Acetylcholinesterase Inhibitiory Activity after *in vitro* gastrointestinal digestion of infusions of *Mentha* species

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ABSTRACT

Aims: to study the acetylcholinesterase inhibitory activity of *Mentha* infusions before and after the gastrointestinal digestion and to correlate this activity with the chemical compounds present in these infusions.

Place and Duration of Study: Fresh *Mentha x piperita*, *M. spicata*, *M. pulegium* were bought in a local supermarket. These plants were composed of leaves, stems and flowers for the identification, which was carried out in Plant Biotechnology Centre, Faculty of Sciences, University of Lisbon. The chemical identification of the infusions and the enzymatic tests were carried out in the Center of Chemistry & Biochemistry, Faculty of Science University of Lisbon from September 2010 till June 2011.

Methodology: The compounds present in the infusions were identified by LC-MS. The enzyme activity was carried out using a spectrophotometric method. The digestive simulation was accomplished using enzymatic juices prepared in the laboratory and Caco-2 cells lines simulating the intestine barrier.

Results: All the *Mentha* infusions contained rosmarinic acid. *M. spicata* infusion contained also eriocitrin and eriodictyol. The IC_{50} values for acetylcholinesterase inhibitory activity of the infusions, before digestion, stayed between 0.72 and 1.9 mg/mL. These activities are statistically different at $p<.05$. These activities can be explained by the presence of the phenolic compounds mentioned. Rosmarinic acid has an IC_{50} equal to 0.439 mg/mL (1.22 mM), eriocitrin and eriodictyol have IC_{50} equal to 0.439 mg/mL (0.29 mM) and 0.256 mg/mL (0.89 mM) respectively. The presence of these two flavonoids, eriocitrin and eriodictyol, can account for the higher activity detected for *M. spicata*. The gastric juice or the pancreatic juices used to simulate the gastrointestinal digestion did not originate any difference in the chemical composition of the infusions (analysed by HPLC-DAD). This was also corroborated by the enzymatic tests. The Caco-2 cells did not originate any modification in the enzymatic activity of the infusions. The analysis of the cell homogenate revealed the presence of rosmarinic acid and the phenolic compounds, although in minor amount.

Conclusion: *Mentha* infusions have the capacity to inhibit acetylcholinesterase, due to the presence of rosmarinic acid, eriocitrin and eriodictyol The composition of the *Mentha* herbal teas was not modified by the gastro-intestinal juices, or by the intestinal cell line.

Keywords: *Mentha* aqueous-extracts, rosmarinic acid, eriocitrin, eriodictyol, anti-acetylcholinesterase, *in vitro* digestion

1. INTRODUCTION

Plants for medicinal purposes have been used for countless years. Plants extracts still have a pivotal part on today's therapy, and are an almost exclusive source of drugs for the majority of the world population.

Plants from *Mentha* species (Lamiaceae) are well disseminated and broadly used in gastronomy, with their infusions being drunk worldwide for its pleasant taste, alone or mixed with other herbs to potentiate their flavor. In the Mediterranean cuisine *Mentha* species are also used to spice soups and stew dishes, in which the herbs are added at the end of the process. The way of preparing the tea or the dishes is similar to infusions. These herbal teas are likewise used for their traditional medicinal properties, mainly for gastrointestinal problems [1]. One of the ways that the infusions can help the digestion is through the increase in the gastrointestinal motility. Alterations in the motility of the digestive tract are associated with many symptoms of gastrointestinal diseases, such as dyspepsia, gastric stasis, vomiting, abdominal pain, paralytic ileus, and constipation [2]. In the stomach there is a neural release of acetylcholine in the region of histamine-secreting cells. The inhibition of AChE activity allows acetylcholine to diffuse to the location of the oxyntic cells and thus to produce an acid secretory response [3]. This enzyme is also present in the intestine epithelial cells where acetylcholine is the primary transmitter of excitatory motor neurons [4]. Alterations in the cholinergic metabolism may have particular importance since the action of this major excitatory neurotransmitter on enteric neurons, smooth muscle, and mucosa plays a major role in normal gut function [5].

There, the extracts may be subject to the effect of gastrointestinal juices or act upon the cells localized on the gastrointestinal surface, eventually helping the digestive process.

Several studies have been developed using the essential oil extracts of distinct *Mentha* species [1,6] but there has not been much focus on the aqueous extracts. *Mentha spicata* [7,8] and *Mentha pulegium* water extracts were studied before [6,7]. These studies focused on antioxidant and inhibition of acetylcholinesterase activity and antimicrobial properties.

The objective of the present work was to investigate whether infusions of *Mentha x piperita* (peppermint), *Mentha spicata* (spearmint) and *Mentha pulegium* (pennyroyal) possess acetylcholinesterase activity even after the gastrointestinal digestion. This is one of the few studies using aqueous extracts *of Mentha* species, which are the most common form of consumption. To the best of our knowledge this is the first report discussing the *in vitro* digestion of aqueous extracts of *Mentha* species and the biological activities after gastrointestinal digestion.

2. MATERIAL AND METHODS

2.1.Chemicals.

All chemicals were of analytical grade. Acetylcholinesterase (AChE) type VI-S, from electric eel 349 U/mg solid, 411 U/mg protein, 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), pancreatin, pepsin, acetylthiocholine iodide (AChI), tris[hydroxymethyl]aminomethane (Tris buffer), trifluoracetic acid, methanol (HPLC grade), rosmarinic acid, eriocitrin, eriodictyol, were obtained from Sigma. The culture media, DMEM (Dubbleco's Modified Eagle Medium), HBSS (Hank's Buffered Salt Solution) and FBS, heat inactivated (Fetal Bovin Serum) were obtained from Lonza, VWR International.

2.2 Plant Material.

Specimens of different *Mentha* species (*Mentha x piperita*, *M. Spicata*, *M. pulegium*) were freshly bought in a local supermarket. These plants were composed of leaves, stems and flowers for the identification, which was carried out by Prof. Dr. Lia Ascensão, Plant Biotechnology Center, Institute of Biotechnology and Bioengineering. Faculty of Science, University of Lisbon. The study was done using only the leaves from the plants.

2.3 Extract Preparation.

Aqueous plant extracts were prepared as infusions, by boiling 10 g of ground fresh plant material for 15 minutes in 1000 mL of distilled water. The infusions were then filtered through Whatman paper. The infusions were subsequently freeze-dried in *Heto Powerdry LL3000*. All the extracts were prepared in triplicates. These freeze-dried extracts were used in all subsequent experiments.

2.4 Composition analysis by HPLC-DAD and MS Analysis.

The HPLC analysis was carried out in a Liquid Chromatograph Finnigan™ Surveyor® Plus Modular LC System equipped with a Purospher® STAR RP-18 column endcapped, 5 µm, 125-4, from Merck, and Xcalibur software. The freeze-dried extracts, 1mg/mL, were analyzed by HPLC, injecting 25 µL and using a gradient composed of solution A (0.5% trifluoracetic acid) and eluent B (methanol) as follows: 0 min, 75% A and 25% B; 20 min, 20% A and 80% B ; 25 min, 75% A and 25% B. The standards were run under the same conditions using 0.1 mg/mL solutions in methanol and the detection was carried out between 200 and 600 nm with a diode array detector. The sample peaks were collected separately and this process was repeated several times when the compound identification was to be carried out by mass spectrometry.

Selected major compounds were collected from the HPLC and analysed using a LCQ Duo ion trap mass spectrometer from Thermo Scientific (San Jose, CA, USA) equipped with electrospray ionization (ESI). Samples were introduced, via a syringe pump at a flow rate of 5

µL min^{-1}, into the stainless steel capillary of the ESI source. The applied spray voltage in the source was 4.5 kV, the capillary voltage was 10 V and the capillary temperature was 220 ºC. All the mass spectrometer parameters were adjusted in order to optimize the signal-to-noise ratios for the ions of interest. Nitrogen was used as nebulising and auxiliary gas in the source. All mass spectrometry data were acquired in the negative ion mode, the full scan spectra were recorded in the range m/z 100–1000 and three micro-scans were averaged. Collision Induced Dissociation (CID) and tandem mass spectrometry (MS^2) experiments were performed with helium as collision gas and by gradually increasing the Normalized Collision Energy (NCE) to promote fragmentation of the isolated ions.

Rosmarinic acid was quantified by HPLC-DAD using as a standard purchased from Sigma. The quantification was carried out using a calibration line based on the area of the several peaks of rosmarinic acid with different concentrations.

2.5 *In Vitro* Metabolism by The Gastric and Pancreatic Juices.

Both assays were adapted from the literature [9]. 2.5 mL of gastric or pancreatic juice were added to 2.5 mL of extract solution (10 mg/mL). The mixture was left to incubate at 37 ºC for 4 h. Samples (100 µL) were taken hourly, added to 900 µL of ice-cold methanol and analyzed by HPLC-DAD. The mixture was centrifuged for 5 minutes at 5000 x *g*, in the case of the pancreatic juice, prior to the HPLC analysis. 700 µL samples of the mixture were also taken, centrifuged 5 min at 5000 x *g* and the supernatant was analysed to determine acetylcholinesterase inhibition, using water instead of extract as a control. The gastric juice consisted of 320 mg of pepsin in a 100 mL solution containing 200 mg NaCl, pH 1.2 (with HCl). 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.

2.6 Metabolism by the Caco-2 Cells.

The assay for the metabolism of plant extracts by Caco-2 cells was done as described in the literature [10]. Caco-2 cells (ATCC#HTB-37), a colorectal adenocarcinoma epithelial cell line, were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 2 mM L-glutamine, at 37 ºC in a atmosphere with 5% CO_2. The culture medium was changed every 48–72 h. For the assays, 2 x 10^4 cells were seeded in 4 cm diameter Petri dishes and grown for 10 days (to approximately 90% confluence). The medium was replaced with 2.5 mL HBSS plus 1 mg/mL of plant extract. The cells were left in the incubator and 100 µL samples of the medium were collected at 0, 1, 2, 4, and 6 h, added to 900 µL of ice-cold water, centrifuged 10 min at 5000 x g, and analyzed by HPLC. Samples (700 µL) were also collected at the same incubation times, centrifuged and analyzed for acetylcholinesterase inhibition, against a blank of HBSS incubated with Caco-2 cells under the same conditions. All assays were done in triplicate. After the 6-h assay, the cells in each Petri dish were washed with 500 µL HBSS and incubated with 1 mL solution of 1:10 TFA (0.05% in methanol) for 30 min. The extract was centrifuged for 10 min at 5000 x *g*, and the supernatant was analyzed by HPLC.

2.7 Antiacetylcholinesterase Activities of the Digested Extracts.

Aliquots from the digestive experiments (section 2.5) and also from culture medium of Caco-2 cells (section 2.6) were withdrawn at the beginning of the experiment and after each hour, as described in literature [10]. The sample was centrifuged at 5000 x *g* for 5 minutes, in the case of the digestive tests, to discard the pellet containing the enzyme, pepsin or pancreatin. The upper phase was used for the determination of acetylcholinesterase inhibition activity using an adaptation of the Ellman method described in Ingkaninan et al (2003) [11]. The culture medium was used directly to the activity test. 90 µL of 50 mM Tris–HCl buffer pH 8, 30 µL sample and 7.5 µL acetylcholinesterase solution containing 0.26 U/mL were mixed in a microplate and left to incubate for 15 min. Subsequently, 22.5 µL of a solution of AChI (0.023 mg/mL) and 142 µL of 3 mM DTNB were added. The absorbance at 405 nm was read when the reaction reached equilibrium. The enzyme activity was measured in the presence (A_{sample}) and in the absence ($A_{control}$) of the extract. All the tests were carried out in triplicate, and the enzyme inhibition was calculated as:

155 inhibition (I)% =100-($A_{sample}/A_{control}$) x 100

156

157 ## 3. RESULTS AND DISCUSSION

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159 In this work we used infusion concentrations similar to those used by the population in general.
160 After lyophilisation, the amount of extract obtained for each *Mentha* was: 298±20 mg/g for
161 *Mentha* x *piperita* (*M. piperita*), 325±18 mg/g for the *Mentha spicata* (*M. spicata*) and 340±15
162 mg/g for *Mentha pulegium* (*M. pulegium*).

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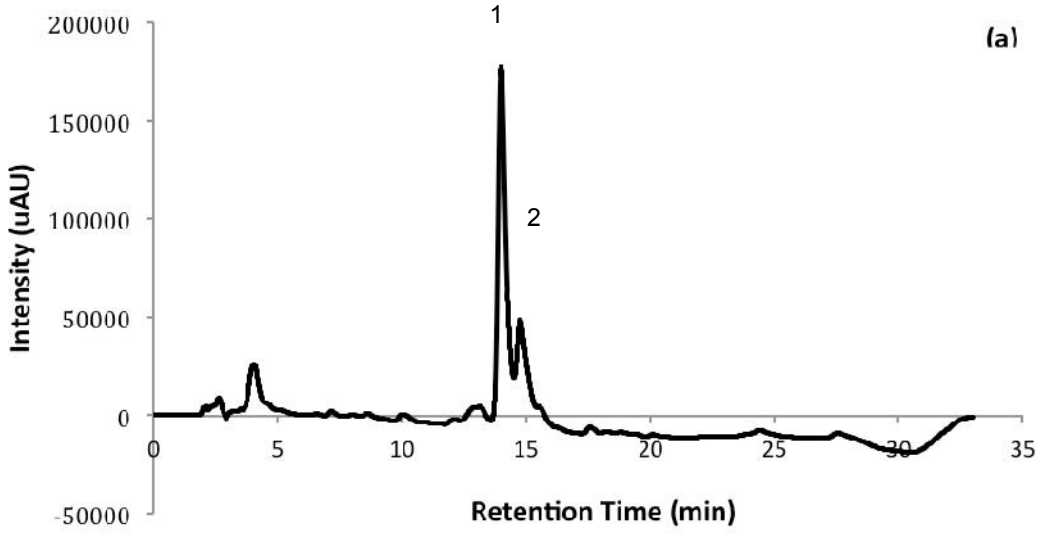
164 ### 3.1. Composition Analysis by HPLC-RP-DAD and MS.

165 The different extracts were analysed by HPLC-RP-DAD and the chromatograms are displayed
166 in **Figure 1a**-**c**, for *Mentha x piperita*, *M.pulegium* and *M. spicata,* respectively. For every extract
167 studied, there is the same major component (Peak 1), which encompasses over 60% of the
168 total area. It was identified as rosmarinic acid (RA), both by comparison with a standard in
169 HPLC-DAD and MS^2 experiments (**Table 1**). This comparison was carried out through retention
170 time, UV-Vis spectra and then by mass analysis. It should be noted that the MS^2 spectrum
171 obtained for the fraction identified as rosmarinic acid is in agreement with the one available on
172 Massbank (record number: PR100686) [12].



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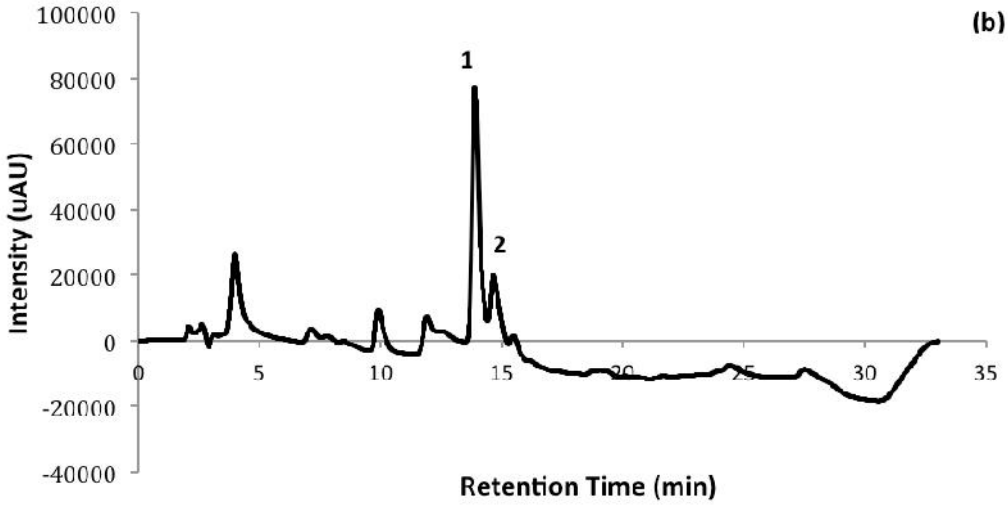

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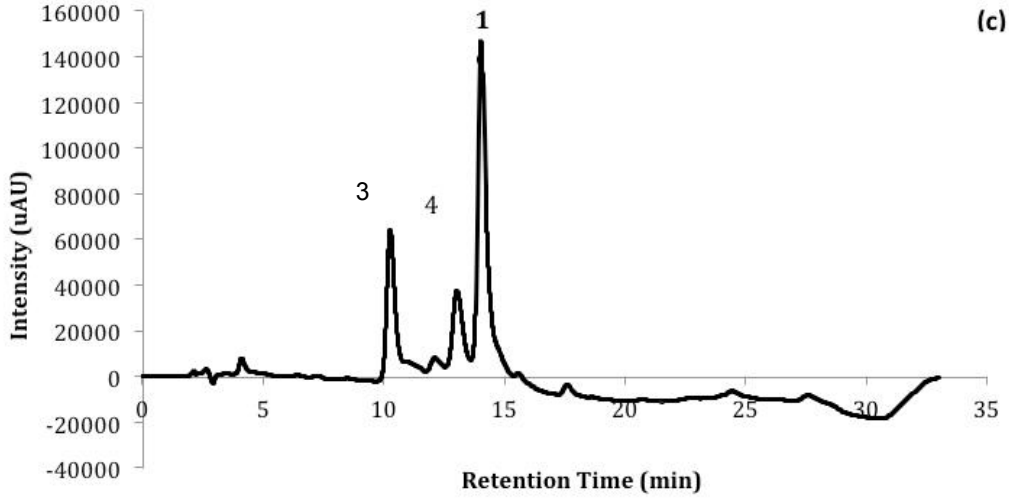

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Figure 1 – RP-HPLC separation of (a) *Mentha x piperita*; (b) *Mentha pulegium*; (c) *Mentha spicata*. Peak identification: (1) Rosmarinic Acid; (2) Lithospermic acid; (3) Eriocitrin; (4) Eriodictyol.

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179 Using a calibration curve, the concentration of rosmarinic acid (RA) in the lyophilized extracts
180 was assessed. *M. piperita* extracts possess the highest concentration, with 81.97 µM of RA
181 (29.5 µg of RA/mg of extract), followed by *M. spicata* with 73.34 µM, (26.4 µg of RA/mg of
182 extract). *M. pulegium* showed the lowest RA concentration, 11.24 µM (4.0 µg of RA/mg of
183 extract).

184 The differences between the extracts composition were centred between 10 and 15 minutes of
185 the run, revealing some compounds that are exclusive for each extract. *M. piperita* and *M.*

pulegium possess a compound (Peak 2) that was not found in *M. spicata*. The infusion of the fraction corresponding to Peak 2 afforded a deprotonated molecule $[M-H]^-$ at *m/z* 537, **Figure 2**, and the fragmentation of its ion is consistent with the structure of lithospermic acid (**Table 1**), a derivative of rosmarinic acid [13]. Lithospermic acid was also detected in *Mentha x piperita* [14] and other Lamiacea family plants [15].

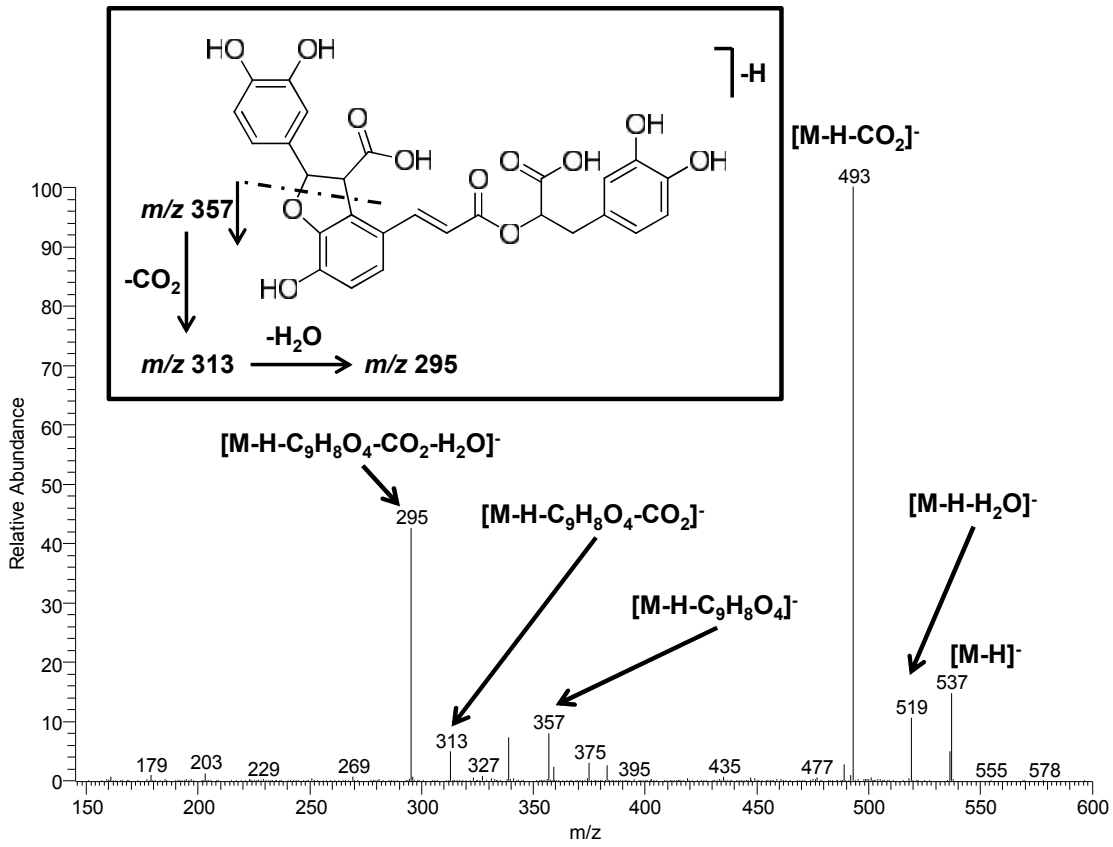


Figure 2: MS^2 spectrum of the compound labelled as Peak 2 (identified as lithospermic acid). The inset shows the fragmentation pathway that explains some of the fragment ions detected.

The fraction corresponding to Peak 3 afforded a deprotonated molecule at *m/z* 595 and the fragmentation pattern (**Table 1**) is consistent with eriocitrin [16]. The fraction corresponding to Peak 4 afforded a deprotonated molecule at *m/z* 287 for which the fragmentation pattern (**Table 1**) is consistent with eriodictyol. For the latter it should be mentioned that the identification was made by comparison with the eriodictyol mass spectrum available at Massbank (record number: PR100639) [12]. There are some ion abundance differences between our spectrum and the one available at Massbank, which can be due to: 1) different mass analysers (we used an ion trap while the data available at Massbank was acquired on a Q-TOF); 2) the collision energy used to acquire the Massbank data was higher than our collision energy. Despite these differences, the comparison of our spectrum with that of eriodictyol at Massbank gave a similarity score of 0.717. Eriocitrin as well as rosmarinic acid have also been identified in *Mentha* extracts [17].

Table 1: MS^2 data for the compounds identified in the HPLC chromatograms (the relative abundances of the precursor and fragment ions are presented between brackets).

Chromatogram Peak	Compound Name	*m/z*	
		Precursor ion (Rel. Ab. %)	Fragment ions (Rel. Ab. %)
1	Rosmarinic acid	359 (44%)	223 (22%); 197 (23%), 179 (30%);

			161 (100%); 133 (2%)
2	Lithospermic Acid	537 (15%)	519 (11%); 493 (100%); 357 (8%); 295 (43%)
3	Eriocitrin/Neoeriocitrin	595 (100%)	287 (19%)
4	Eriodictyol	287 (100%)	151 (66%); 135 (1%)

Besides rosmarinic acid, a *M. spicata* extract solution (1 mg/mL) also contains eriocitrin and eriodictyol in concentrations of 298 µM and 233 µM, respectively.

3.2 Metabolization of the Extracts by *in vitro* Enzymatic Digestion.

3.2.1 Acetylcholinesterase-inhibitory activities of Mentha Extracts and their Components

The biological activity chosen was the inhibition of acetylcholinesterase (AChE) activity involved in the gastrointestinal motility and previously found to be the target of the *Mentha* aqueous extract components [7]. *M.spicata* and *M. pulegium* showed previously IC_{50} values of 0.721±0.001, 1.581±0.053 mg/mL [7] and *Mentha x piperita* had an IC_{50} value of 1.93±0.11 mg/mL. *M. pulegium* and *Mentha x piperita* showed similar activities, due to the presence of the rosmarinic acid, mainly. Rosmarinic acid is able to inhibit AChE, with an IC_{50} value of 0.439±0.025 mg/mL (1.22±0.07 mM) [18]. *M. spicata* showed the highest activity as AChE inhibitor due to the presence of the flavonoid derivatives, besides rosmarinic acid. Eriocitrin and eriodictyol have IC_{50} values of 0.439±0.089 mg/mL (0.289±0.059 mM) and 0.256±0.006 mg/mL (0.888±0.021 mM), respectively, and, therefore, are stronger AChE inhibitors than rosmarinic acid itself.

The main components of *M. spicata*, rosmarinic acid, eriocitrin and eriodictyol, were mixed in the concentrations found in the plant extract in the IC_{60} value for acetylcholinesterase inhibition. The inhibition of AChE of the mixture was 64.4±3.2 %. The inhibition by each of the components was determined individually, at the same concentration, and was 51.5±0.6 % for eriocitrin, 31.6±0.2 % for eriodictyol, and 24.3±1.9 % for rosmarinic acid. The sum of the individual inhibitions is higher than the inhibition of the full extract, probably because all the compounds inhibit AChE by binding to the same binding sites in active gorge of the enzyme [19]. The inhibition by the *M. spicata* extract seems to be due predominantly to the eriocitrin content.

There are many studies of extracts' activities *in vitro*, but very few tackle the problem of gastrointestinal digestion. It is well known that after the intake, the extracts suffer modifications in the digestive tract, making it necessary to evaluate the final biological activity [20]. With this in mind, studies of *in vitro* enzymatic digestion, mimicking the stomach and small intestine environments were carried out. All the extract digestive *in vitro* studies were done with concentrations similar to the IC_{50} values found in a previous study [7].

3.2.2 Enzymatic Digestion of *Mentha* Extracts by Gastric Juice.

The low pH environment and presence of the proteolytic enzyme pepsin causes a degradation of certain polyphenol polymers in their constituent monomers [20]. The results obtained in the present work indicated that the main compounds present in the *Mentha* extracts were not modified; this confirms the fact that the compounds detected are monomers. Usually, the acidic pH of the gastric juice hydrolysis oligomers to its monomers [20]. Therefore, a low pH did not cause any degradation, as confirmed by HPLC-DAD, **Figure 3a**. These results suggest that due to the lack of variation in chemical structure, the digested extract should keep approximately the same antiacetylcholinesterase inhibition activity (**Table 2**). In fact, it was noticed a slight decrease in the acetylcholinesterase inhibitory activity of the digested extracts when compared to the control, nevertheless these differences were not statistically significant at 95% confidence level (**Table 2**). These results are in accordance with those previously obtained for the digestion of *Plectranthus barbatus* water extract, also containing rosmarinic acid as the main compound [10], in which no decrease in the activity by the gastric juice was observed.

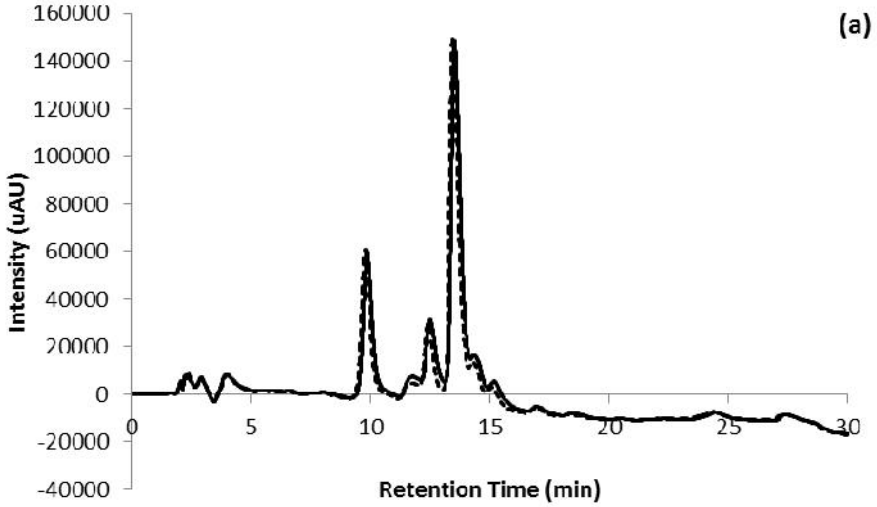
Table 2: Inhibition of AChE activity of the water extracts of different *Mentha* species after 4h *in vitro* digestion. Values (%) relatively to the initial activity.

Extract	Gastric juice	Pancreatic juice	Caco-2
M. piperita	90.4±11.9	88.9±2.4	92.4±2.6
M.pulegium	81.5±5.8	96.8±9.5	122.9±3.7
M.spicata	82.4±7.3	79.8±3.4	83.7±0.1

Results are represented as the mean ± SD from at least 3 experiments. The differences between the treatments were not significant at p<.05.

3.2.3. Enzymatic digestion of *Mentha* Extracts by Pancreatic Juice.

The pancreatic juice contains pancreatin, which is a mixture of several enzymes: amylase, lipase and protease at pH 8.0. The metabolization of the *Mentha* extracts' constituents was analysed by HPLC-DAD. The chromatograms obtained during the digestive process did not reveal any changes in the composition. The results for the infusion with higher activity, *M. spicata*, is shown in **Figure 3 b.** The biological activity, AChE inhibition, after 4h digestion varied between 3 and 20% decrease (**Table 2**). These differences may be explained by artefacts during the experimental set-up. Variance analysis did not indicate any statistically significant difference. Our previous work using also a Lamiaceae plant [10] indicated a decrease of 50% in the enzyme inhibition activity after the pancreatic juice digestion. However, the compounds that were modified were neither the rosmarinic acid, nor the flavonoid derivatives, but the abietane diterpenoids present in the water extract of the other Lamiaceae herb, *P. barbatus*.



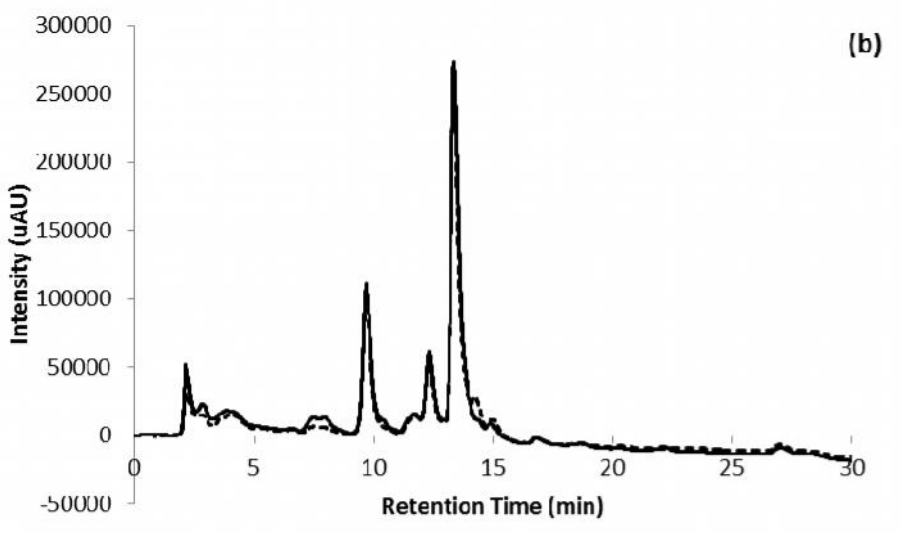


Figure 3 – RP-HPLC analysis of *Mentha spicata* after enzymatic digestion by (a) gastric juice, and (b) pancreatic juice. The chromatograms at 0h (dashed lines) and at 4h (full lines) digestion are shown.

3.2.4. Metabolization of extracts by Caco-2 cells

The use of Caco-2 cell line is an elegant way of simulating the intestinal barrier, because these cells are morphologically and functionally similar to the intestinal enterocytes [21]. The incubation of the extracts with this cell line will show whether there is any metabolization of the extracts prior to their absorption. The HPLC-DAD analyses revealed that there were no changes in the chemical composition of the extracts, results not shown. The activity as AChE inhibitor of the metabolized extracts showed that there were no significant alterations in the extract composition (**Table 2**). The increased activity found in the *M. pulegium* extract (122%), could be explained by an increase in the concentration of the extract during the cell tests. The variance analysis made to the results presented in **Table 2** indicated that they were not statistical different at $p<.05$. The lack of modifications was confirmed by HPLC-DAD, where the chromatogram did not indicate any modifications. The results were similar to those previously obtained in which these cells were not able to metabolise the compounds that were outside the cell membrane [10].

These results indicated that when reaching the intestine, the herbal infusions from *Mentha* still have the initial compounds and retain approximately the activity found in the initially prepared *Mentha* beverage.

The search for the presence of the polyphenols inside the Caco-2 cells revealed the presence of rosmarinic acid, as observed before [18]. Eriocitrin and eriodictyol could not be detected inside the cells, probably because they were below the detection limit of the analytical system.

4. CONCLUSION

In conclusion, our results show that the herbal teas of mint species revealed the presence of rosmarinic acid, but *M. spicata* also showed eriocitrin and eriodictyol which were the main responsible compounds for the biological activities of this extract. The biological effects of the aqueous extracts of *Mentha* species, used in the concentration commonly consumed, may be kept throughout the digestive system. All the extracts inhibited acetylcholinesterase activity even after the *in vitro* simulation of the gastrointestinal digestion. As these herbal teas are pleasant drinks and are usually consumed in high quantities, they have high probability to be useful in the treatment of some gastrointestinal problems.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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