio of catechin/acetaldehyde/anthocyanin–pyruvate of 50:25:1. After 10 days of reaction, the solution was analyzed by HPLC-DAD and the portisin extract was isolated by Toyopearl gel column chromatography by elution with water/ethanol 85% (v/v), following the procedure previously described (Mateus, Oliveira, Santos-Buelga, Silva, & De Freitas, 2004). All the extracts were freeze-dried and stored at −20 °C until used. Two milligrams of each extract were re-dissolved in 10 ml of methanol for further analysis.

2.3. Polyphenolic characterization of the extracts

Total polyphenol content, the total flavonoid content and the concentration of the anthocyanins and anthocyanin-derived pigments of the extracts were determined as previously described (Faria et al., 2005).

2.4. HPLC conditions

All extracts were analyzed by HPLC (Knauer K-1001) on a 250 × 4.6 mm i.d. reversed-phase C18 column (Merck, Darmstadt); detection was carried out at 511, 528 and 570 nm using a diode array detector (Knauer K-2800). The solvents were A:H_2O/HCOOH (9:1), and B:H_2O/CH_3CN/HCOOH (6:3:1). The gradient consisted of 20–85% B for 70 min at a flow rate of 1.0 ml min^{-1}. The

column was washed with 100% B for 20 min and then stabilized at the initial conditions for another 20 min. Extract III was also analyzed by HPLC using the same conditions with a different solvent, B:$CH_3CN/H_2O/CH_3COOH$ (8:1.95:0.05).

2.5. LC–MS conditions

A liquid chromatograph (Hewlett-Packard 1100 series) equipped with an AQUA™ (Phenomenex, Torance, CA, USA) reversed-phase column (150 × 4.6 mm, 5 μm, C18), thermostatted at 35 °C, was used. Solvents were (A) aqueous 0.1% trifluoroacetic acid, and (B) acetonitrile, establishing the gradient as reported elsewhere (Pissarra et al., 2003). Double online detection was done in a photodiode spectrophotometer and by mass spectrometry. The mass detector was a Finnigan LCQ (Finnigan Corporation, San Jose, USA) equipped with an API source, using an electrospray ionisation (ESI) interface. Both the auxiliary and the sheath gas were a mixture of nitrogen and helium. The capillary voltage was 3 V and the capillary temperature 190 °C. Spectra were recorded in positive ion mode between m/z 120 and 1500. The mass spectrometer was programmed to do a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and a MS–MS of the most intense ion using relative collision energies of 30 and 60.

2.6. Cells and culture conditions

The Caco-2 cell line was obtained from the American Type Culture Collection (ATCC37-HTB, Rockville, MD, USA) and was used from passage number 53–73. Caco-2 cells were maintained in a humidified atmosphere of 5% CO_2-95% air and were grown in Minimum Essential Medium (Sigma, St. Louis, MO, USA) supplemented with 15% fetal bovine serum, 25 mmol l^{-1} of HEPES, 100 units ml^{-1} of penicillin, 100 μg ml^{-1} of streptomycin and 0.25 μg ml^{-1} of amphotericin B (all from Sigma). Culture medium was changed every 2–3 days and the culture was split every 7 days. For subculturing, the cells were removed enzymatically (0.25% trypsin-EDTA, 5 min, 37 °C), split 1:3, and subcultured in plastic culture dishes (21 cm^2; ∅ 60 mm; Corning Costar, Corning, NY). For the experiments, the Caco-2 cells were seeded on 24-well plastic cell culture clusters (2 cm^2; ∅ 16 mm; Corning Costar); 24 h prior to the experiment, the cell medium was free of fetal bovine serum. Uptake studies were generally performed 9–11 days after the cells formed a monolayer. Each square centimetre contained about 600–1000 μg of cell protein.

2.7. Transport studies

2.7.1. General

The transport experiments were performed in Hanks' medium with the following composition (in mmol l^{-1}): (137) NaCl, (5) KCl, (0.8) $MgSO_4$, (1.0) $MgCl_2$, (0.33) Na_2HPO_4, (0.44) KH_2PO_4, (0.25) $CaCl_2$, (0.15) Tris–HCl, and (1.0) sodium butyrate, pH 7.4.

Initially, the growth medium was aspirated and the cells were washed with Hanks' medium at 37 °C; then the cell monolayers were pre-incubated in Hanks' medium at 37 °C. Transport studies were performed in cells cultured on plastic supports, ^{3}H-MPP^+ being applied to the medium facing the apical cell membrane. Uptake was initiated by the addition of 0.3 ml of medium at 37 °C containing 200 nmol l^{-1} ^{3}H-MPP^+. Incubation was stopped after 5 min by placing the cells on ice and rinsing them with 0.5 ml of ice-cold Hanks' medium. The cells were then solubilized with 0.3 ml 0.1% (v/v) triton X-100 (in 5 mM Tris–HCl, pH 7.4) at room temperature overnight. Radioactivity in the cells was measured by liquid scintillation counting.

2.7.2. Effect of compounds

Compounds to be tested were present during both the preincubation and incubation periods. Controls for all treatments were run in the presence of ethanol for all the extracts, ferulic acid, caffeic acid, *p*-coumaric acid, tannic acid, gallic acid and phloroglucinol and in the presence of H_2O for vinylphenol and catechol.

2.8. Statistical analysis

Values are expressed as the arithmetic means ± SEM. Statistical significance of the difference between various groups was evaluated by one-way analysis of variance (ANOVA), followed by the Bonferroni test. For comparison between two groups, the Student's *t*-test was used. Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Extracts

Blueberry (*V. myrtillus*) extract, and two other derivative extracts, are being developed, aiming to be further applied in the food industry as food colorants. The major interest of the putative use of these extracts lies in their unusual colours in acidic conditions (more orange and bluish hues) when compared with their natural anthocyanin precursors. Extracts I, II and III were obtained as described in Section 2.

The blueberry extract I is comprised of anthocyanins, whilst extract II is mainly comprised of anthocyanin–pyruvic acid adducts and extract III is comprised of bluish anthocyanin-derived pigments, named portisins (Mateus et al., 2003). The identities of these compounds were previously confirmed by HPLC–MS (Faria et al., 2005) and the general structures are presented in Fig. 1. The pigment extract contents were characterized (Table 1) and their antioxidant properties have been investigated in a previous work (Faria et al., 2005).

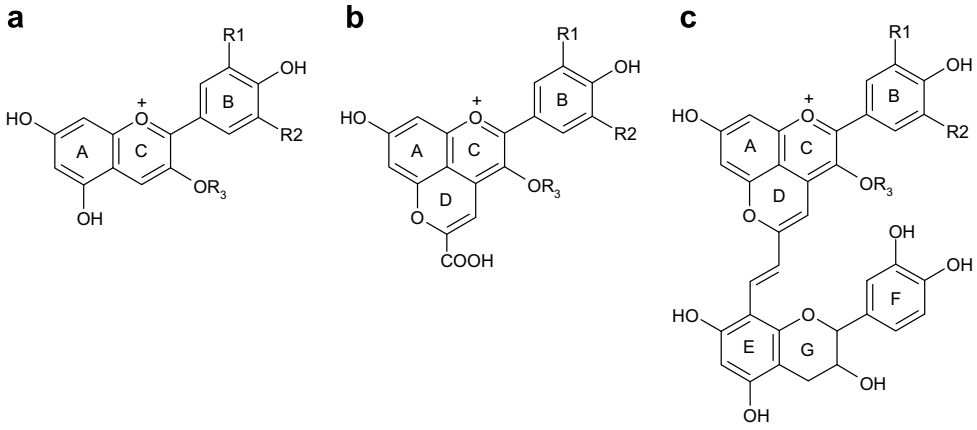


Fig. 1. General structure of blueberry (*Vaccinium myrtillus*) anthocyanins (a), anthocyanin–pyruvic acid adducts (b) and portisins (c), present in extracts I, II and III, respectively. R1 and R2, independently of each other, are H, OH or OMe. R3 is glucose, galactose or arabinose.

Table 1
Contents of total phenolics, total flavonoids and total pigments of a blueberry (*Vaccinium myrtillus*) extract (I) and its respective anthocyanin–pyruvic acid adducts (II) and vinylpyranoanthocyanin–catechin (III) extracts (from Faria et al., 2005).

	Total phenolics (mg l^{-1})	Total flavonoids (mg l^{-1})	Total pigments (mg l^{-1})
Extract I	258 ± 8.5^{a}	153 ± 9.3^{a}	78.8 ± 4.0^{a}
Extract II	320 ± 7.5^{b}	149 ± 7.1^{a}	39.8 ± 2.0^{b}
Extract III	318 ± 13.8^{b}	287 ± 8.7^{b}	101 ± 5.0^{c}

The mean values followed by different letters in each column are significantly different at $p < 0.001$.

3.2. Modulation of ^{3}H-MPP^{+} intestinal uptake

Most nutrients and xenobiotics pass the intestinal barrier as organic cations and ^{3}H-MPP^{+} (*N*-[methyl-^{3}H]-4-phenylpyridinium acetate) has been used as a model to study the intestinal uptake of organic cations (Grundemann, Liebich, Kiefer, Koster, & Schomig, 1999; Russ, Gliese, Sonna, & Schomig, 1992; Sitte et al., 2001). ^{3}H-MPP^{+} was used as a substrate and, as previously reported by Martel, Calhau, and Azevedo (2000), its uptake into Caco-2 cells is linear in time for up to 5 min of incubation. Therefore, in order to determine initial rates of uptake, cells were incubated in the presence of ^{3}H-MPP^{+} (200 nmol l^{-1}) for 5 min.

Caco-2 cells were pre-incubated with 100 μg ml^{-1} of the blueberry extract (extract I), for 60 min, and incubated with ^{3}H-MPP^{+} for 5 min. Extract I did not show any effect on ^{3}H-MPP^{+} uptake. The two extracts derived from anthocyanins, containing anthocyanin–pyruvic acid adducts (extract II) and vinylpyranoanthocyanin–catechins (extract III) were also tested. Extract III did not show any effect on ^{3}H-MPP^{+} uptake; however, extract II, the anthocyanin–pyruvic acid adducts extract, significantly decreased ^{3}H-MPP^{+} uptake (Fig. 2). The different results may be explained by the different structures present in the extracts. The major structural difference between the compounds present in extract II, comparatively to the other extracts, is the presence of a carboxylic group in ring D (Fig. 1).

Molecular size could be an important issue but, since the smaller (extract I) and the larger molecules (extract III) were those that had no effect on ^{3}H-MPP^{+} uptake; this parameter was thought not to interfere with the transport. The hypothesis that the presence of the carboxylic group may play an important role in ^{3}H-MPP^{+} uptake was suggested. In order to confirm this hypothesis, some phenolic acids were tested and compared with structurally similar phenolic molecules (Fig. 3). Some interesting trends were observed on ^{3}H-MPP^{+} uptake when the cells were pre-incubated for 60 min with these compounds: (1) *para*-coumaric acid significantly inhibited ^{3}H-MPP^{+} uptake (Fig. 4), but curiously, vinylphenol (whose structure is similar to

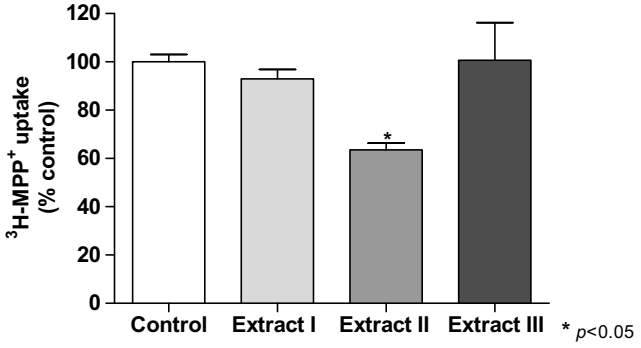


Fig. 2. Effect of 100 μg ml^{-1} of a blueberry (*Vaccinium myrtillus*) extract (I) and its respective anthocyanin–pyruvic acid adducts (II) and vinylpyranoanthocyanin–catechin (III) extracts on ^{3}H-MPP^{+} apical uptake by Caco-2 cells. Confluent Caco-2 monolayers were pre-incubated at 37 °C for 60 min and then incubated with 200 nmol l^{-1} ^{3}H-MPP^{+} for 5 min in the presence of EtOH (control) or the tested compounds. Each value represents the mean ± SEM ($n = 6$–21).

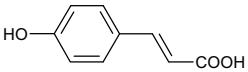
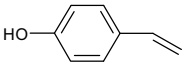
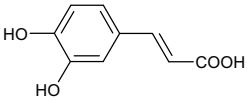
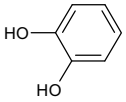
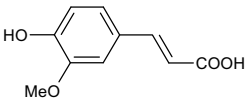
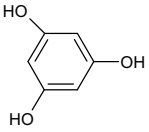
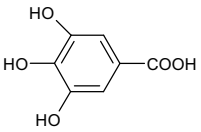
Compound	Structure	Compound	Structure
p-Coumaric acid		Vinylphenol	
Caffeic acid		Catechol	
Ferulic acid		Phloroglucinol	
Gallic acid			

Fig. 3. Structures of phenolic acids and vinylphenol, catechol and phloroglucinol.

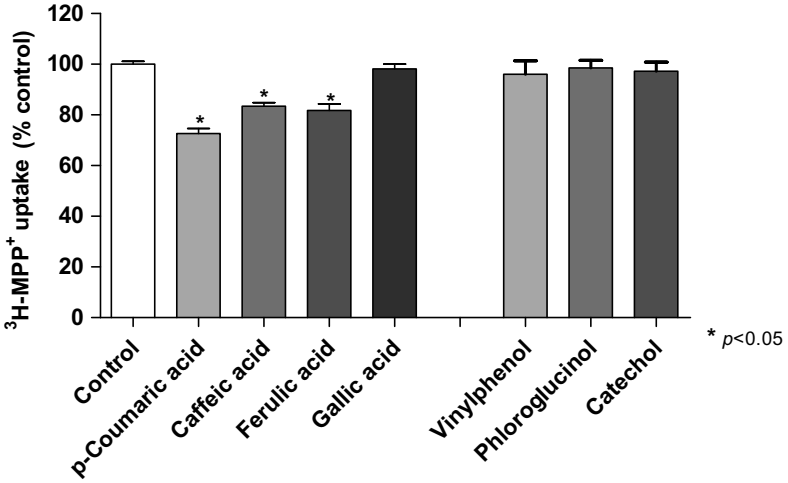


Fig. 4. Effects of 250 μg ml^{-1} of different phenolic acids, vinylphenol, phloroglucinol and catechol on ^{3}H-MPP$^+$ apical uptake by Caco-2 cells. Confluent Caco-2 monolayers were pre-incubated at 37 °C for 60 min and then incubated with 200 nM ^{3}H-MPP$^+$ for 5 min in the presence of the solvent (control) or the tested compounds. Each value represents the mean ± SEM ($n = 6$–12).

para-coumaric acid without the carboxylic group) did not have a significant effect; (2) caffeic acid did also significantly decrease ^{3}H-MPP$^+$ uptake but catechol, a similar molecule without the carboxylic group and the vinyl bond, had no effect on ^{3}H-MPP$^+$ uptake (Fig. 4); (3) gallic acid and phloroglucinol were also tested and did not show any effect on ^{3}H-MPP$^+$ uptake under the tested conditions.

Overall, these data may suggest an influence of the presence of the carboxylic group in the phenolic structure. On the other hand, all the phenolic acids bearing a vinylphenolic group, in which a higher electronic conjugation is likely to occur, induced a decrease of ^{3}H-MPP$^+$ uptake.

3.3. A structure–activity approach

The structures present in both extracts I and II include *ortho*-dihydroxyl groups in ring B, have been related to some beneficial properties (such as antioxidant activity, antiradical activity, reducing power and heavy metal chelating ability) displayed by the flavonoids (including anthocyanins) (Fernandez, Mira, Florencio, & Jennings, 2002; Rice-Evans, Miller, & Paganga, 1996). Extract III has more *ortho*-dihydroxyl groups than have extracts I and II, as well as an additional hydroxyl group in ring C of the catechin moiety. If these characteristics had influence on organic

cation uptake in intestinal epithelia, extract III would be expected to show the highest effect, which did not occur. Instead, the results obtained seem to indicate a relationship between ^{3}H-MPP$^+$ uptake and the presence of a carboxylic group with high π electron conjugation in the phenolic structure. This outcome arises from the observation of the results obtained with extract II and the phenolic acids tested.

Several hypotheses may be advanced for the interaction of these compounds with the substrate (^{3}H-MPP$^+$) or with the transporters involved in the intestinal uptake. In fact, the observed effect could result from ionic interactions between the tested compounds and ^{3}H-MPP$^+$, since the substrate has a positive charge and the acids, at the pH used for the experiment, are partially dissociated yielding a negative charge.

Also, all the pH values of the preincubation media were checked and set to pH 7.4, assuring that the effects perceived did not arise from pH variations since the transporters involved on ^{3}H-MPP$^+$ are pH-dependent (Martel et al., 2000).

On the other hand, regarding the transporters involved in ^{3}H-MPP$^+$ uptake, a competitive inhibition was also considered as a possibility for the observed effect since a concentration dependence of the inhibition of organic cation uptake was observed for extract II (Fig. 5). However, considering that experiments were performed in neutral pH, the anthocyanin derivative pigments are not expected to acquire any charge in solution, so it was unlikely that OCTs (organic cation transporters) were involved in intestinal absorption of these compounds. Furthermore, it has been reported that some isoforms of MCT (monocarboxylic acid transporter) are involved on intestinal uptake of cinnamic and benzoic acids (Konishi, Kubo, & Shimizu, 2003; Konishi & Shimizu, 2003; Watanabe, Yashiro, Tohjo, & Konishi, 2006). Nevertheless, the results obtained with extract II can be explained by some events: (i) interference of the tested molecule with the transporter itself, that could lead to changes in the transporter conformation, affecting the affinity of the transporter for its substrate and/or (ii) involvement in transporter regulation mechanisms.

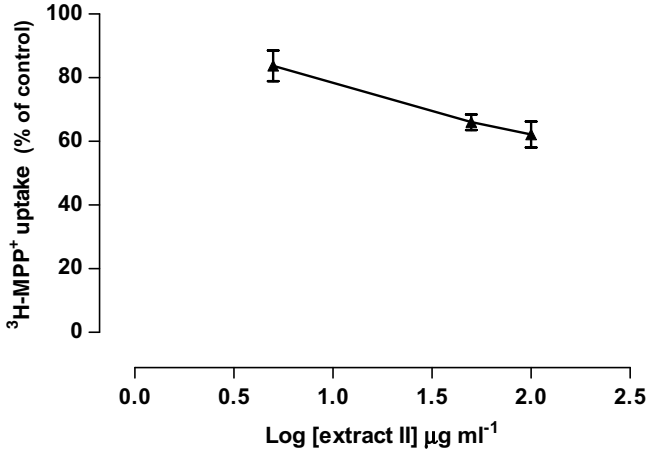


Fig. 5. Effect of different concentrations of extract II (5; 50; 100 μg ml^{-1}) on ^{3}H-MPP$^+$ apical uptake by Caco-2 cells. Confluent Caco-2 monolayers were pre-incubated at 37 °C for 60 min and then incubated with 200 nmol l^{-1} ^{3}H-MPP$^+$ for 5 min in the presence of the solvent (control) or the tested compound. Each value represents the mean ± SEM (n = 9–12).

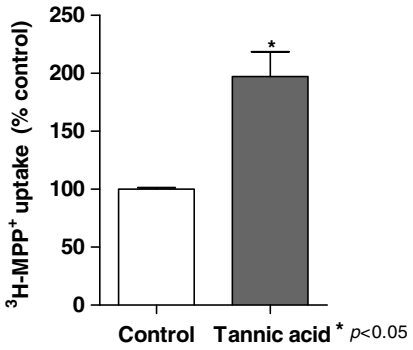


Fig. 6. Effect of 250 μg ml^{-1} of tannic acid on ^{3}H-MPP$^+$ apical uptake by Caco-2 cells. Confluent Caco-2 monolayers were pre-incubated at 37 °C for 60 min and then incubated with 200 nmol l^{-1} ^{3}H-MPP$^+$ for 5 min in the presence of the solvent (control) or the tested compound. Each value represents the mean ± SEM (n = 6–9).

3.4. Tannic acid influence on ^{3}H-MPP$^+$ uptake

As described above, gallic acid showed no effect on ^{3}H-MPP$^+$ uptake. However, since tannic acid has a structure based mainly on glucose esters of gallic acid, this acid was also tested and an interesting result was obtained. Tannic acid increased ^{3}H-MPP$^+$ uptake in Caco-2 to 197 ± 22% of control at the concentration tested (Fig. 6).

There are already some reports regarding the interaction of tannic acid with transport of several substances (Cai & Bennick, 2006; Liu et al., 2005; Naus et al., 2007). The disparities in molecular weight between gallic and tannic acids may in part be responsible for the observed effect. In fact, this effect was similar to those observed with more complex flavonoids tested under the same conditions – procyanidins (Faria et al., 2006). This previous work has shown that procyanidins increase ^{3}H-MPP$^+$ uptake in Caco-2 cells, this effect being more pronounced with higher molecular weight structures.

4. Conclusion

In conclusion, the anthocyanin–pyruvic acid adducts extract (extract II) decreased organic cation uptake, probably due to the presence of a carboxylic group with extended electronic conjugation in the phenolic structures. This effect was also perceived with phenolic acids bearing the same structural feature. The putative use of these compounds in the food industry must be attempted cautiously since interactions with organic cations present in the diet, as nutrients or xenobiotics, may affect their absorption and bioavailability. Type and complexity of the ingested

compounds are important factors in this regard, and should be taken into consideration. Further studies with purified individual molecules will be helpful for clarifying a putative structure–activity relationship.

Acknowledgments

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