

Antispasmodic activity of aqueous extracts from *Mentha x piperita* native from Trás-os-Montes region (Portugal)

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ABSTRACT

The wild mint *Mentha x piperita* (*Mentha rotundifolia* Huds.), has been used by the local people in a northern region of Portugal to prepare infusions for treating digestive pain and spasms, as an appetite stimulant and for treating headache and migraine. This suggests that these aqueous extracts have analgesic; antispasmodic and stomachic properties. In the present study the antioxidant potential of aqueous extracts of *Mentha x piperita* is investigated since natural antioxidants can scavenge the reactive oxygen species, ROS, and thus might attenuate inflammation pathways. The antispasmodic activity was also checked.

The antioxidant potential of the extract was evaluated by the DPPH[•] method, by the quantification of the total phenolic compounds and by characterization of the main phenolic compounds. The antispasmodic effects were investigated by performing pharmacological assays using the distal ileum of guinea pigs.

The aqueous extract exhibits antioxidant properties that may be due to its phenolic content. The main phenolic compounds were quinic, caffeic, rosmarinic and chlorogenic acids. The antispasmodic effects are observed after electrical stimulation of segments of distal ileum of guinea pigs and may be attributed, at least, to a α_1 blockage.

Keywords- *Mentha x piperita*, phenolic content, antispasmodic activity, cytotoxicity, antioxidant activity.

1. INTRODUCTION

Medicinal plants have been a classical source of pharmacological active molecules since early times. An estimation from WHO (World Health Organisation) shows that 80% of the world population uses plants as the main resource in basic health care, either as an option, or as a sole alternative [1-2]. Many of the species used empirically, lack a scientific validation for efficacy and safety. Thus, it has become necessary to evaluate the true medical potential of the plants used in traditional medicine. This can be achieved by the search for the pharmacologically active principles, and by the comparative study of the therapeutic action of the herbal extracts and the isolated drugs, so as to determine whether the presence of the other extract components has a potency or inhibitory effect on the activity/toxicity of the plant active principle.

Peppermint (*Mentha piperita* L.) is a perennial herb native of Europe and cultivated in many parts of the world. Best known for its flavouring and fragrance properties, peppermint leaves (fresh and dried) and the essential oil extracted from the leaves are used in many foods, cosmetic and pharmaceutical products. Several of pharmacological activities of peppermint have been documented: the antioxidant, antiviral, antibacterial, fungicidal and even anti-tumour activities were proved by in vitro assays; the antispasmodic, hepatic, renal, antiallergenic and anti-inflammatory actions were studied in test animals [3]. Studies in humans further confirmed the gastrointestinal, respiratory tract and analgesic actions [3], but these actions were mostly studied in essential oil extracts which are the most commonly used commercial routes.

Mentha x piperita L., a hybrid of spearmint (*M. spicata* L.) and water mint (*M. aquatica* L.), is a popular herb used to prepare tisanes, either with medicinal purposes or as a degustation beverage. In a previous ethnopharmacological survey concerning plants of the northern region of Portugal, the local people stated that infusions of “mentrasto” could be used for “digestive pain as tonic and as an appetite stimulant; against migraine and spasms” suggesting that aqueous extracts of this wild plant is traditionally used as an antispasmodic (spasms of the bile duct, gallbladder and gastrointestinal (GI) tract) and in the treatment of gastrointestinal ailments, as dyspepsia, enteritis, flatulence, gastritis or intestinal colic, and also as antiseptic, analgesic and antiastenic [4-5].

The aims of this work are to assess the anti-inflammatory potential, the anti-spasmodic activities and the toxicity of the aqueous extracts of the endemic *Mentha x piperita* plant which grows in Trás-os-Montes region, situated in the north of Portugal.

A determination of the anti-oxidant activity, the total phenolic content and the identification of the major phenolic substances were made in order to have a basic

chemical characterization of the antioxidant profile of the sample. In fact, the composition of various extracts of mentha is well established and studied, but small changes in composition can be expected due to the different climatic conditions and soil composition in which the plant grows. The anti-oxidant capacity of the sample was determined by the disappearance of DPPH[•] free radical, the total phenolics content by Folin-Ciocalteu reagent, using gallic acid as a standard, and the identification of the major phenolic substances present in the extract by was made by liquid chromatography with diode array detector (DAD) UV detection, connected with electrospray mass spectrometry, LC-DAD-MS and MS/MS.

The antispasmodic activity of the extract was tested in vitro by in the isolated ileum of guinea pigs. This activity was tested both in parasymphatic and sympatric systems.

2. MATERIALS AND METHODS

2.2 Solvents, reagents and standards

All solvents and reagents from various suppliers were of the highest purity needed for each application. The Folin-Ciocalteu reagent was from Merck. Materials and reagents for HPLC analysis: methanol (HPLC gradient grade) and formic acid (HPLC gradient grade) were obtained from Merck (Darmstadt, Germany). Water from a Milli-Q purification system (Simplicity- UV, Millipore Corp., France) was used for sample preparation and LC-DAD-MS analysis. Gallic acid, quinic acid, chlorogenic acid, rosmarinic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Caffeic acid was from Merck (Darmstadt, Germany). Hydroxytyrosol, *p*-hydroxybenzoic acid, and butylated hydroxytoluene (BHT) butylated Hydroxyanisole (BHA) and DPPH[•] (1,1-Diphenyl-2-Picrylhydrazyl) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) from Sigma-Aldrich. All standards were prepared as stock solutions in methanol. Working standards were made by diluting stock solutions in 62.5% aqueous methanol containing 1 g.L⁻¹ BHT and 6 M HCl to yield concentrations ranging between 0.5 and 25 mg.L⁻¹. Stock/working solutions of the standards were stored in darkness at -18 °C.

2.2 Plant Material

The plant was collected in “Local da Torre” in Chaves council (belonging to Trás-os-Montes region, north of Portugal (June of 2009)). The plant was dried in the air at room temperature, in the dark. The identification of the was done at the Department of Biology of The University of Trás-os-Montes and Alto Douro, who provided the voucher of the specimen. Dried leaves of *Mentha x piperita* were used to make the aqueous extracts. The national rules concerning the biodiversity rights were followed during the collection of the samples.

2.3 Extraction

For the preparation of the aqueous extract, one hundred grams of chopped and dried plant material were placed in a 2000 mL Schot flask and 1000 mL water were added. The flask was capped and the plant was macerated for 48 h at room temperature in dark. The water fraction was removed by filtration through Whatman filter paper and then through 450 mm carbonates filters, lyophilized and stored at 4° C until used. Before the determination by HPLC, the samples were filtered quickly through a 0.45 μ m membrane filter (Millex-HV).

2.4 DPPH[•] Assay

The antiradical activity of the extracts was determined spectrophotometrically in a Microplate Reader by monitoring the decrease of DPPH[•] concentration at 515 nm. A dilution series (twelve different concentrations) was prepared in a 96 well plate. The reaction mixtures in the sample wells consisted of 25 μ L of extract and 200 μ L of 150 μ M DPPH[•] (freshly dissolved in methanol). The reaction was conducted at room temperature, until no variation of the absorbance was observed. Four experiments were performed in triplicate. BHT and BHA were used for comparison. Inhibition of free radical by DPPH[•] in percent (I %) was calculated in following way:

$$I = 100 \times \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \quad (\text{eq. 1})$$

where A_{control} is the absorbance of the control reaction and A_{sample} is the absorbance of the aqueous extract. The antiradical activity was expressed in terms of the amount of antioxidants necessary to decrease the initial DPPH[•] absorbance by 50 % (IC₅₀). The IC₅₀ value for each extract was obtained from linear and/or non linear regression analysis, by plotting the percentage of DPPH[•] scavenging as a function of extract concentration.

2.5 Total Phenolics Assay

Total phenolics were estimated using the Folin-Ciocalteu colorimetric method described previously [6] with a little modification. Briefly, 300 μ L of the aqueous extract (1.04 mg.mL⁻¹) was diluted with 2 mL of water in a 10.0 mL volumetric flask, to which 1.0 mL undiluted Folin-Ciocalteu reagent was added. Immediately, 5.0 mL 20% (w/v) Na₂CO₃ were added and the volume was made up to 10 mL with H₂O. After 30 minutes incubation at room temperature, the absorbance was measured at 735 nm and compared to a pre-prepared gallic acid calibration curve. Determinations were performed in duplicate. Results were expressed as milligram of gallic acid (GA) per milligram of lyophilized. All determinations were performed in triplicate (n = 3).

2.6 Phenolic content analysis

LC-DAD-MS and MS/MS analysis were carried out in an LCQ Advantage ThermoFinnigan mass spectrometer equipped with an electrospray ionisation source and using an ion trap mass analyser. It was controlled by Xcalibur software (ThermoFinnigan). It was coupled to an HPLC

(Surveyor ThermoFinnigan) system with a photodiode array detector (DAD) (Surveyor ThermoFinnigan) and an auto sampler. The conditions of analyses were: capillary temperature 300 °C; source voltage 4.5 kV, source current 100.0 μ A, and capillary voltage -15.0 V in negative mode. It was used a reversed-phase Zorbax Eclipse XDB-C18 analytical column, 150×2.1 mm (length×I.D.) and 3.1 μ m particle size (Agilent Technologies, Germany). The mobile phase was: Solvent A: methanol; Solvent B: water with formic acid (0.5%). A gradient program was adopted as follows: linear from 0 to 20% of solvent A (0-30 min), 20 to 100% of solvent A (30-40 min). LC analyses were performed at room temperature; the injection volume was 25 μ L, and the flow-rate was 0.3 mL/min; the DAD detector was scanned from 200 to 500 nm, and the chromatographic profile was recorded at 254 nm.

2.7 Antispasmodic Activities

For antispasmodic activities assays guinea pigs of both sexes were used with average weight of 350 g. The animals were kept on the Bioterium (from the animal house) of Department of Physiology and Pharmacology of the Faculty of Pharmacy of Oporto University. The guinea pigs received water and normal diet. They were maintained under control of lighting (cycles of 12 h light/dark) and under temperature of 23 \pm 2 °C. This part of the research was conducted in accordance with the internationally accepted principles for laboratory animal use and care, as found in the European Community guidelines.

After sacrifice by cervical dislocation followed by bleeding a midline incision of the abdominal cavity was made and a segment of distal ileum was removed and immediately placed nutrient of Krebs-Henseleit solution, at room temperature. The intestinal content was carefully removed with liquid nutrition and the intestinal segment was transferred to new nutrient solution in order to remove the mesenteric adhesions and cut it into fragments of approximately 4 cm in length. The longitudinal coat of smooth muscle was separated from the ileum and each fragment of the isolated muscle was fixed to a rod holder and placed between two platinum wires of the electrode and the other end was attached to lever of isometric myography. Preparations were placed in organ bath (40 ml of nutritive liquid continuously oxygenated with carbogen at 37 °C) and subjected to a baseline tension of 1.5 g. Preparations were left for a stabilization period of 30 to 45 minutes with renewal of the nutrient solution at intervals of 10 minutes.

Electrical stimulation of the tissue was induced by a stimulator connected to an analog-digital converter PowerLab/400 (ADInstruments, Australia). The data acquisition between PowerLab and computer was made via a cable connected to a SCSI card and the software Chart for Windows. Electrical stimulation was used as follows: intensity 110V, frequency of 0.2 Hz, duration of 2 ms. After the stabilization of the preparation electrical stimulation has been started and the responses obtained by the effect of the drugs and the sample recorded.

3. RESULTS AND DISCUSSION

The commercial importance of *Mentha* spp. extracts arise essentially from their antioxidant and anti-septic properties. In order to compare the anti-oxidant capacity of the present sample with those native to other regions the quantification of total phenolics of the extracts have been done as well as the determination of the main phenolic constituents of the sample. Nevertheless the main purpose of the study is the evaluation of the anti-spasmodic activity of the aqueous extracts. As mentioned before *Mentha x piperita* L. (peppermint), a hybrid of spearmint that grows widely together with other species of mint so there is a high possibility that the local people collect it and thinks it to be the original wild mint, since rigorous classification requires the expertise of a botanist. Despite the morphological resemblances between the original and hybrids spearmints, the pharmacological active components may differ.

3.1 Anti-oxidant properties

3.1.1 Anti-oxidant properties

The most common and reliable methods for the evaluation of the anti-oxidant potential of plant extracts are radical sequestering methods such as DPPH• radical. In addition, Folin-Ciocalteu reagent, using gallic acid as a standard is an easy and widely used method for the determination of the total phenolics.

The reductive capacity of DPPH• radical was evaluated analytically by Uv-Vis spectroscopy using BHA and BHT as standards. Results for total phenolic content and antioxidant capacity for *Mentha x piperita* extracts are presented in Table 1. The values for IC₅₀ follow the order: BHA < BHT < *Mentha x piperita* showing that the extract has a moderate capacity to sequester the free radical. In fact the antioxidant activity of *M. piperita* plant is extensively documented as an essential oil extract [7-8] and as methanol or organic solvents extracts [9]. As far as aqueous extracts are concerned there is much less published data [10-12] but it appears that about 75% of the polyphenolic compounds present in the leaves are extracted in an infusion (750 mg.L⁻¹) [13].

Table 1

Values obtained for CI₅₀ (mg mL⁻¹) and total phenolic content for the aqueous extracts of leaves of *Mentha x piperita*

Compo und	DPPH (linear)	DPPH (exp.)	Total Phenolics
	CI ₅₀ (mg·mL ⁻¹)	CI ₅₀ (mg·mL ⁻¹)	% (m/m) ^a
<i>Mentha</i> <i>x</i> <i>piperita</i>	0.049 ± 0.003 (R ² = 0.9958)	0.048 ± 0.007	0.467± 0.014
BHT	0.021 ± 0.006 (R ² = 0,9961)	0.0181± 0.0008	
BHA	-----	0.0053± 0.0001	

^a mg(gallic acid)/mg (lyophilized)

3.1.2. Total phenolic content and major phenolic compounds

The total phenolic content of *Mentha x piperita* used in this study is about 0.467 [mg (gallic acid)/ mg (lyophilised)]. The results obtained in this study show that *Mentha x piperita* can be used as source of natural anti-oxidants.

The identification of the major phenolic substances was made by the LC-DAD-MS and MS/MS analysis of the *Mentha x piperita* extract enabling the preliminary identification of some compounds. The chromatogram obtained at 254 nm (Figure 1) showed several peaks, which can be attributed to the natural phenolic compounds present in the plant. The identification of some compounds was achieved by comparing the peak on-line UV and mass spectra, with those reported in the literature (references in table 2). Additionally, full scan mode MS analyses followed by MS/MS experiments, in the negative mode, were performed to elucidate the compounds' structure. The results obtained are presented in Table 2. The identity of the main phenolic constituents of *Mentha x piperita* used in this study is caffeic, rosmarinic, quinic and chlorogenic phenolic acids whose structures are presented in Figure 2. Caffeic acid acid has been documented as having strong anti-oxidant properties [14].

Table 2

Identification of some compounds present in the aqueous extract of *Mentha piperitae* by HPLC-DAD and HPLC-ESI-MS and MS/MS.

Rt (min)	Compound Identity	[HPLC-DAD] □ _{max} (nm)	[ESI full scan MS (□)] m/z	[ESI-MS ² (□)] m/z
.02	Quinic acid [15,16]	248 280sh	191 [M-H] ⁻ 237 [M+HCOO] ⁻	191 [M-H] ⁻ 127 [M-CO-2H ₂ O] ⁻
1.18	Caffeic acid [17]	237 303sh 323	179 [M-H] ⁻ 225 [M+HCOO] ⁻	179 [M-H] ⁻ 135 [M-CO ₂ -H] ⁻
18.19	5-O-Caffeoylquinic acid [17,18] (Chlorogenic acid)	241 305sh 326	353 [M-H] ⁻ 707 [2M-H] ⁻	191 [M-C ₉ H ₆ O ₃ -H] ⁻ 179 [Caffeic-H] ⁻
24.77	Rosmarinic acid [19]	290 330	359 [M-H] ⁻	197 [M - H - Caffeic acid] ⁻ 179 [Caffeic acid - H] ⁻ 161 [M - H - Caffeoyl moiety] ⁻

According to Duband et al. [13] the total polyphenolic content of peppermint leaves is approximately 19–23%. This main composition includes 59–67% eriocitrin and rosmarinic acid (combined), 7–12% luteolin 7-O-rutinoside, 6–10% hesperidin, and smaller quantities of 5,6-dihydroxy-7,8,3,4-tetramethoxyflavone, phebrellin, gardenin B and apigenin. *Mentha piperita* is rich in antioxidants such as caffeic acid, rosmarinic acid, eugenol and α -tocopherol [20], which is in accord with the qualitative composition of the studied extract except that which concerns quinic and chlorogenic acids. Moreover, caffeic, chlorogenic and quinic acid are known for showing effects in the biliary flow in rats [21]. Olthof and co-workers [22] suggested that approximately one third of chlorogenic acid and almost all of the caffeic acid is absorbed in the small intestine of humans. This being so, part of chlorogenic acid from ingestion will enter into the blood circulation, but most will reach the colon. According to Rodriguez de Sotillo [23], chlorogenic acid was found to improve glucose tolerance and to modify plasma and liver lipids in Zucker rats. Also Frank et al, [24] reported that the hydroxycinnamate caffeic acid and chlorogenic acid had effects on cholesterol blood concentration: they concluded that caffeic and chlorogenic acid may elevate cholesterol rates in Sprague–Dawley Rats. More recently, some investigations have shown that chlorogenic acid might have an antagonistic effect on glucose transport in sense that it may attenuate intestinal glucose absorption rates and shift the site of glucose absorption to more distal parts of the intestine [25]. Thus the reports suggest that these acids act in glucose tolerance but as regards the effects on lipid blood concentrations further investigations would have to be done in order to find whether these acids raise or lower the lipid blood concentrations and under which conditions they do so.

3.2. Antispasmodic activities.

The spasmolytic activity was tested on the longitudinal coat of smooth muscle of the ileum of guinea pigs. The substances used for testing and its concentrations are listed in Table 3. After testing the contractile response of the isolated organ to acetylcholine, induced by electrical stimulation (period I in figure 3), the contractile signal was reduced in the presence of atropine (the acetylcholine

competitive agonist) and norepinephrine (refer to figure 3, period after III and IV, respectively). The period after II in figure 3 shows the variation of the contractile response to the presence of the crude extract. In fact, after addition of the crude extracts of *Mentha x piperita* a reduction the contractile response induced by electrical stimulation (period I of the same figure) is recorded. However it is not as significant as when atropine and norepinephrine are added.

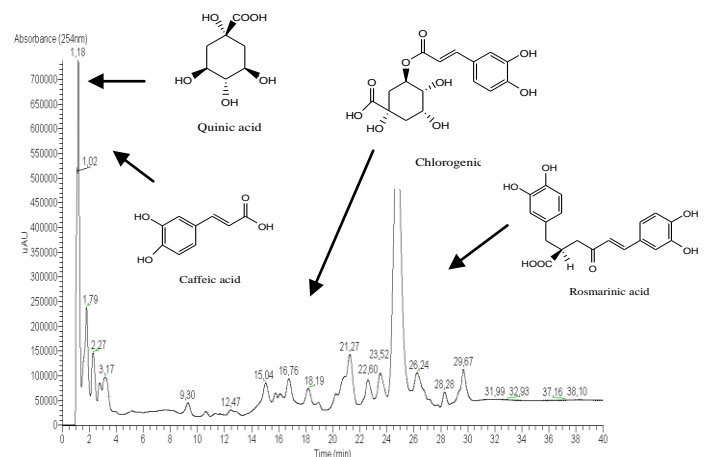


Fig.1. LC-DAD chromatogram of the aqueous extract of *Mentha piperitae*. Column: Zorbax Eclipse XBD-C18. Elution conditions: Solvent A: methanol; Solvent B: water with formic acid (0.5%). Gradient program was adopted as follows: linear from 0 to 20% of solvent A (0-30 min), 20 to 100% of solvent A (30-40 min).

As with any extract of a biological material, an aqueous extract of solution of *Mentha x piperita* may have a complex composition. Therefore, the reduction of contractile response may be related to a blockage of the cholinergic receptor, to the stimulation of the adrenergic receptor or to both. Also the presence of substances which induce the contraction of the smooth muscle cannot be excluded.

Table.3

The substances used for testing the anti-spasmodic activities of the extract and its concentrations.

Substance	Main action	Bath conc.	Dose
Acetylcolin (Ach)	Endogen agonist of cholinergic receptors	5 μ M / 40 ml	100 μ l
Atropine	Antagonist of cholinergic receptors	1,4 μ M / 40 ml	100 μ l
Norepinephrine	Endogen agonist of adrenergic receptors	3,94 μ M / 40 ml	100 μ l
Prazosine	Antagonist α_1 of adrenergic receptors	96 μ M / 40 ml	100 μ l
Yohimbine	Antagonist α_2 of adrenergic receptors	1 μ M / 20 ml	100 μ l
Phenylephrine	Agonist α_1 of adrenergic receptors	5 μ M / 40 ml	100 μ l

In order to verify whether the solution *Mentha x piperita* showed noradrenergic activity, the solution was tested in the presence of solutions of prazosin (refer to figure 4, moment V) and yohimbine (refer to figure 4, moment VI), the α_1 and α_2 antagonists adrenergic receptors, respectively. The blockage of the receptors prevents the decrease in the intestinal motility due to a sympathetic response. When the crude extract was added to the solution with the α_1 antagonist (Figure 4, period after II) a change in the contractile response was indeed recorded suggesting that a cholinergic antagonists or a α_2 adrenergic agonists may be present in the sample extract. Nevertheless it is worthy to note that the decrease of the

contractile response given by the extract is faster in the presence of the adrenergic antagonists (whether α_1 or α_2) as compared to its action alone and also that the contractile response reaches the same limit, suggesting that, although a cholinergic antagonist might be the responsible for the antispasmodic activity, a sympathetic agonist may also be present of in the extract. So, experiments with phenylephrine that stimulates selectively the α_1 receptors were further performed.

The electric stimulation of the tissue in the presence of this phenylephrine raises the baseline and increases the muscle strain (Figure 5) pointing out for a α_1 stimulation. When the test solution of the *Mentha x piperita* extract is added after the addition of phenylephrine a reduction of muscle contraction is observed. Nevertheless the characteristic effect of baseline level increase due to the phenylephrine is no longer observed. Therefore, the results (Figure 5) suggest that the crude extract of *M. piperita* has some chemical component that acts on the α_1 receptors as agonist. It would be interesting to test the solution with clonidine to check if the delay in the response observed with yohimbine may also be due to a potentiation of the effect of α_2 receptor.

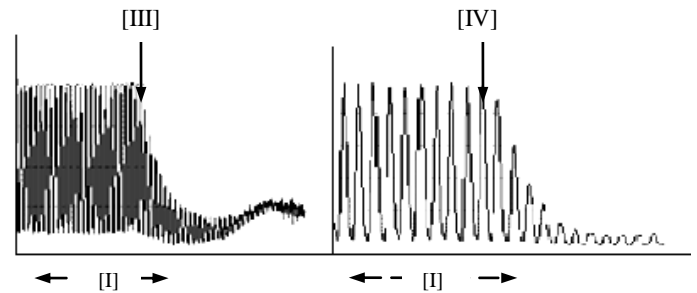
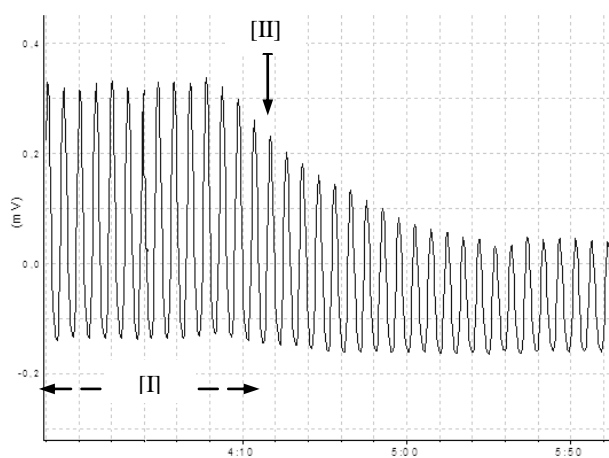


Figure 2. The contractile response of the longitudinal coat of smooth muscle of the ileum of guinea pig to acetylcholine, induced by electrical stimulation (period I). Top: after addition of the crude extracts of *Mentha x piperita* made on II.

Bottom left: after the addition of atropine made on III.
Bottom right: after the addition of norepinephrine made on IV.

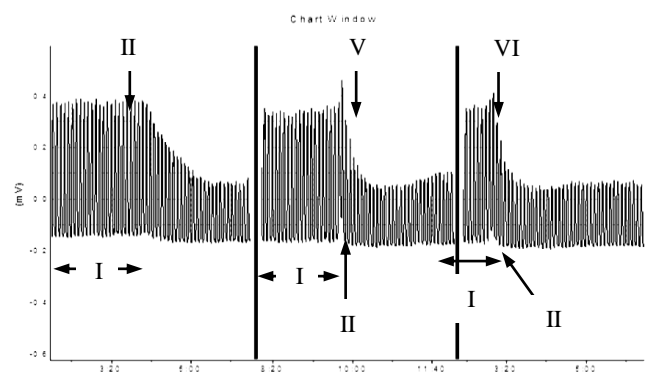


Figure 3 – The contractile response of the longitudinal coat of smooth muscle of the ileum of guinea pig to acetylcholine, induced by electrical stimulation (period I). Instant II refers to an addition of 150 μ l of the crude extracts of *Mentha x piperita*. Instant V refers to an addition of 100 μ L of prazosine. Instant VI refers to an addition of 100 μ L of yohimbine.

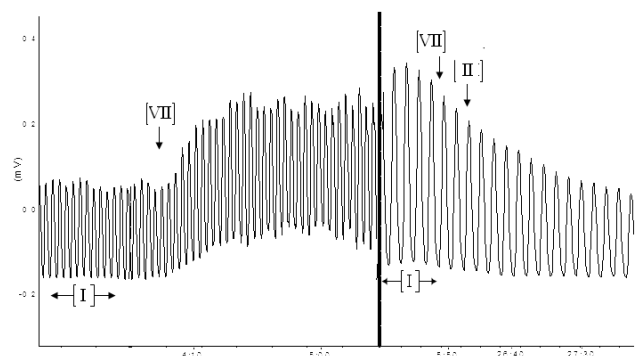


Figure 4 - The contractile response of the longitudinal coat of smooth muscle of the ileum of guinea pig to acetylcholine, induced by electrical stimulation (period I). Instant II refers to an addition of 150 μ l of the crude extracts of *Mentha x piperita*. Instant VII refers to an addition of 100 μ L of phenylephrine.

Finally the effects of the extract solution was tested with a 24 hours storage solution in order to compare them with those obtained using a freshly prepared extract solution. It was found that the activities recorded for the extract with 24 hours of storage were similar but in a lower or higher in proportional amount, from those recorded with the fresh prepared solution. This could be the result of the modification of substances in solution, inactivation or overlap of activities of the various components of the crude extract.

4. CONCLUSIONS

Infusions of wild *Mentha x piperita* are used traditionally in the northern region of Portugal for treatment of digestive disorders. The major phenolic compounds present in the aqueous extracts are rosmarinic, caffeic and chlorogenic acids. Chlorogenic acid and almost all of the caffeic acid is absorbed in the small intestine of humans and are known for showing effects in the biliary flow, on the glucose tolerance and on the plasma and liver lipids in rats models. In humans, some investigations have shown that chlorogenic acid may attenuate intestinal glucose absorption rates and shift the site of glucose absorption to more distal parts of the intestine. Thus the results of phenolic content suggest that the infusions may actually contribute for delay on glucose absorption and in the modification of lipid blood concentrations. In addition, antispasmodic effects of the extract were observed after electrical stimulation of segments of distal ileum of guinea pigs and may be attributed, at least, to a α_1 blockage.

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