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# *Mentha spicata* L. infusions as sources of antioxidant phenolic compounds: emerging reserve lots with special harvest requirements

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*Mentha spicata* L., commonly known as spearmint, is widely used in both fresh and dry forms, for infusion preparation or in European and Indian cuisines. Recently, with the evolution of the tea market, several novel products with added value are emerging, and the standard lots have evolved to reserve lots, with special harvest requirements that confer them with enhanced organoleptic and sensorial characteristics. The apical leaves of these batches are collected in specific conditions having, then, a different chemical profile. In the present study, standard and reserve lots of *M. spicata* were assessed in terms of the antioxidants present in infusions prepared from the different lots. The reserve lots presented the highest concentration in all the compounds identified in relation to the standard lots, with 326 and 188 μg $mL^{-1}$ of total phenolic compounds, respectively. Both types of samples presented rosmarinic acid as the most abundant phenolic compound, at concentrations of 169 and 101 μg $mL^{-1}$ for reserve and standard lots, respectively. The antioxidant activity was higher in the reserve lots which had the highest total phenolic compounds content, with $EC_{50}$ values ranging from 152 to 336 μg $mL^{-1}$. The obtained results provide scientific information that may allow the consumer to make a conscientious choice.

## 1. Introduction

In a world of constant change, innovation, and development, the tea market has acquired a large number of fans, and has been turned to a highly sophisticated and competitive sector. Indeed, this is reflected in the increased worldwide tea consumption, valued at more than three million cups per day.[1,2]

Currently, several products with added value are emerging, and consumers have at their disposal a wide range of different lots with distinct compositions of the same plant for infusion preparation.[2] As an outstanding example, the standard lots have evolved to reserve lots, with special harvest requirements and, therefore, different compositions that confer them with faultless organoleptic and sensorial characteristics. These lots are produced from the younger parts (apical leaves) of the plant collected in the hot summer months and have become a target of high demand by consumers. Beyond the enhanced flavor, the functional properties of these beverages are also of growing interest once infusions have been proved, for a long time, to possess health promoting and disease prevention properties.[3]

Among the most economically important species, *Mentha spicata* L., commonly known as spearmint and belonging to the Lamiaceae family, is widely used in both fresh and dry forms, for infusion preparation[4] or in European and Indian cuisines[5] due to its exceptional taste. Furthermore, it is an important source of antioxidants, such as carotenoids[6] and phenolic compounds.[7,8] These secondary metabolites of plants are well-known for their beneficial properties by acting as protective agents against oxidative damage and as substrates for oxidative browning reactions through enzymatic and chemical mechanisms.[9]

Several studies have focused on the bioactive properties of various plant species, including *M. spicata*, but most of them were performed on dry material,[5,10] and since the most consumed form is the infusion, in the present work we aimed to evaluate and compare the antioxidant activity as well as the bioactive compounds of *M. spicata* infusions prepared from standard and reserve lots (emerging added value products), in order to provide scientific information concerning the potential differences between both batches and allow the consumer to make a conscientious choice.

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# 2. Materials and methods

## 2.1. Samples and sample preparation

Different lots of *M. spicata* (standard and reserve lots) were obtained from *Cantinho das Aromáticas*, organic farmers from Vila Nova de Gaia (Portugal), as dry material for infusion preparation. The main difference is that reserve lots have special harvest requirements, being produced from younger parts (apical leaves) of the plant collected in the hot summer months.

The infusions were prepared by adding 200 mL of distilled water at 90 °C (the ideal temperature according to the label) to 600 mg of dry material, and left to stand for 5 minutes. A certain volume of these infusions was further diluted to evaluate the antioxidant activity, whereas an aliquot of the infusion was filtered through 0.2 μm nylon filters for the phenolic compounds analysis.

## 2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid was purchased from Prolabo (VWR International, Fontenay-sous-Bois, France). Phenolic standards were from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Sigma (St Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

## 2.3. Phenolic compounds analysis

The lyophilized infusions were dissolved in water and analysed using a Hewlett-Packard 1100 chromatograph (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 $C_{18}$, 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents were: (A) 0.1% formic acid in water, and (B) acetonitrile. The elution gradient established was isocratic 15% for 5 min, 15% B to 20% B over 5 min, 20–25% B over 10 min, 25–35% B over 10 min, 35–50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL $min^{-1}$. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system *via* the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 °C, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at −4500 V in the negative mode. The MS detector was programmed for recording in two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in the sample. Settings used were: declustering potential (DP) −450 V, entrance potential (EP) −6 V, collision energy (CE) −10 V. The EPI mode was used in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP −50 V, EP −6 V, CE −25 V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1500.

The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard compounds, when available. Otherwise, compounds were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compounds from the same phenolic group. The results were expressed in μg per mL of infusion. For each formulation, the analyses were carried out in triplicate.

## 2.4. Evaluation of antioxidant activity

**2.4.1. DPPH radical-scavenging activity.** This methodology was performed using an ELX800 microplate Reader (Bio-Tek Instruments, Inc.; Winooski, USA). The reaction mixture on a 96 well plate consisted of the sample solutions (30 μL) and methanolic solution (270 μL) containing DPPH radicals ($6 \times 10^{-5}$ mol $L^{-1}$). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where $A_S$ is the absorbance of the solution containing the sample, and $A_{DPPH}$ is the absorbance of the DPPH solution.

**2.4.2. Reducing power.** The sample solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol $L^{-1}$, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in a 48-well plate, the same with deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in the Microplate Reader mentioned above.

**2.4.3. Lipid peroxidation inhibition by TBARS assay.** Porcine brains were obtained from official slaughtered animals, dissected, and homogenized with Polytron in an ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1 : 2 w/v brain tissue homogenate which was centrifuged at 3000*g* for 10 min. An aliquot (100 μL) of the supernatant was incubated with the sample solutions (200 μL) in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1 mM; 100 μl) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 μL), followed by thiobarbituric acid (TBA, 2%, w/v, 380 μL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000*g* for 10 min to

remove the precipitated protein, the color intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = $[(A - B)/A] \times 100\%$, where $A$ and $B$ are the absorbance of the control and the sample solution, respectively.

**2.4.4. $EC_{50}$ value calculation.** The results obtained in the three antioxidant activity assays were converted to $EC_{50}$ values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay), calculated from the graphs of DDPH radical scavenging percentage, absorbance at 590 nm and lipid peroxidation inhibition percentage, respectively, *versus* sample concentration. Trolox was used as a positive control.

### 2.5. Statistical analysis

For all the experiments three samples were analysed and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using Student's *t*-test with $\alpha = 0.05$. This treatment was carried out using the SPSS v. 22.0 program.

## 3. Results and discussion

### 3.1. Phenolic compounds

Fig. 1 shows the phenolic compounds profile recorded at 280 nm of a *M. spicata* reserve lot aqueous extract, prepared by infusion. Data of retention time, $\lambda_{max}$, pseudomolecular ion, main fragment ions in $MS^2$, and tentative phenolic compound identification are presented in Table 1.

Compounds **4**, **6**, **11** and **14** were positively identified as caffeic acid, luteolin-7-*O*-rutinoside, rosmarinic acid and luteolin-7-*O*-glucoside, respectively, by comparison with authentic standards, as also by their MS fragmentation patterns, retention times and UV-vis characteristics. With the exception of luteolin-7-*O*-rutinoside, these compounds were previously identified in several studies regarding this species.[7,8,11]

Compound **1** ($[M - H]^-$ at *m*/*z* 353) was identified as 3-*O*-caffeoylquinic acid based on its $MS^2$ fragmentation, yielding the base peak at *m*/*z* 191 and the ion at *m*/*z* 179 with an intensity of 80% relative to the base peak, considered characteristic of 3-acyl-chlorogenic acids as reported by Clifford *et al.*[12] Compound **3** was easily distinguished by its base peak at *m*/*z* 173 [quinic acid $- H - H_2O]^-$, accompanied by a secondary fragment ion at *m*/*z* 179 with approximately 75% abundance of the base peak, which allowed identifying it as 4-*O*-caffeoylquinic acid according to the fragmentation pattern described by Clifford *et al.*[12] To the best of our knowledge these compounds have never been reported as such in *M. spicata*, although many researchers[8,11,13–15] describe the presence of chlorogenic acid in this plant without indicating the particular caffeoylquinic acid they are referring to.

Compound **2** presented a UV spectrum and product ions similar to *p*-coumaric acid. The presence of *p*-coumaric acid was previously reported in this species by different authors,[8,11,13,14] although that identity was discarded in our case, since the compound detected herein eluted earlier than a standard of *p*-coumaric acid. Therefore, peak 2 was just tentatively assigned as a *p*-coumaric acid derivative.

Compounds **8**, **9**, **12**, **15**, **16** and **17** were also identified as hydroxycinnamoyl derivatives, namely caffeic acid oligomers. Compound **8** showed a pseudomolecular ion $[M - H]^-$ at *m*/*z* 719 and an $MS^2$ majority fragment at *m*/*z* 359 corresponding to $[M - 2H]^{2-}$; these mass characteristics coincided with those of sagerinic acid, a rosmarinic acid dimer previously reported by us in other Lamiaceae.[16,17] Compounds **12** and **17** presented the same pseudomolecular ion $[M - H]^-$ at *m*/*z* 493, and characteristic $MS^2$ fragment ions at *m*/*z* 313, 295 and 197 and UV spectra, which allowed their identification as salvianolic acid A isomers.[18,19] Based on their relative order of elution as described by Ruan *et al.*,[19] these compounds were tentatively identified as isosalvianolic acid A and salvianolic acid A, respectively. Compounds **9**, **15** and **16** also presented the same pseudomolecular ion ($[M - H]^-$ at *m*/*z* 717) and a fragmentation pattern consisting of successive losses of 198 u (3-(3,4-dihydroxyphenyl)lactic acid, danshensu) or 180 u (caffeic acid).

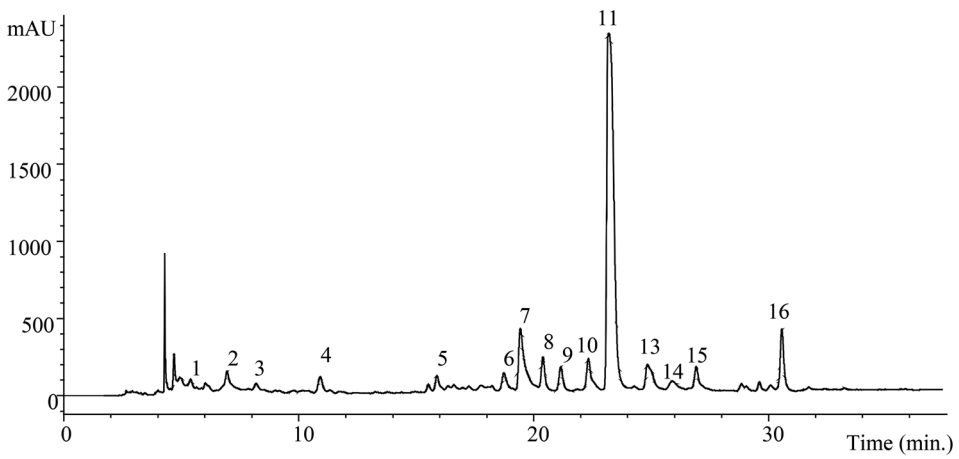


**Fig. 1** Phenolic compounds profile of *M. spicata* reserve lot aqueous extract, prepared by infusion, recorded at 280 nm.

 

**Table 1** Retention time (Rt), wavelengths of maximum absorption in the visible region ($\lambda_{max}$), mass spectral data, identification and quantification (μg mL$^{-1}$) of phenolic compounds in *Mentha spicata* standard and reserve lots

| Peak | Rt (min) | $\lambda_{max}$ (nm) | Pseudomolecular ion $[M-H]^-$ (*m*/*z*) | $MS^2$ (*m*/*z*) | Tentative identification | Standard lot | Reserve lot | Student's *t*-test *p*-value |
|---|---|---|---|---|---|---|---|---|
| 1 | 5.3 | 328 | 353 | 191(100), 179(80), 173(6), 161(4), 135(28) | 3-*O*-Caffeoylquinic acid | 3.48 ± 0.05 | 4.5 ± 0.2 | 0.005 |
| 2 | 7.2 | 312 | — | 163(100), 119(50) | *p*-Coumaric acid derivative | 2.45 ± 0.03 | 6.13 ± 0.07 | <0.001 |
| 3 | 8.4 | 328 | 353 | 191(38), 179(75), 173(100), 161(5), 135(68) | 4-*O*-Caffeoylquinic acid | 5.0 ± 0.1 | 2.60 ± 0.04 | <0.001 |
| 4 | 11.3 | 326 | 179 | 135(100) | Caffeic acid | 1.6 ± 0.2 | 4.5 ± 0.2 | <0.001 |
| 5 | 16.3 | 284, 336sh | 595 | 287(100) | Eriodictyol-*O*-deoxyhexosyl-hexoside | 0.96 ± 0.05 | 6.7 ± 0.3 | <0.001 |
| 6 | 19.3 | 348 | 593 | 285(100) | Luteolin-7-*O*-rutinoside | 5.2 ± 0.1 | 8.6 ± 0.5 | 0.003 |
| 7 | 20.1 | 348 | 461 | 285(100) | Luteolin-*O*-glucuronide | 5.1 ± 0.1 | 40.9 ± 0.3 | <0.001 |
| 8 | 21.1 | 284 | 719 | 359(71), 197(21), 179(14), 161(57), 135(7) | Sagerinic acid | 5.4 ± 0.2 | 13.1 ± 0.1 | <0.001 |
| 9 | 21.8 | 284, 338sh | 717 | 537(21), 519(54), 493(21), 339(24), 321(27), 313(9), 295(100), 277(18) | Salvianolic acid E | 13.3 ± 0.4 | 9.8 ± 0.1 | 0.002 |
| 10 | 22.9 | 284, 336sh | 609 | 301(100) | Hesperetin-*O*-deoxyhexosyl-hexoside | 2.7 ± 0.1 | 4.7 ± 0.2 | <0.001 |
| 11 | 23.9 | 330 | 359 | 197(50), 179(27), 161(100), 135(21) | Rosmarinic acid | 101 ± 2 | 169 ± 1 | <0.001 |
| 12 | 24.4 | 326 | 493 | 313(12), 295(100), 197(11), 179(7), 161(17), 135(25) | Isosalvianolic acid A | 12.8 ± 0.3 | nd | — |
| 13 | 25.6 | 348 | 607 | 299(100), 284(48) | Chrysoeriol-7-*O*-rutinoside | 10.6 ± 0.3 | 16.2 ± 0.4 | <0.001 |
| 14 | 26.6 | 348 | 461 | 285(100) | Luteolin-7-*O*-glucoside | 3.5 ± 0.1 | 6.9 ± 0.2 | <0.001 |
| 15 | 27.6 | 289, 330sh | 717 | 519(95), 339(13), 321(100), 295(22), 277(7) | Salvianolic acid B | 7.8 ± 0.4 | 16.2 ± 0.3 | <0.001 |
| 16 | 27.9 | 289, 332sh | 717 | 519(100), 339(27), 321(87), 295(13), 277(33) | Salvianolic acid L | 6.2 ± 0.4 | nd | — |
| 17 | 31.1 | 324 | 493 | 359(48), 313(5), 295(4), 197(15), 179(19), 161(100), 135(4) | Salvianolic acid A | 4.4 ± 0.3 | 21.6 ± 0.2 | <0.001 |
| | | | | | Total phenolic acids | 164 ± 3 | 248 ± 1 | <0.001 |
| | | | | | Total flavonoids | 28.0 ± 0.3 | 84 ± 1 | <0.001 |
| | | | | | Total phenolic compounds | 191 ± 3 | 332 ± 1 | <0.001 |

tr – traces; nd – not detected.

These characteristics could match the identities of salvianolic acid B (also known as lithospermic acid B), E or L. Salvianolic acid B contains a furan ring in its structure that is lacking in salvianolic acids E and L.[19] According to Liu *et al.*,[20] salvianolic acid B would be more prone to lose a danshensu unit (−198 u), owing to the easy loss of the carboxyl connected with the furan ring, so that peak 9 that showed lower abundance of the product ion at *m*/*z* 519 (−198 u) than peaks 15 and 16 should not correspond to salvianolic acid B. Based on this assumption and the relative elution order described for those compounds by the same authors, compounds **9**, **15** and **16** might be tentatively identified as salvianolic acids E, B and L. To the best of our knowledge, all these caffeoyl oligomers are reported in *M. spicata* for the first time.

The four remaining compounds corresponded to flavonoids. UV spectra of compounds **5** ($[M-H]^-$ at *m*/*z* 595) and **10** ($[M-H]^-$ at *m*/*z* 609) suggested that they were flavanones. In both cases, only one $MS^2$ fragment was released from the loss of a deoxyhexosyl-hexoside moiety (−308 u) leading to the production of ions at *m*/*z* 287 ($[\text{eriodictyol}-H]^-$) and 301 ($[\text{hesperetin}-H]^-$), so that the compounds were tentatively identified as eriodictyol-*O*-deoxyhexosyl-hexoside and hesperetin-*O*-deoxyhexosyl-hexoside, respectively. Eriocitrin (eriodictyol-7-*O*-glucoside) and eriodictyol have been reported in the water-soluble extracts of *M. spicata*,[7] but hesperetin glycosides, as far as we know, have not been reported in this species.

Compound **7** presented a pseudomolecular ion $[M-H]^-$ at *m*/*z* 461 releasing a fragment ion at *m*/*z* 285 ($[M-176]^-$, loss of a glucuronyl moiety), allowing its assignment as luteolin-*O*-glucuronide. Compound **13** presented a pseudomolecular ion $[M-H]^-$ at *m*/*z* 607, yielding fragment ions at *m*/*z* 299 (−308 u; loss of a deoxyhexosyl-hexoside residue), which allowed its assignment as chrysoeriol-7-*O*-rutinoside, taking into account its previous identification in another *Mentha* species, such as *Mentha* × *villosa*.[21] The presence of luteolin-7-*O*-glucoside and luteolin has been reported in *M. spicata*,[7,8,13,14,22] although, to the best of our knowledge, the two compounds detected herein have not been reported in *M. spicata*.

The *M. spicata* reserve lot presented a higher concentration of all the identified compounds than the standard lot, and both types of samples presented rosmarinic acid as the most abundant phenolic compound. Moreover, compound **12** (isosalvianolic acid A) and compound **16** (salvianolic acid L) were not found in the reserve lot.

Table 2 Antioxidant activity, expressed in $EC_{50}$ values (μg $mL^{-1}$), of *Mentha spicata* standard and reserve lots

| Samples | Standard lot | Reserve lot | Student's *t*-test *p*-value |
|---|---|---|---|
| DPPH scavenging activity | 546 ± 17 | 336 ± 3 | <0.001 |
| Reducing power | 301 ± 3 | 198 ± 2 | <0.001 |
| Lipid peroxidation inhibition (TBARS assay) | 173 ± 5 | 152 ± 5 | <0.001 |

### 3.2. Antioxidant activity

The results obtained for the antioxidant activity of the infusions of *M. spicata* standard and reserve lots are presented in Table 2. The reserve lot revealed stronger antioxidant activity than the standard lot, presenting lower $EC_{50}$ values in all the assays performed. Both lots revealed the best results in the lipid peroxidation inhibition TBARS assay, followed by the reducing power and the DPPH scavenging activity assays. In previous studies, a water-soluble extract from *M. spicata* revealed an $EC_{50}$ value of 65.2 μg $mL^{-1}$ (ref. 7) and its ethanol extracts showed an $EC_{50}$ value of 16.2 μg $mL^{-1}$ (ref. 8) in the DPPH scavenging activity assay. Mata *et al.*[23] also reported the DPPH scavenging activity of water and ethanol extracts obtained from this plant with $EC_{50}$ values of 5.7 and 65.2 μg $mL^{-1}$, respectively. In all cases, higher antioxidant activity was found than the one obtained in our study, which might be due to the different solvents and extraction conditions used.

The antioxidant capacity of the studied infusions of *M. spicata* was higher on the sample that revealed the highest total phenolic compounds content, the reserve lot, which is in accordance with the well-known bioactivity of these compounds.

Overall, the results obtained in the present study highlight the importance of plant harvest conditions, by demonstrating differences in the bioactive molecule composition of infusions prepared from different lots, namely phenolic acids and flavonoids. The enhanced antioxidant capacity of the extracts obtained from the reserve lot might be a real asset in the choice of the best material for infusion preparation.

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