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Antioxidant and anti-acetylcholinesterase activity of commercially available medicinal infusions after *in vitro* gastrointestinal digestion

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***Cynara cardunculus*, *Fraxinus angustifolia* and *Pterospartum tridentatum* are plants commercialized in Portugal for digestion-related ailments, among others. The aim of this study was to identify the chemical composition of the infusions and determine the inhibition of acetylcholinesterase, the antioxidant activity, and to evaluate if the chemical composition and activities remained after *in vitro* gastrointestinal digestion. These activities can be related with the traditional uses of these plants. The toxicity of the infusions was also tested with Caco-2 cells, a model of intestinal cells. The composition of the plant extracts revealed mainly flavonoids and phenolic acids. The antioxidant activity measured as half maximal effective concentration (EC_{50}) values ranged from 19 to 120 µg/ml and the acetylcholinesterase inhibition activity of the three extracts determined as half inhibitory concentration (IC_{50}) was between 1 and 2.5 mg/ml. The composition and the biochemical activities remained after the *in vitro* gastrointestinal digestion. The infusions showed no toxicity against Caco-2 cell lines. The antioxidant activity and inhibition of acetylcholinesterase were maintained during the digestive process, and therefore can be important mechanisms for the therapeutic effects attributed to the infusions under evaluation, namely to treat digestive problems. Even though the infusions presented biochemical activities, they were not toxic for the intestine cell line.**

Key words: Antioxidant activity, acetylcholinesterase inhibition, chlorogenic acid, flavonoid, gastrointestinal problems, *Cynara cardunculus*, *Fraxinus angustifolia*, *Pterospartum tridentatum*.

INTRODUCTION

Plant infusions have a long tradition in the treatment of problems related with the digestive tract. In Portuguese local shops and pharmacies, the three plants under evaluation have been recommended as infusions for the

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"digestive system": Artichoke bracts (*Cynara cardunculus* var. scolymus, Asteraceae), ash tree leaves (*Fraxinus angustifolia*, Oleaceae), and "carqueja" flowers (*Pterospatum tridentatum*, Fabaceae).

According to the Portuguese ethnobotanic literature, all these plants are used in treatments related with the gastrointestinal tract and liver (Proença da Cunha et al., 2003). *C. cardunculus* bract infusions are used as digestives, to treat nausea, and to reduce levels of cholesterol and triglycerides in the bloodstream (Proença da Cunha et al., 2003). Infusions of leaves of *F. angustifolia* are used as laxative, anti-inflammatory, and to lose weight (Proença da Cunha et al., 2003). Infusions of flowers of *P. tridendatum* are used to treat constipation, stomach aches, feeling of unwellness after eating and to decrease blood cholesterol levels (Oliveira and Neiva, 2004).

Acetylcholinesterase (AChE) inhibitors can be used to increase gastrointestinal motility since acetylcholine is the major excitatory neurotransmitter responsible for the peristaltic contractions (Holzer and Maggi, 1994). Acetylcholine is also responsible for controlling the ion transport in gut epithelial cells and, therefore, water secretion for gut hydration (Hirota and Mckay, 2006). Gut hydration is important in establishing a proper aqueous environment for the enzymatic digestion and absorption of nutrients, and to provide surface lubrication to propel intestinal contents by the peristaltic movements (Hirota and McKay, 2006). Conditions that may be associated with disturbances of the gastrointestinal motility and treated with AChE inhibitors include dysphagia, gastric stasis achalasia, abdominal pain, paralytic ileus, vomiting and constipation (Sasho et al., 1995).

The AChE inhibitor neostigmine is used to treat conditions related with impairment of gastrointestinal motility such as colonic pseudo-obstruction (Ponec et al., 1999) and post-operative impairment after colorectal surgery (Kreis et al., 2001). Other AChE inhibitors are also used to treat gastric motility dysfunctions such as metochlopramide and vinitidine (Sasho et al., 1995).

Some gastrointestinal disturbances are related with inflammation, such as inflammatory bowel diseases. The inflammation in these cases is related with free radicals such as reactive oxygen and nitrogen species (ROS and RNS), and therefore the use of antioxidant compounds, namely radical scavengers may be helpful (Zhu and Li, 2012). Research has demonstrated a direct protective role of dietary antioxidants on intestinal mucosa through local antioxidant and anti-inflammatory activities (Gálvez et al., 2001).

The objective of this work is to contribute to the knowledge of the biochemical mechanism by which commercial herbal infusions of *C. cardunculus*, *F. angustifolia* and *P. tridentatum* may facilitate the digestive action.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade. Thiazolyl tetrazolium bromide (MTT), chlorogenic acid, dicaffeoylquinic acid, acetylcholinesterase (AChE), acetylthiocholine (AChI), pepsin, pancreatin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), rutin, chlorogenic acid, luteolin, apigenin and quercetin were obtained from Sigma (Barcelona, Spain). Dubelco's modified eagle's medium (DMEM), Hank's balanced salt solution (HBSS) with and without phenol red, glutamine, Pen-strep (penicillin and streptomycin mixture), phosphate buffered saline (PBS) and foetal bovine serum (FBS) were bought from Lonza (Verviers, Belgium). High-performance liquid chromatography (HPLC) grade water, methanol and trifluoroacetic acid were obtained from Merck (Darmstadt, Germany).

Plant extract preparation

Bracts of *C. cardunculus* var. *scolymus* L., leaves of *F. angustifolia* Vahl, and flowers of *P. tridentatum* L. were purchased from DIÉTICA® in January, 2011. The plants were cultivated in Portugal and the product is commonly found in Portuguese pharmacies and natural product shops. The plant extracts were prepared as infusions using 10 g of plant material in 100 ml of freshly boiled distilled water. After 10 min of "infusion", the extract was filtered through a grade 1 Whatman paper and lyophilized. The yield of extraction was 77.7 mg of extract/g of plant for *C. cardunculus*, 98.5 mg of extract/g of plant for *F. angustifolia*, and 102.5 mg of extract/g of plant for *P. tridentatum*.

High-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS) analysis

The HPLC analysis was carried out in an Elite LaChrom® VWR Hitachi liquid chromatograph equipped with a Column oven L-2300 and Diode array detector L-2455 (VWR, USA). A column LiChroCART® 250-4 LiChrospher® 100 RP-8 (5 µm) was used. The extracts were analysed by HPLC injecting 25 µl (1 mg/ml) with an auto injector, and using a gradient composed of solution A (0.05% trifluoroacetic acid), and solution B (methanol) as follows: 0 min, 80% A, 20% B; 20 min 20% A, 80% B; 25 min, 20% A, 80% B. For the extract of *F. Angustifolia*, a different gradient was used: 0 min, 95% A, 5% B; 20 min 50% A, 50% B; 23 min, 30% A, 70% B; 25 min, 30% A, 70% B. For both methods, the flow was 1 ml/min and the detection was carried out between 200 and 500 nm with a diode array detector. To estimate concentrations of phenolic compounds, calibration curves were made, with peak areas for chlorogenic acid, cynarin, rutin, quercetin, apigenin and luteolin, ranging from 100 to 1 µM. As some of the extract flavonoid derivatives are not commercially available, the approximate concentrations were estimated based on their aglycons, or in structurally similar compounds, with similar UV spectra. When isolated compounds were needed, the same methods were applied but 25 µl of a 10 mg/ml solution were injected, and the compounds were collected after detection. This process was repeated till the desired amount was obtained and the solvent was sublimated by lyophilisation. The LC-MS and LC-MS/MS analysis were carried out on a liquid chromatograph Surveyor Plus Modular LC system connected to a LCQ Duo ion trap mass spectrometer equipped with an electrospray ionisation (ESI) source, from Thermo Scientific (Bremen, Germany). The column used was a LiChroCART® 250-4

LiChrospher® 100 RP-8 (5 µm) column (Merck, Darmstadt, Germany). The extracts were analysed by injection of 25 µl at a concentration of 10 mg/ml and using a linear gradient composed of solution A (1.0% formic acid), and solution B (methanol) as follows: 0 min, 70% A, 30% B; 20 min 20% A, 80% B; 25 min, 20% A, 80% B. The mass spectrometer was operated in both positive and negative ion modes in the range 120 to 1000 m/z, and the parameters were adjusted in order to optimize the signal-to-noise ratios (S/N) for the ions of interest.

Briefly, the nebulizing and auxiliary gas (nitrogen) flow rates were 40 and 20 (arbitrary units) and the capillary temperature was set to 250°C. Collision induced dissociation (CID) experiments were performed by isolating the ions within the ion trap and accelerating them in order to suffer multiple collisions, with the background gas present in the ion trap (helium) using a data dependent acquisition mode. The ions of interest were activated by applying a percentage of a supplementary a.c. potential in the range of 0.75 to 1.75 Vp-p (peak-to-peak) to the end cap electrodes of the ion trap at the resonance frequency of the selected ion (referred to as the normalized collision energy, NCE). The injection times were 50 ms in a full scan and 200 ms in an MS/MS scan. Xcalibur™ software from thermo scientific was used to acquire and process the data.

Acetylcholinesterase inhibition and antioxidant activity

Acetylcholinesterase enzymatic activity was measured using adaptations of the method described by Ingkaninan et al. (2003). Briefly, 325 µl of 50 mM Tris buffer (or 50 mM HEPES, when analysing the standard dissolved in methanol) pH 8, 100 µl of sample (several quantities of extract, all dissolved in water, in order to calculate IC_{50} values) and 25 µl acetylcholinesterase solution containing 0.26 U/ml were mixed in a spectrophotometer cell and allowed to incubate for 15 min at 25°C. Subsequently, 75 µl of a solution of AChI (0.023 mg/ml) and 475 µl of 3 mM Ellmen's reagent (DTNB) were added. The absorbance at 405 nm was read during the first 5 min of the reaction and the initial velocity was calculated. A control reaction which was considered to have 100% activity was carried out using the same volume of water instead of sample. Percentage inhibition was calculated as:

$$I\ (\%) = 100 - (V_{sample} / V_{control}) \times 100$$

Where I is the percent inhibition of acetylcholinesterase, V_{sample} is the initial velocity of the extract containing reaction and $V_{control}$ is the initial velocity of the control reaction. Tests were carried out in triplicate and a blank with buffer instead of enzyme solution was used. When different volumes of sample were used (maximum 200 µl), the final volume was corrected to 1 ml by adjusting the amount of buffer added in the beginning, and control reactions were carried out in the same conditions. Antioxidant activity was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, as described by Falé et al. (2009). To a 2.5 ml solution of DPPH (0.002% in methanol), 25 µl of plant extract were added. A control cell consisted of DPPH solution and 25 µl of the same solvent used in the extract (water). The mixture was incubated for 30 min at room temperature. The absorbance was measured at 517 nm against a corresponding blank. The antioxidant activity was calculated as:

$$AA\ (\%) = 100 \times (A_{DPPH} - A_{sample}) / A_{DPPH}$$

Where AA is the antioxidant activity, A_{DPPH} is the absorption of the control DPPH solution against the blank, A_{sample} is the absorption of and the extract concentration providing 50% of antioxidant activity (IC_{50}) was obtained by plotting the antioxidant activity against the plant extract concentration.

In vitro gastro-intestinal digestion

In vitro metabolism by the gastric juice

The assay was adapted from Yamamoto et al. (1999). The gastric juice (100 ml) consisted of 320 mg of pepsin, 200 mg NaCl, pH 1.2 (with HCl) juice. Two and a half millilitres of gastric juice were added to 2.5 ml of extract dissolved in water. The mixture was left to incubate at 37°C for 4 h. Samples (100 µl) were taken hourly, added to 900 µl of ice-cold methanol and analysed by HPLC. Assays were done in triplicate.

In vitro metabolism by pancreatic juice

The assay was adapted from Yamamoto et al. (1999). Two and a half millilitres of pancreatic juice were added to 2.5 ml of extract dissolved in water. The mixture was left to incubate at 37°C for 4 h. Samples (100 µl) were taken hourly, added to 900 µl of ice-cold methanol, and centrifuged for 5 min at 5000 *g*. The supernatant was analysed by HPLC. The pancreatic juice consisted of 250 mg of pancreatin in 10 ml of potassium-phosphate buffer 50 mM, pH 8. Assays were done in triplicate. Two hundred microlitre samples were taken at the same time, centrifuged for 5 min at 5000 *g* and the supernatant was analysed for acetylcholinesterase activity against a blank with water instead of plant extract. Assays were done in triplicate and the concentration used corresponded to the IC_{50} value for AChE inhibition.

Cytotoxicity studies in Caco-2 and HeLa cells

Caco-2 (ATCC#HTB37), a human colorectal epithelial adenocarcinoma cell line, and HeLa (ATCC#CCL-2), a human cervical adenocarcinoma cell line, were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine at 37°C in an atmosphere with 5% CO_2. HeLa cells were passaged every 48 to 72 h. The medium of Caco-2 cells was changed every 48 to 72 h, and the cells were passaged before reaching confluence. Cytotoxicity studies were performed using the MTT viability test (Mosmann, 1983). Briefly, 5,000 cells/well were seeded in 96-well plates and incubated for 48 h at 37°C in an atmosphere with 5% CO_2. The medium was replaced by new medium, containing 10% FBS and several concentrations of the plant extracts, and incubated for 24 h in the same conditions before applying the MTT reagent.

Data analysis

The software used was Microsoft® Excel 2010, and the results were expressed as means ± standard deviation. Additional analysis of variance (ANOVA) was performed with $p = 0.05$ and $p = 0.1$.

RESULTS AND DISCUSSION

Composition of the herb infusions

The infusions of *C. cardunculus*, *F. angustifolia* and *P.*

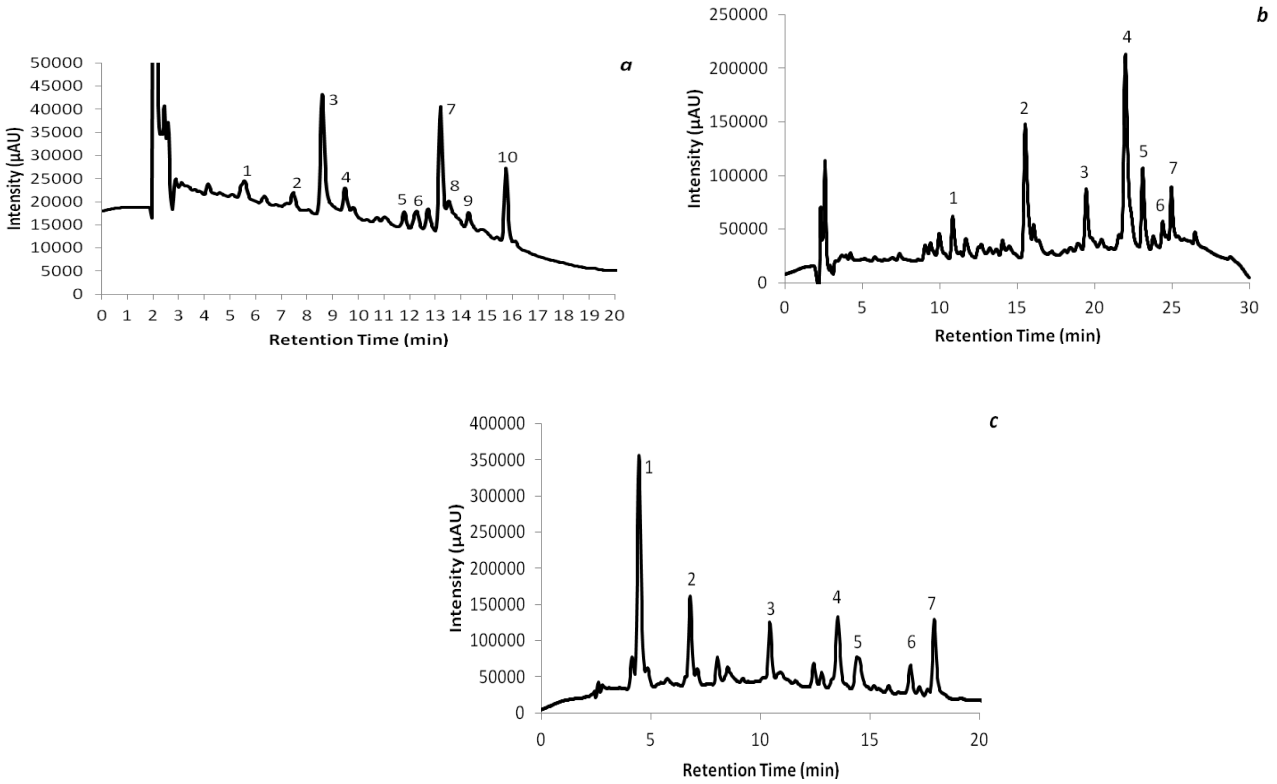

Figure 1. Chromatograms of the infusions of: (a) C. *cardunculus*; (b) F. *angustifolia*; (c) P. *tridentatum*.

tridentatum were analysed by HPLC and LC-MS. The HPLC chromatograms are shown in Figure 1a to c, respectively, and the LC-MS identification of the main peaks can be seen in Table 1 to 3. The HPLC analysis was performed with the same concentration of plant extract, 1 mg/ml, but the scale of the chromatograms in Figure 1 is different.

The extract of *C. cardunculus* was mainly composed of caffeoylquinic and dicaffeoylquinic acids, (Table 1). Peaks 4, 5, 6 and 9 were generically identified as dicaffeoylquinic acid isomers since the fragmentation did not allow an unequivocal identification. Compound 7 was identified as 1,3-dicaffeoylquinic acid (cynarin). This is in agreement with the reports in the literature where cynarin is one of the main compounds of this plant (Wang et al., 2003). Peaks 1, 2 and 3 were identified as caffeoylquinic acid isomers. There are reports in the literature that state that the most abundant caffeoylquinic acid in artichoke is chlorogenic acid (3-*O*-caffeoylquinic acid) (Pandino et al., 2012). Peak 3 was identified as chlorogenic acid. Compound 8 was identified as luteolin-7-*O*-glucoside, according to the fragmentation pattern, also as a known constituent of artichoke. Compound 10 was identified as a malonylglucoside, specifically a luteolin-7-O-(6''-malonylglucoside). There are reports in the literature according to which this compound has been detected in artichoke (Pinelli et al., 2007).

The infusion of *F. angustifolia* also contained caffeoylquinic acid isomers, the compounds 1 and 2, which were identified as 1-caffeoylquinic acid and 3-caffeoylquinic acid (chlorogenic acid) respectively (Table 2). The mass spectrum of compound 3 can be attributed to isorhamnetin-3-O-glucoside-7-O-rhamnoside (Vukics and Guttman, 2010). Compound 4 can be identified as rutin (quercetin 3-*O*-rutinoside), and such attribution is also corroborated by the data available in the literature (Cuyckens et al., 2001). The MS spectrum of compound 5 showed an ion m/z of 539, the deprotonated molecule did not present a typical fragmentation of a flavonoid derivative. Oleuropein was proposed according to the literature (Del Boccio et al., 2003).

Compound 6 presents the typical fragmentation of a flavonoid glycoside. The mass spectrum indicated kaempferol-3-*O*-rutinoside. Compound 7, similar to what was observed for compound 5, did not present the typical fragmentation of a flavonoid glycoside. The fragmentation pattern is consistent with a ligstroside (Silva et al., 2006). The composition of the extract of *F. angustifolia* leaves was very similar to the composition of the extracts of other *Fraxinus* species (Sanz et al., 2012) and *Olea europea*

Table 1. Main components of the infusions of *C. cardunculus*.

HPLC peak	Compound	Ion (*m/z*)	Product ions *m/z* (Rel. Ab. %)
1	Caffeoylquinic acid	353	191 (100), 179 (63), 135 (7)
2	Caffeoylquinic acid	353	191 (13), 179 (100), 135 (5)
3	Chlorogenic acid	353	191 (100), 179 (9)
4	Dicaffeoylquinic acid isomer		353 (100), 335 (36), 179 (19)
5	Dicaffeoylquinic acid isomer	515	353 (100), 335 (20), 317 (59), 299 (60), 255 (19), 203 (59)
6	Dicaffeoylquinic acid isomer		353 (100), 335 (10), 317 (5), 299 (9), 203 (8), 179 (6)
7	Cynarin	515	447 (1), 353 (100), 335 (5), 191 (5)
8	Luteolin-7-*O*-glucoside	447	327 (4), 285 (100) [MS^3 285: 267 (1), 257 (1), 243 (4), 241 (6), 217 (3), 199 (3), 175 (2), 151 (1)]
9	Dicaffeoylquinic acid isomer	515	353 (100), 317 (4), 299 (11), 255 (2), 203 (5), 179 (3) [MS^3 353: 191 (12), 179 (100), 173 (43)]
10	Luteolin-7-*O*-(6'' malonylglucoside)	533	489 (100) [MS^3 489: 327 (4), 285 (100)]

Ab Rel (%): relative abundance (%).

Table 2. Main components of the infusions of *F.angustifolia*.

HPLC peak	Compound	Ion (*m/z*)	Product ions *m/z* (Rel. Ab. %)
1	Caffeoylquinic acid isomer	353	191 (100), 179 (88), 135 (6)
2	Chlorogenic acid	353	191 (100), 179 (7)
3	Isorhamnetin-3-*O*-glucoside-7-*O*-rhamnoside	623	507 (2), 461 (100), 315 (1) [MS^3 461: 315 (100), 297 (8), 195 (2), 179 (3), 161 (46), 153 (4), 135 (66)]
4	Rutin	609	343 (6), 301 (100), 271 (4), 255 (3), 179 (2) [MS3 301: 271 (77), 255 (43), 229 (4), 193 (4), 179 (100), 151 (38)]
5	Oleuropein	539	507 (2), 403 (2), 377 (100), 345 (6), 307 (34), 275 (15) [MS^3 377: 345 (22), 307 (100), 275 (41), 149 (3)]
6	Kaempferol-3-*O*-rutinoside	593	327 (2), 285 (100), 257 (3), 229 (1), 213 (1) [MS^3 285: 285 (63), 267 (38), 257 (100), 255 (27) 241 (27), 239 (11), 229 (35), 213 (15), 199 (9), 197 (8), 163 (8), 151 (3)]
7	Ligstroside	569[a]	523 (83), 385 (100), 223 (18), 205 (1)

[a]The ion corresponds to $[M+HCOO]^-$.

(olive tree) leaf extracts (Fu et al., 2010) which also belongs to the family Oleaceae and Tribe Oleeae.

The infusion of flowers of *P. tridentatum* was composed mainly of C-glycosilated flavonoids such as 1, 2 and 3 (Table 3). It also contained the O-glicosilated flavonoids 4, 5 and 6, and the flavonoid aglycon 7. Compound 1 can be identified as taxifolin-6-*C*-glucoside based on the MS^2 and MS^3 data (Vukics and Guttman, 2010). The MS^2 of the deprotonated molecule of compound 2 indicates that it is myricetin-6-*C*-glucoside (Paulo et al., 2008). Taking into account the MS^2 and MS^3 data, compound 3 can be identified as genistein-8-*C*-glucoside. This compound has been identified in a plant of the same family (*Fabaceae*), *Genista tenera* being one of its major constituents (Rauter et al., 2009). Compound 4

Table 3. Main components of the infusions of *P. tridentatum*.

HPLC Peak	Compound	Ion (*m/z*)	Product ions *m/z* (Rel. Ab. %)
1	Taxifolin-6-*C*-glucoside	465	447 (3), 375 (11), 345 (100) [MS3 345: 327 (100), 317 (15), 301 (6), 283 (2), 221 (6), 177 (2), 167 (13), 151 (1)
2	Myricetin 6-*C*-glucoside	479	389 (3), 359 (100), 331 (1) [MS3 359: 341 (45), 331 (100), 315 (10), 313 (12), 300 (20), 298 (6), 273 (4), 221 (69), 207 (9), 194 (23), 167 (22)]
3	Genistein-8-*C*-glucoside	431	341 (2), 311 (100), 283 (1) [MS3 311: 311 (8), 283 (100)]
4	Isoquercitrin	463	343 (2), 301 (100), 179 (1) [MS3 301: 271 (68), 257 (12), 255 (31), 239 (3), 229 (2), 193 (2), 179 (100), 151 (39)]]
5	Apigenin 5,7-dimethyl ether 4'-galactoside/unidentified	505	297 (100)
		519	-
6	Biochanin A glucoside	491[a]	445 (21), 283 (100) [MS3 283: 283 (19), 268 (100)]
7	Biochanin A	283	283 (30), 268 (100) [MS3 268: 268 (40), 267 (100), 240 (92), 226 (13), 224 (64), 212 (7), 196 (2)]
		285	270 (1), 253 (1), 229 (1), 170 (0.1), 153 (0.2), 149 (1), 123 (1)

[a]The ion corresponds to $[M+HCOO]^-$.

can be identified as isoquercitrin. This conclusion is supported by literature data since this compound has already been identified in *P. tridentatum* extracts (Paulo et al., 2008).

Regarding the peak marked as 5 in *P. tridentatum*, the HPLC-DAD chromatogram (Figure 1) clearly shows that this chromatographic peak is rather broad, thus allowing us to assume that there is co-elution of two or more compounds. The LC-MS data confirms this assumption since two distinct *m/z* values were obtained for this peak (*m/z* 519 and 505). The MS^2 data of the ion at *m/z* 505 (see Table 3) allowed us to identify this compound as apigenin 5,7-dimethyl ether 4'-galactoside. The fragmentation pattern of compound 6 showed close resemblance to biochanin A glucoside (Silva et al., 2006), a glycosylated isoflavone that has already been identified in *P. tridentatum* extracts (Paulo et al., 2008). Compound 7 was analysed in both negative and positive ion modes. The positive ion MS data enabled us to identify this compound. Indeed, the fragmentation of the protonated compound 7 is consistent with the fragmentation of biochanin A, available in the literature (Madeira et al., 2010). Furthermore, the MS^2 spectrum of the deprotonated molecule of compound 7 is similar to published data (Kang et al., 2007), thus confirming the conclusions drawn. The compounds found in the infusion of *P. tridentatum* were previously found in extracts from the same species (Paulo et al., 2008) and in extracts of *Cytisus multiflorus* (Pereira et al., 2012), which also belongs to the Family Fabaceae and Tribe Genisteae.

Activities of *C. cardunculus, F. angustifolia* and *P. tridentatum* infusions

The extracts of the three plants inhibited acetylcholinesterase (AChE) activity and showed also antioxidant activity (Table 4). The extracts of *F. angustifolia* and *P. tridentatum* showed IC_{50} values for AChE inhibition of around 1 mg/ml, while *C. cardunculus* was a less potent inhibitor with an IC_{50} value of 2.5 mg/ml (Table 4). The extracts of *F. angustifolia* and *P. tridentatum* were also better antioxidants, with IC_{50} values around 20 µg/ml, than *C. cardunculus*, which presented an IC_{50} value of 120 µg/ml (Table 4). The IC_{50} values for the inhibition of AChE by the plant infusions are within the values found in the literature for other active plant aqueous extracts (Falé et al., 2012a, b). The lowest activity presented by the extract of the *C. cardunculus* extract may be due to the lower amount of phenolic compounds extracted by gram of plant.

Analysing the HPLC chromatogram and calculating the amount of caffeoylquinic acid isomers (1, 2, 3), dicaffeoylquinic acid isomers (4,

Table 4. IC_{50} values for acetylcholinesterase (AChE) inhibition and DPPH scavenging activity of the infusions of *C. cardunculus*, *F. angustifolia* and *P. tridentatum*, and remaining activities after 4h digestion with gastric and pancreatic juices.

Species	IC_{50} (µg/ml)		Remaining activity after digestion (%)			
			Gastric		Pancreatic	
	AChE	DPPH	AChE	DPPH	AChE	DPPH
C. cardunculus	2505±253	123.1±5.7	105.7±11.9	96.7±4.5	107.6±16.1	108.9±4.7
F. angustifolia	1066±19	22.2±0.6	99.6±2.9	113.0±18.0	107.8±13.9	103.9±9.6
P. tridentatum	1090±4	18.6±0.7	105.8±5.5	107.5±2.5	103.5±17.3	105.1±5.0

Table 5. Toxicity of the three infusions in Caco-2 and HeLa cell lines. Results expressed in percentage (%) of mortality with 2 mg/mL. Values statistically different from the control ($P<0.05$) are marked with "*".

Species	Caco-2	HeLa
C. cardunculus	30.5±8.1*	58.6±4.5*
F. angustifolia	1.3±0.5	2.8±2.1
P. tridentatum	7.2±5.3	14.7±2.4*

5, 6, 7, 9), and luteolin derivatives (8, 10), an amount of phenolic compounds of 88 µmol/g was estimated. For *F. angustifolia* just the amount of the main components (1, 2, 4) amounts 254 µmol/g, and for *P. tridentatum,* the total amount a flavonoids was estimated as 180 µmol/g.

The EC_{50} values for the antioxidant activity of *F. angustifolia* and *P. tridentatum* were in the same magnitude of those found for other plant extracts (Falé et al., 2009; Hernandez et al., 2010), and also for the commercial antioxidant BHT, which showed an EC_{50} value of 12 µg/ml (Mata et al., 2007). The components of the infusions in this study were shown as being active in the inhibition of AChE activity, such as the main compounds of *C. cardunculus* extract, chlorogenic acid (3), with an IC_{50} value of 553.2 ± 22.6 µM (Hernandez et al., 2010) and cynarin (7) with IC_{50} value of 149.9 ± 3.2 µM. The concentration of the compounds was estimated by HPLC, and in the IC_{50}, the extract contained 147.4 µM of chlorogenic acid (3) and other caffeoylquinic acids isomers (1, 2), which explains 27% of the activity. It also contains 61.5 µM of cynarin (7) and other dicaffeoylquinic acid isomers (4, 5, 6, 9), which explains 41% of its activity. The amount of luteolin derivatives was estimated as 12.7 µM. Although the IC_{50} value for luteolin derivatives is not known, the IC_{50} value for luteolin is 92.1 µM (Falé et al., 2012b), and based on this value, luteolin derivatives may explain approximately 14% of the inhibition of the extract.

The main compound of *F. angustifolia* infusion, rutin (4), showed an IC_{50} value of 149.0 ± 6.6 µM, determined previously by our group (Hernandez et al., 2010). The other main component of this infusion is chlorogenic acid (2). The amounts of these compounds found in the infusion at the IC_{50} were 73.5 and 48.0 µM of rutin and chlorogenic acid, respectively. These values can explain 28% of the activity detected. Other reports have also shown that verbascoside, a hydroxytyrosol derivative similar to oleuropein (5) and ligstroside (7) also inhibits AChE activity approximately 50% with 320 µM (Georgiev et al., 2011).

The infusion of *P. tridentatum* was composed of glycosylated flavonoids. Its components were isolated by HPLC and tested for acetylcholinesterase activity in the same concentration as found in the plant extract at the IC_{50}. The inhibitions obtained for the compounds isolated by HPLC were very low, while the mixture (extract) at the same concentration inhibited 52.4 ± 2.5%. Estimating the approximate concentrations of the flavonoid glycosides by HPLC analysis, at the IC_{50} value, and summing all the concentrations, a value of 196 µM was found. This value is just slightly higher than the IC_{50} values found for several commercially available standard flavonoid glycosides, 141, 142, 136 and 138 µM for rutin, hyperoside, isoquercitrin and quercitrin, respectively (Hernandez et al., 2010). This suggests that all the flavonoids may contribute to the inhibition caused by the extract as a whole, which is also supported by a previous report that showed that different flavonoids may bind to the active site of AChE by interacting with the same binding sites (Falé et al., 2012b).

In vitro digestion of *C. cardunculus, F. angustifolia* and *P. tridentatum* infusions

The herbal infusions were subject to the action of digestive juices during 4 h. The biochemical activities were determined and the identification of the chemical constituents was verified by HPLC-DAD. It could be seen that these activities did not change after artificial gastric and pancreatic digestions (Table 2). This result was confirmed by the HPLC analysis which indicated no differences

from the chromatograms shown in Figure 1. The flavonoids present in these infusions and their glycosylated derivatives, were stable under gastric and pancreatic conditions. This observation is in agreement with other reports for this class of compounds (Bouayed et al., 2012).

The flavonoid C-glycosides were stable during the *in vitro* digestion, as expected for their stronger bond between sugar and aglycon. As far as we know, this is the first report concerning the digestion of the flavonoid C-glycosides. The stability of esters in pancreatic conditions is usually highly variable in the literature. Chlorogenic acid, for instance, is stable at low pH as agreed by most reports (Gumienna et al., 2011; Siracusa et al., 2012), but in pancreatic conditions its stability may range from 41 to 71% (Bouayed et al., 2012) or 31 to 91% (Gumienna et al., 2011). In the present study, the activities were kept constant during the whole test.

Toxicity of the infusions of *C. cardunculus, F. angustifolia* and *P. tridentatum*

The toxicity of the infusions was tested in the human cell lines Caco-2 and HeLa, using several concentrations of plant extract in order to calculate the cell viability. The value established as the maximum concentration was 2 mg/ml and it is 20 times higher than the limit of IC_{50} values considered usually as toxic (Oonsivilai et al., 2008). All the plant extracts decreased the cell viability in less than 50% with 2 mg/ml (Table 5), except for *C. cardunculus* that showed an IC_{50} value of 1.9 ± 0.3 mg/ml for HeLa cells, which was approximately the maximum concentration tested (2 mg/ml). Toxicity studies in Caco-2 cells with plant aqueous extracts have suggested that IC_{50} values of 0.147 mg/ml may be considered as toxic (Oonsivilai et al., 2008), but usually values higher than 0.1 mg/ml are considered non-toxic to human cell lines (Okonogi et al., 2007). The IC_{50} toxicity values shown by the plant infusions in the present study were always much higher than 0.1 mg/ml. Only *C. cardunculus* showed an IC_{50} slightly inferior to 2 mg/ml with HeLa cells, although a non-toxic value.

Conclusions

The commercially available infusions of *C. cardunculus*, *F. angustifolia* and *P. tridentatum* have shown mild to high activities as acetylcholinesterase inhibitors and antioxidants. These activities are due to the extracts being composed mainly by active compounds, such as phenolic acids (chlorogenic acid and cynarin) and glycosylated flavonoid derivatives. The chemical composition of the infusions, and their activities, are maintained throughout the digestive process. Even though the extracts were able to modulate biochemical activities, they did not compromise the viability of human cell lines, showing no toxicity.

Therefore the infusions of *C. cardunculus*, *F. angustifolia* and *P. tridentatum* may be effective in the treatment of digestive problems due to their content in acetylcholinesterase inhibitors and antioxidant compounds, and did not show any toxicity under the concentrations tested.

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