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1 **Challenges of traditional herbal teas: plant infusions and their**

2 **mixtures with bioactive properties**

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17 **Abstract**

18 In order to characterize and study the bioactivities of individual plant species and to
19 determine how these characteristics are modified when preparing blends, five different
20 plant species were selected: *Erica australis* L., *Genista tridentata* L., *Melissa officinalis,*
21 L., *Mentha spicata* L., and *Prunella vulgaris* L.. Infusions prepared from each plant
22 species and from three selected mixtures were analyzed in terms of nutritional value,
23 phenolic composition, and bioactive properties (antioxidant, antimicrobial, cytotoxic, and
24 anti-inflammatory activity). The major detected compound in *M. officinalis*, *M. spicata*,
25 and *P. vulgaris* infusions was rosmarinic acid, whilst in *E. australis* and *G. tridentata*
26 flavonoid derivatives such as quercetin and genistein were identified. *P. vulgaris* and *M.*
27 *officinalis* presented the best results in TBARS and OxHLIA assays, respectively. *M.*
28 *spicata* and all mixtures presented anti-inflammatory activity. *M. spicata* showed the best
29 cytotoxic properties and antimicrobial activity, and none of the infusions showed
30 hepatotoxicity for non-tumour cells.

31

32 *Keywords:* Plant infusions; plant mixtures; phenolic compounds; bioactive properties.

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34 **1. Introduction**

35 Herbal teas, particularly infusions with both medicinal and nutritional purposes, have a
36 long history of traditional use among different cultures and regions. There are numerous
37 aromatic and medicinal plants consumed by people over the centuries and referenced in
38 folk medicine for the treatment of various diseases. In the last decades, science has been
39 proving the bioactive characteristics of several popular plants also recognized for their
40 aromatic properties and characteristic flavor[1-3]. Some studies have been mixing different
41 species of plants in order to study the possible synergic effect, and intent to increase the
42 nutritional levels as well as the aromatic properties and flavour[4, 5]. These plant species are
43 featured by the production of a significant and diversified number of secondary
44 metabolites, particularly the phenolic compounds, which has been attracting great
45 research interest due to their potential commercial value in several areas, such as food,
46 textile and pharmaceutical industries[6]. One of the most studied features in natural matrices
47 is their chemical composition, since the content in phytochemicals is correlated with
48 different biological properties, in particular its antioxidant activity. These compounds
49 present outstanding actions in the prevention of diverse diseases like cancer, infections,
50 and degenerative diseases[7]. More recently, several studies have been conducted in order
51 to explore the potential application of plant extracts in different food matrices with the
52 objective of, not only extend their shelf life, but also functionalize them, bringing benefits
53 to consumers' health[8, 9]. Taking into account the widespread consumption of different
54 herbal teas by the general population and all the beliefs of the folk medicine related to
55 their consumption, it becomes interesting, from a scientific point of view, to characterize
56 each one regarding their nutritional and chemical properties as well as to study their
57 bioactivity, for consumers' elucidation. Additionally, with the growing interest of food
58 and pharmaceutical industries, among others, in the search for natural matrices with

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59 potential of application in the development of new products, the knowledge and
60 validation of these plants becomes essential.
61 Considering all this interest and demand, this study aims the chemical characterization of
62 five plant species *Erica australis* L. (flowering tips), *Genista tridentata* L. (flowers),
63 *Melissa officinalis,* L. (leaves), *Mentha spicata* L. (inflorescences), and *Prunella vulgaris*
64 L. (leaves) and three mixtures using different combinations of these plants. In addition, it
65 was studied and compared some bioactive properties, such as the antioxidant,
66 antimicrobial, and anti-inflammatory activities, in order to further expanded the scientific
67 knowledge of each plant species *de per se*, but as well to understand eventual synergic
68 effects between these plants in innovative mixtures, which can improve the health
69 beneficial effects of consumers and the added value of these products.
70
71 **2. Material and Methods**
72 **2.1 Samples**
73 Dry samples of each plant material from the studied species were provided by Ervital®,
74 an organic and sustainable farming company based in Castro Daire (Portugal).
75 The samples consisted of: *Erica australis* L. flowering tips, e.g. pink to purple bell-shaped
76 flowers in late Spring with few tiny needle-like leaves; *Melissa officinalis* L. fully
77 expanded leaves; *Mentha spicata* L. inflorescences, e.g. bracts, axes and flowers in
78 slender spikes, in anthesis; *Genista tridentata* L. flowers, e.g. masses of small, pea-like
79 yellow blooms in late Spring; and *Prunella vulgaris* L. fully expanded leaves (**Figure 1**).
80 Botanical identification was confirmed by professor Ana Maria Carvalho of the
81 Polytechnic Institute of Bragança (Trás-os-Montes, Portugal).
82 Plant mixtures were prepared following the proportions and combinations based on folk
83 uses and the sensory characteristics of the selected species: mixture 1: 20% *P. vulgaris*,

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84 40% *G. tridentata*, and 40% *M. spicata*; mixture 2: 30% *E. australis*, 30% *P. vulgaris*,
85 and 40% *M. officinalis*; and mixture 3: 30% *P. vulgaris*, 35% *M. officinalis*, and 35% *M.*
86 *spicata*.
87
88 **2.2 Infusions preparation**
89 For the preparation of the infusions it was followed, a protocol described by Pereira et al.
90 [10]. For each infusion, 1 g of dry sample was added to 200 mL of boiled distilled water and
91 left to stand at room temperature during 5 min.
92 The infusions of the plant mixtures were prepared following the extraction conditions:
93 mixture 1 (10 g/L, 90ºC, 7-9 min), mixture 2 (10 g/L, 85ºC, 7-9 min) and mixture 3 (4
94 g/L, 80ºC, 5 min).
95 All the samples were filtered through Whatman No. 4 paper, frozen at -20 ºC and freeze-
96 dried afterwards to obtain a dry extract.
97
98 **2.3 Nutritional value**
99 The infusions were analysed for their content in fat, carbohydrates, ash, protein, and
100 energetic value using recommended AOAC procedures [11]. The free sugars were analysed
101 by HPLC (Knauer, Smartline system 1000) coupled to a refractive index detector (RI
102 detector, Knauer Smartline 2300) according to the method described by Barros et al. [12].
103 Quantification was achieved using calibration curves obtained for the individual sugars
104 using the internal standard method (IS, melezitose). The results were expressed in g per
105 100 mL of infusion. The energetic value was calculated according to the following
106 equation: Energy (cal) = 4 × (mg proteins + mg carbohydrates) + 9 × (mg lipids).
107
108 **2.4 Phenolic compounds composition**

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109 The dry extracts were re-suspended in aqueous ethanol (50%, v/v), at a concentration of
110 10 mg/mL, and filtered (0.2 µm). The phenolic profile of the infusions was determined
111 by liquid chromatography (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose,
112 CA, USA), with diode-array detector (280, 330 and 370 nm wavelengths) linked to an
113 electrospray ionization mass spectrometry working in negative mode (Linear Ion Trap
114 LTQ XL, Thermo Scientific, San Jose, CA, USA)[13]. The phenolic compounds were
115 identified according to their chromatographic characteristics, by comparison to the
116 obtained standard compounds and with literature. For quantitative analysis, were used
117 calibration curves prepared with appropriate standards. The results were expressed in mg
118 per g of dry extract (mg/g) as mean ± standard deviation of three independent analyses.
119
120 **2.5 Evaluation of biological activities**
121 *Antioxidant activity assays*. The lipid peroxidation inhibition in porcine (*Sus scrofa*) brain
122 homogenates was evaluated by the decrease in thiobarbituric acid reactive substances
123 (TBARS) following the protocol described by Pinela et al. [14]. The results were expressed
124 in EC_{50} values (sample concentration providing 50% of antioxidant activity, µg/mL). The
125 anti-haemolytic activity of the extracts was evaluated by the oxidative haemolysis
126 inhibition assay (OxHLIA), as previously described by Lockowandt et al. [15]. The results
127 were expressed as the inhibitory concentration (IC_{50} value, µg/mL) able to promote a Δt
128 haemolysis delay of 60 and 120 min. Trolox was used as positive control for both assays.
129 *Anti-inflammatory activity:* The LPS-induced NO production by Murine macrophage
130 (RAW 264.7) cell lines was determined as nitrite concentration in the culture medium [16].
131 Dexamethasone (50 µM) was used as positive control and the results were expressed as
132 IC_{50} values (µg/mL).

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133 *Cytotoxic activity in tumour and non-tumour cells:* The cytotoxicity was determined
134 using four human tumour cell lines, HeLa (cervical carcinoma), HepG2 (hepatocellular
135 carcinoma), MCF-7 (breast adenocarcinoma), and NCI-H460 (non-small cell lung
136 cancer), and a non-tumour cells primary culture PLP2 (porcine liver) for hepatotoxicity.
137 A phase contrast microscope was used to monitor the growth of cell cultures, which were
138 sub-cultured and plated in 96-well plates (density of 1.0×10^4 cells/well). Dulbecco's
139 modified Eagle's medium (DMEM) supplemented with FBS (10%), penicillin (100 U/ml)
140 and streptomycin (100 μg/mL) were used[17]. The results were expressed as GI_{50} values
141 (sample concentration that inhibited 50% of the net cell growth, in μg/mL) and ellipticin
142 was used as positive control.
143 *2.5.2. Antimicrobial activity:* Antibacterial activity was evaluated according to a
144 previously described methodology[18] using Gram (+) bacteria (*Bacillus cereus* (food
145 isolate), and *Listeria monocytogenes* (NCTC 7973) as well as Gram (-) bacteria
146 (*Escherichia coli* ATCC 25922 and *Salmonella typhimurium* ATCC 13311). The
147 minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were
148 determined and streptomycin and ampicillin were used as positive controls. On the other
149 hand, the antifungal activity was evaluated following the protocol described by [19], using
150 *Aspergillus niger* (ATCC 6275), *Aspergillus versicolor* (ATCC 11730), *Penicillium*
151 *funiculosum* (ATCC 36839), and *Penicillium verrucosum* var. *cyclopium* (food isolate).
152 The MIC and minimum fungicidal concentration (MFC) were determined. Ketokonazole
153 and bifonazole were used as positive control. The microorganisms are deposited at
154 Mycological laboratory, Department of Plant Physiology, Institute for Biological
155 Research "Siniša Stanković", University of Belgrade, Serbia.
156

157 ***2.6.*** **Statistical analysis**

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158 For each species, three samples were analyzed and all the assays were carried out in
159 triplicate. The results were analyzed using one-way analysis of variance (ANOVA) post-
160 hoc Tukey and are expressed as mean values and standard deviation (SD). When less than
161 three samples were present, the results were analysed by *t*-Student test, with p = 0.05.
162 This treatment was carried out using the SPSS v.22.0 program.
163
164
165 ## *3.* Results and Discussion
166 ### *3.1. Nutritional Composition*
167 In the present study, five plant species and threes containing different proportions of these
168 plants were assessed. The blends were prepared according to the sensory characteristics
169 aimed for each one, bearing in mind that smells and tastes can lead us to places or
170 memories. Thus, mixture 1 was created to drink as hot as possible, promoting the
171 memories of the countryside, containing 40% of *G. tridentata* and *M. spicata* with a
172 smaller amount of *P. vulgaris* (20%). Mixture 2 invokes mountain features, combining
173 the roughness and a fairly bitter taste of *E. australis,* with the gentle flavor of *P. vulgaris*
174 (30%) and the refreshing sweetness of *M. officinalis* (40%). Finally, mixture 3 brings us
175 to a unique sensation through the mixture of *P. vulgaris* (30%), *M. officinalis* (35%) and
176 *M. spicata* (35%) in close proportions, taking advantage of their profusely aromatic
177 profile and long history of traditional use.
178 Since the results of our analyses on the nutritional composition of individual infusions
179 and mixtures revealed an absence of ash, protein and fat, **Table 1** only shows the results
180 for free sugars and energy. While *P. vulgaris* infusion did not reveal any free sugars, *E.*
181 *australis* showed two molecules, fructose and glucose, while all the other individual
182 infusions and mixtures additionally presented sucrose in their composition. *G. tridentata*

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183 and *M. officinalis* infusions showed the highest content of total sugars (69±3 and 59±2
184 mg/100 mL, respectively), which is reflected in their greater energetic contribution
185 (276±16 and 236±9 cal/100 mL, respectively). On the other hand, *E. australis* that only
186 revealed two free sugars in its composition, was the infusion with the lowest total content
187 (16.0±0.3 mg/100 mL), followed by mixture 3 infusion (18.8±0.8 mg/100 mL), thus also
188 presenting the lowest energetic values (64±1 and 75±5 cal/100 mL, respectively). It was
189 observed that the mixtures have shown a reduced amounts of free sugars, which could be
190 caused by the synergism between plants. These results are in agreement with Pinela et al.
191 [20] and Dias et al. [21] who evaluated the nutritional composition of *G. tridentata* and *M.*
192 *officinalis*, respectively. Several studies have shown that the cultivation conditions
193 (temperature, soil, light exposure, and others) can cause considerable changes in the
194 chemical and nutritional composition of various plants [22]. This may justify the fact that
195 in a study developed by Carocho et al. [23], trehalose was detected in a *M. officinalis*
196 aqueous extract, in opposite to what happened in the present work. Moreover, it did not
197 reveal the presence of glucose, which in the present work appeared as the most abundant
198 free sugar [23]. On the other hand, in a study involving several plants, where *M. spicata* was
199 assessed as infusion and as condiment, the infusion composition in terms of free sugars
200 was similar to the same one obtained herein, while the dry plant also presented trehalose.
201 Regarding the infusion of *M. spicata*, although presenting a similar relevance of each
202 sugar content in the total amount, with sucrose as the major sugar and very similar
203 contributions of fructose and glucose, the total free sugars content detected in the present
204 study was much higher (28.1±0.4 mg/100 mL) than that reported in the referred study
205 (13.20±0.85 mg/100 mL) [24], which can possibly be explained by the different origin or
206 harvest conditions.

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207 Regarding the blends, mixture 1 showed the major amount of fructose (27.6±0.3 mg/100
208 mL), followed by mixture 2 (12.0±0.7 mg/100 mL) and finally mixture 3 (4.7±0.4 mg/100
209 mL). The values found offer a combined balance between sweet/bitter flavor, especially
210 in mixture 1, which has a greater amount of *G. tridentata.* Mixture 2 showed the highest
211 levels of glucose (7.95 ± 0.07 mg/100 mL). Mixture 3 revealed the lowest content in
212 sugars (18.8 ± 0.8 mg/100 mL), which could be the most recommended blend for
213 consumers with diabetes, even so, its use should be with caution.
214
215 *3.2. Phenolic compounds characterization*
216 **Table 2** presents the peak characteristics (retention time, wavelength of maximum
217 absorption and mass spectral data) and tentative identification of the phenolic compounds
218 present in the infusions of *Erica australis* L., *Genista tridentata* L.*, Melissa officinalis,*
219 L. *Mentha spicata* L., and *Prunella vulgaris* L. and for the tree mixtures. In turn, the
220 quantification of these compounds obtained by HPLC-DAD analysis is presented in
221 **Table 3**. An exemplification phenolic profile is shown in **Figure 2**.
222 *E. australis* revealed the presence of thirteen compounds (peaks 5, 6, 7, 16, 20, 23, 26,
223 33, 40, 42, 45, 47 and 49; **Table 2**), being some of the identified molecules (peaks 16, 20,
224 23, 26, and 40) previously identified by some of the authors in a similar plant species
225 (*Calluna vulgaris* (L.) Hull), obtained by using different organic solvents and aqueous
226 extracts (infusion and decoctions) [25]. However, in the present work eight new compounds
227 were detected, among them two phenolic acids (namely peaks 5 and 7) and six flavonoids
228 (6, 33, 42, 45, 47 and 49), being all these compounds previously identified in other *Erica*
229 species [26-28]. The major group of compounds present in this sample were flavonoids, being
230 acetylquercetin-*O*-rhamnoside (12.7±0.1 mg/g extract) the main molecule.

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231 *G. tridentata* revealed the presence of fifteen flavonoids (peaks 1, 4, 8, 12, 21, 23, 26, 28,
232 _ENREF_531, 35, 38, 46, 48, 51, and 52 **Table 2**), especially isoflavones, flavonols and
233 flavanonols. Our research group has previously studied this plant species revealing a very
234 similar phenolic profile [29], with the exception of two compounds (peaks 8 and 12), which
235 were tentatively identified as genistein derivatives, such as genistein-*O*-dihexoside (peak
236 8) and genistein derivative (peak 12, no full identification of this compound was
237 obtained). According to [30], isoflavonoids are among the main secondary metabolites of
238 Genista *species.* Nevertheless, dihydroquercetin-6-*C*-hexoside (45±1 mg/g extract) was
239 the main compound present in *G. tridentata*.

240 Additionally, ten phenolic compounds were detected in *M. officinalis* infusion (9-11, 19,
241 27, 32, 36, 37, 39, and 41; **Table 2**), nine of which phenolic acids and one flavonoid. A
242 similar phenolic profiles has been previously identified by us in *M. officinalis* aqueous
243 (infusions and decoction) and hydroalcoholic extracts [23, 31, 32]. Thus, all compounds were
244 identified accordingly and likewise, rosmarinic acid (53.9±0.5 mg/g extract) was also the
245 main phenolic compound present in this studied plant species.

246 *M. spicata* presented fourteen phenolic compounds in its profile (3, 9, 14, 15, 22-24, 27,
247 29, 32, 34, 36, 37, and 43; **Table 2**), being eight tentatively identified as phenolic acids,
248 five flavonoids and one lignan. Some of the authors have previously studied this species
249 (infusion and hydroalcoholic extracts) and the herein studied sample presented a similar
250 phenolic composition [33, 34], thus three new compounds were identified (peaks 14, 34 and
251 37). These compounds were tentatively identified as salvianolic acid I, medioresinol-*O*-
252 hexoside and lithospermic acid A, respectively. Compounds 14 and 37 showed the same
253 pseudomolecular ion ($[M-H]^-$ at *m/z* 537), thus their fragmentation pattern match the
254 structure of different compounds, as previously identified by [23, 31, 32, 35, 36]. Peak 34 ([M-
255 H]$^-$ at *m/z* 549) yielded a fragment at *m/z* 387, corresponding to a medioresinol, losing a

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256 hexosyl moiety, therefore being assigned as medioresinol-*O*-hexoside, as also by the fact
257 that this compound was previously reported in *Mentha* species [37]. Rosmarinic acid (90±2
258 mg/g extract) was also the main compound present in this sample.
259 Twelve phenolic compounds were detected in *P. vulgaris* (2, 9, 13, 17, 18, 21, 25, 27, 30,
260 32, 37 and 50 **Table 2**), nine phenolic acids, three flavonoids, and one unknown
261 compound. The phenolic composition of this plant species was not previously identified
262 by us; nevertheless, peaks 9, 21, 27, 32, and 37 were identified in the other four studied
263 samples, therefore the assumption were also taken into account for *P. vulgaris*, being
264 identified as caffeic acid, quercetin-3-*O*-rutinoside, sagerinic acid, rosmarinic acid, and
265 lithospermic acid A isomer 3, respectively. Additionally, peaks 2, 13, 18, 25, 37, and 50
266 were also tentatively identified as caffeic acid derivatives (glycoside, caffeic acid dimers
267 and trimers), while peaks 30 and 17 were assigned as flavonoid derivatives. Peak 2 ([M-
268 H]$^-$ at *m/*z 341) released a MS^2 fragment at *m/z* 179 (caffeic acid), presenting the loss of
269 -162u (loss of hexosyl moiety), being identified as caffeic acid hexoside. Peak 13 and 37
270 ([M-H]$^-$ at *m/z* 537) were associated to caffeic acid trimers, taking into account the
271 findings mentioned in the other studied samples [23, 31, 32, 36, 38], these compounds were
272 identified as caffeic acid trimer and lithospermic acid A, respectively. Peak 18 ([M-H]$^-$ at
273 *m/z* 571) releasing various fragments characteristics to those described for yunnaneic acid
274 E [23, 39], identity that was tentatively associated to this compound. Peak 25 ([M-H]$^-$ at *m/z*
275 717) presented the same fragmentation patter as peak 19 (salvianolic acid B isomer 1),
276 which was previously identified in *M. officinalis*, thus it was tentatively assigned as
277 salvianolic acid B isomer 2. Peak 50 ([M-H]$^-$ at *m/z* 715), revealed a MS^2 fragment at *m/z*
278 535, which might be attributed to the loss of caffeic acid (-180 mu). Further loss of 44
279 mu (carboxyl group moiety) from the ion at *m/z* 535 would release the fragment at *m/z*
280 491 (salvianolic acid C). Regardless of these observations, no definite structure could be

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281 assigned to this peak, thus it was tentatively assigned as salvianolic acid C derivative.
282 Peak 30 ($[M-H]^-$ at *m/z* 549) released a MS^2 fragment at *m/z* 301 ($[M-H-162-86]^-$, loss of
283 a malonylhexoside moiety), being tentatively identified as quercetin-*O*-malonylhexoside.
284 Peak 17 ($[M-H]^-$ at *m/z* 555) could be assigned as a apigenin derivative owing to the
285 product ion observed at *m/z* 269 and UV spectra (λ_{max} around 334 nm), hence, the exact
286 identity of this compound was not achieved.
287 Twenty one phenolic compounds (1, 2, 4, 9, 12, 13, 15, 17, 21-24,25, 27, 29, 32, 37, 43,
288 48, 50 and 52; **Table 2**) were detected in mixture 1 (20% *P. vulgaris*, 40% *G. tridentata,*
289 40% *M. spicata*), fourteen (2, 9-11, 17, 19, 25, 28, 32, 36, 37, 39, 41 and 50; **Table 2**) in
290 mixture 2 (30% *E. australis*, 30% *P. vulgaris*, 40% *M. officinalis*), and eighteen (3, 9, 10,
291 13, 17, 19, 21, 22, 24, 25, 27, 32, 36, 37, 39, 41, 43 and 50; **Table 2**) in mixture 3 (30%
292 *P. vulgaris*, 35% *M. officinalis*, 35% *M. spicata*). In common, all these mixtures present
293 in their composition *P. vulgaris*, which may justify some similarities found in the
294 mixtures, namely the presence of some phenolic compounds. The major compound
295 detected in all mixtures was rosmarinic acid with 40.3±0.9, 52±2, and 65.0±0.4 mg/g
296 extract in mixtures 1, 2, and 3, respectively. Caffeic, sagerinic and rosmarinic acids were
297 present not only in *P. vulgaris*, but also in the three mixtures, which can be justified by
298 the presence of this plant in all mixtures. Overall, *M. spicata* infusion presented the
299 highest total phenolic compounds amount (164±2 mg/g extract), with the highest
300 contribution of phenolic acids (127±1 mg/g extract), whereas *G. tridentata* showed the
301 highest concentration of flavonoids (107±2 mg/g extract) and did not present phenolic
302 acids in its composition. In the mixtures, the highest values of total flavonoids and
303 phenolic compounds were found in mixture 1 (68±1 and 128±1 mg/g extract,
304 respectively), and mixture 3 revealed to be the most concentrated in phenolic acids
305 (98.3±0.1 mg/g extract).

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307 *3.3. Bioactive properties*

308 Regarding bioactive properties, the results of the antioxidant and anti-inflammatory
309 activity and cytotoxicity of each analysed sample are presented in **Table 4**. The
310 antioxidant activity of the infusions was evaluated by two methods: the lipid peroxidation
311 inhibition (TBARS) and the oxidative haemolysis inhibition (OxHLIA). In the TBARS
312 assay, *P. vulgaris* infusion presented the lowest EC_{50} value (2.56±0.04 μg/mL), thus
313 presenting the highest antioxidant activity, followed by *M. spicata* and *E. australis*
314 (4.2±0.1 and 4.4±0.2 μg/mL, respectively). Regarding mixtures, as expected, the EC_{50}
315 values were higher than the one revealed by *P. vulgaris* infusion and lower than those
316 presented by single plant infusions, once all mixtures are partially composed by this plant,
317 which possesses a strong activity. This fact can also help explaining the lowest
318 antioxidant capacity of mixture 1, since it is the one containing the lowest proportion
319 (20%) of *P. vulgaris*, along with the fact that this is the only mixture containing the less
320 active plant, *G. tridentata*.

321 Regarding OxHLIA assay, nor *P. vulgaris* nor the mixtures showed activity. *M. officinalis*
322 exhibited the highest antioxidant activity, with the lowest EC_{50} values (24.8±0.3 and
323 46.6±0.4 μg/mL, for 60 and 120 min respectively). *M. spicata* and *G. tridentata* revealed
324 a similar activity for 60 min of haemolysis delay (38.3±0.6 and 37.7±0.9 μg/mL,
325 respectively), but *M. spicata* was not able to protect the erythrocytes population for 120
326 min. On the other hand, despite the lower activity of *E. australis* compared to this latest,
327 it presented the capacity of delaying oxidative haemolysis for 120 min (230±11 μg/mL).
328 It is interesting to note that the activity of the mixtures seems once again related to that
329 of *P. vulgaris*, once none of these infusions revealed inhibition capacity. The antioxidant
330 capacity of *E. australis* aqueous extracts was also previously reported by Nunes and

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331 Carvalho [40], who obtained in radical scavenging assay the IC_{50} values of 296.8 ± 8.8
332 µg/mL.
333 The anti-inflammatory activity of all the samples tested are presented in **Table 4**. From
334 the analysis of the presented results it is possible to verify that in the individual plant
335 species only the infusion prepared from *M. spicata* showed anti-inflammatory activity.
336 Concerning the mixtures, only mixture 1 did not show anti-inflammatory activity, being
337 mixture 2 the most active one (241±3 µg/mL), which was unexpected, once this is the
338 only mixture that did not present *M. spicata* in its composition. Moreover, even though
339 none of the remaining individual infusions presented anti-inflammatory activity, mixtures
340 2 and 3 showed better results. In the case of mixture 3, this activity could be ascribed to
341 its 35% of *M. spicata*, nevertheless, it could not explain the remaining results, which
342 clearly suggests synergistic effects occurring in the mixtures. On the other hand, the
343 activity of the mixtures can also be related to rosmarinic acid present in *M. spicata*, *M*
344 *officinalis* and *P. vulgaris*, which showed, in previous studies, anti-inflammatory activity
345 [41, 42].
346 In the cytotoxic activity assay, which results are presented in **Table 4**, none of the
347 infusions showed toxicity for non-tumour cells (PLP2). *M. spicata* infusion was the only
348 one presenting toxicity in all tumour cell lines, in concentrations ranging from 251±6 to
349 322±15 µg/mL, which, apart from that, was only observed for mixture 3 (249±6 to
350 292±11 µg/mL). On the other hand, *E. australis* and *P. vulgaris* only showed
351 antiproliferative activity against HepG2 (278±21 µg/mL) and HeLa (359±10 µg/mL) cell
352 lines, respectively. Except for MCF-7 cell line, in which neither mixture 1 nor mixture 2
353 revealed activity, and HepG2, where mixture 2 revealed a lower GI_{50} value, the results
354 obtained for the mixtures were not statistically different, meaning similar cytotoxic
355 activity. These results are in accordance with Berdowska et al. [43], who obtained more

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356 toxic against the MCF-7. Previous studies performed on plants of the Lamiaceae family
357 characterized them as containing high amounts of rosmarinic acid, presenting
358 antioxidant, antimicrobial, and antitumour properties [42].
359 *M. officinalis* is one of the best-known plants in the Lamiaceae family and has been used
360 since ancient times in traditional medicine because it has been demonstrating
361 antibacterial, antifungal, and anti-inflammatory effects capable of acting at the level of
362 several diseases [31, 44]. For this reason, it has been extensively studied, and its bioactivity
363 has been associated with the presence of phenolic compounds, namely rosmarinic acid [45].
364 *M. spicata* is also a plant of the same family consumed worldwide that is described in the
365 literature as being an important source of antioxidants recognized for its beneficial
366 properties for consumer's health [46], often attributed to rosmarinic acid that stands out as
367 being the most abundant phenolic compound in this herb [33]. Finally, **Table 5** presents the
368 results of the antimicrobial activity against a panel of bacteria and fungi selected
369 according to their importance in public health. In general, *M. spicata* was the sample that
370 showed the lowest MIC values for bacteria (both gram-negative and gram-positive),
371 presenting even lower MBC (0.5 mg/mL) than ampicillin (1.20 mg/mL) for *Salmonella*
372 *Typhimurium*. For gram-positive bacteria, *E. australis* presented the same MIC and MBC
373 values as *M. spicata* (0.5 and 1 mg/mL, respectively). These values are comparable to
374 those obtained with the infusions of the same species in a recent research work [47].
375 Regarding mixtures, mixture 3 clearly stands out for providing the worst antibacterial
376 activity, with the highest MIC and MBC values (4 and 8 mg/mL, respectively). Relatively
377 to antifungal activity (**Table 5**), it is possible to verify that *E. australis* and *M. spicata*
378 presented, generally, the lowest MIC and MFC values, similar to those obtained for
379 ketoconazole for some tested fungi. Thus, *M. spicata* can be pointed out as the most active
380 both in antibacterial and antifungal assays. These results are in agreement with those

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381 described in the literature that highlight the plants of the Lamiaceae family for presenting
382 high antimicrobial properties[48].
383

384 **4. Conclusions**

385 Overall, the results obtained in the present study highlight the interest of these plant
386 infusions in diets, by demonstrating their richness in bioactive molecules such as phenolic
387 acids and flavonoids. Moreover, the enhanced biological activities and chemical
388 composition of blends prepared with different plants and different parts of the plants,
389 might be an asset in the choice of the best mixture for infusion preparation. On the other
390 hand, the addition of these plant extracts to foodstuff can also bring benefits as increased
391 nutritional value and shelf life.
392

393 **Conflict of Interest**

394 The authors state no conflict of interest regarding this manuscript.
395

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Figure 1. Morphological features of dried plant samples and respective mixtures studied provided by Ervital®.

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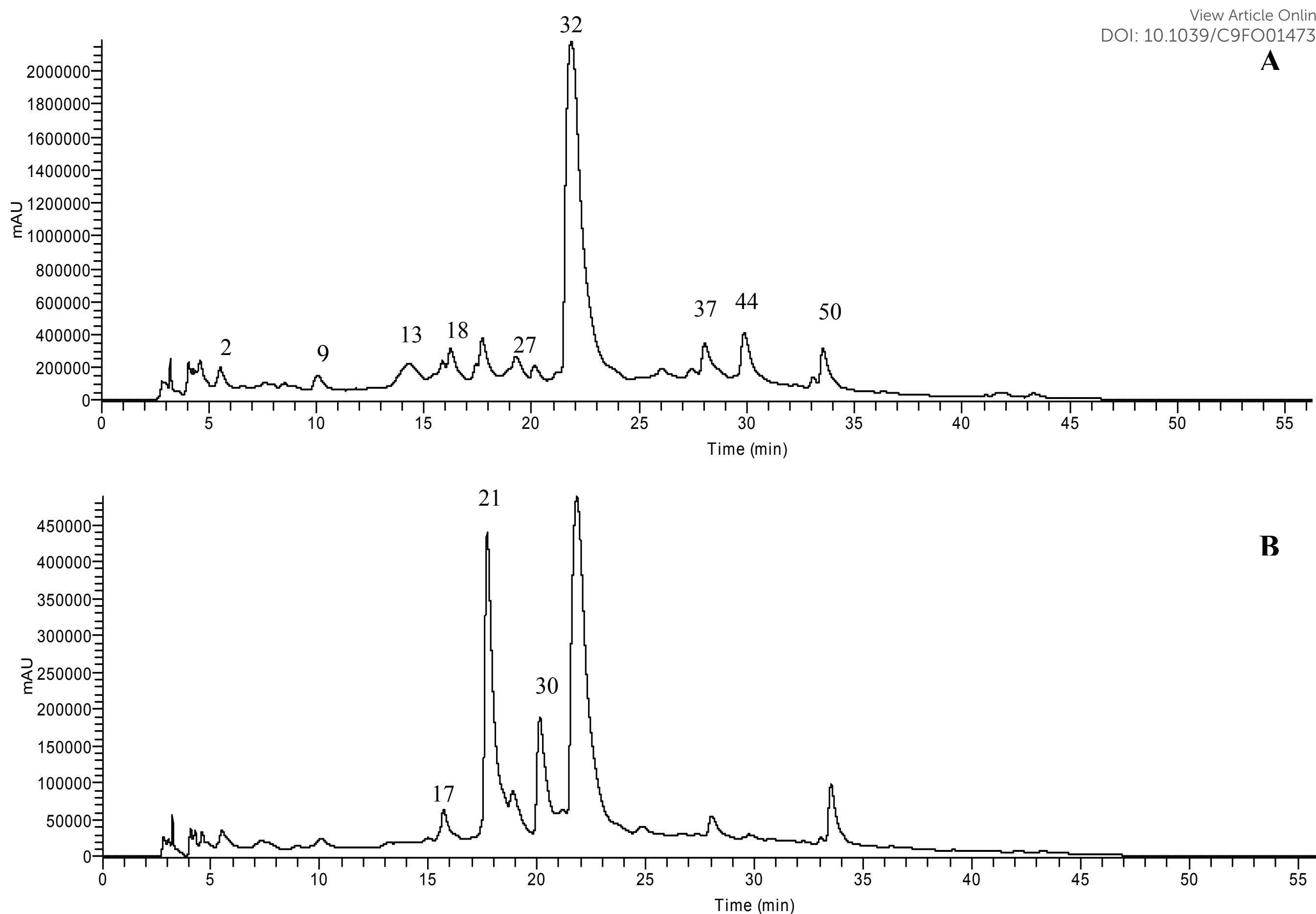

Figure 2. Phenolic profile of *Prunella vulgaris* recorded at 280 nm (A), 370 nm (B). Peak numbering is indicated as defined as in **Table 1.** Nutritional value, free sugars (mg/100 mL) and energy (cal/100 mL) of *E. australis, M. officinalis, M. spicata, P. vulgaris*, *G. tridentata,* and mixtures 1, 2, and 3 infusions (mean±SD).

	E. australis	*G. tridentata*	*M. officinalis*	*M. spicata*	*P. vulgaris*	Mi
Free sugars (mg/100 mL)						
Fructose	9.15 ± 0.07^{c}	47 ± 1^{a}	12.8 ± 0.9^{b}	5.6 ± 0.4^{d}	nd	27.
Glucose	6.8 ± 0.4^{b}	9.9 ± 0.5^{a}	6.2 ± 0.4^{b}	5.25 ± 0.07^{c}	nd	6.
Sucrose	nd	12 ± 1^{c}	40.1 ± 0.9^{a}	17.2 ± 0.9^{b}	nd	14.
Total	16.0 ± 0.3^{d}	69 ± 3^{a}	59 ± 2^{b}	28.1 ± 0.4^{c}	nd	48.
Carbohydrates (mg/100 mL)	16.0 ± 0.3^{d}	69 ± 3^{a}	59 ± 2^{b}	28.1 ± 0.4^{c}	nd	48.4
Energy (cal/100 mL)	64 ± 1^{d}	276 ± 16^{a}	236 ± 9^{b}	112 ± 4^{c}	nd	193

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Protein, ash and fat contents were zero; carbohydrates content was obtained by the total of sugars; results expressed as medium value ± standard deviation (SD), nd – not detected. Mixture 1: 20% *P. vulgaris*, 40% *G. tridentata,* 40% *M. spicata;* Mixture 2: 30% *E. australis*, 30% *P. vulgaris*, 40% *M. officinalis*; Mixture 3: 30% *P. vulgaris*, 35% *M. officinalis*, 35% *M. spicata*. The statistical treatment was performed comparing the individual plants (small letters) and comparing the mixtures (capital letters), therefore in each row different letters mean statistically significant differences ($p<0.05$).

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Table 2.

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Table 1. Nutritional value, free sugars (mg/100 mL) and energy (cal/100 mL) of *E. australis, M. officinalis, M. spicata, P. vulgaris*, *G. tridentata,* and mixtures 1, 2, and 3 infusions (mean±SD).

	E. australis	***G. tridentata***	***M. officinalis***	***M. spicata***	***P. vulgaris***	**Mixture 1**	**Mixture 2**	**Mixture 3**
Free sugars (mg/100 mL)								
Fructose	9.15 ± 0.07^{c}	47 ± 1^{a}	12.8 ± 0.9^{b}	5.6 ± 0.4^{d}	nd	27.6 ± 0.3^{A}	12.0 ± 0.7^{B}	4.7 ± 0.4^{C}
Glucose	6.8 ± 0.4^{b}	9.9 ± 0.5^{a}	6.2 ± 0.4^{b}	5.25 ± 0.07^{c}	nd	6.1 ± 0.7^{B}	7.95 ± 0.07^{A}	1.05 ± 0.01^{C}
Sucrose	nd	12 ± 1^{c}	40.1 ± 0.9^{a}	17.2 ± 0.9^{b}	nd	14.7 ± 0.4^{B}	16.6 ± 0.5^{A}	13.0 ± 0.2^{C}
Total	16.0 ± 0.3^{d}	69 ± 3^{a}	59 ± 2^{b}	28.1 ± 0.4^{c}	nd	48.4 ± 0.8^{A}	36.6 ± 0.1^{B}	18.8 ± 0.8^{C}
Carbohydrates (mg/100 mL)	16.0 ± 0.3^{d}	69 ± 3^{a}	59 ± 2^{b}	28.1 ± 0.4^{c}	nd	48.4 ± 0.8^{a}A	36.6 ± 0.1^{B}	18.8 ± 0.8^{C}
Energy (cal/100 mL)	64 ± 1^{d}	276 ± 16^{a}	236 ± 9^{b}	112 ± 4^{c}	nd	193.6 ± 0.6^{A}	146.2 ± 0.6^{B}	75 ± 5^{C}

Protein, ash and fat contents were zero; carbohydrates content was obtained by the total of sugars; results expressed as medium value ± standard deviation (SD), nd – not detected. Mixture 1: 20% *P. vulgaris*, 40% *G. tridentata,* 40% *M. spicata;* Mixture 2: 30% *E. australis*, 30% *P. vulgaris*, 40% *M. officinalis*; Mixture 3: 30% *P. vulgaris*, 35% *M. officinalis*, 35% *M. spicata*. The statistical treatment was performed comparing the individual plants (small letters) and comparing the mixtures (capital letters), therefore in each row different letters mean statistically significant differences ($p<0.05$).

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Table 2. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and identification of phenolic compounds in *E. australis, M. officinalis, M. spicata, P. vulgaris*, *G. tridentata,* and mixtures 1, 2, and 3 infusions.

Peak	Rt	λmax	[M-H] m/z	MS2	Tentative Identification	References
1	4.59	292,342sh	465	447(25),375(79),357(21),345(100),327(86),317(42),167(63)	Dihydroquercetin 6-*C*-hesoxide	29
2	5.53	327	341	179(31),135(100)	Caffeic acid hexoside	49
3	6.54	328	353	191(38),179(75),173(100),161(5),135(68)	4-*O*-Caffeoylquinic acid	33
4	6.81	294,348sh	465	447(7),375(54),357(13),345(100),327(50),317(23),167(38)	Dihydroquercetin-6-C-hesoxide	29
5	8.45	310	337	191(10),173(100),163(21),155(5),137(5),119(3)	*cis* 4-*p*-Coumaroylquinic acid	50
6	9.69	280	289	245(100)	(-)-Epicatechin	50
7	9.72	310	337	191(10),173(100),163(21),155(5),137(5),119(3)	*trans* 4-*p*-Coumaroylquinic acid	50
8	9.82	260,332sh	593	431(100),269(10)	Genistein-*O*-dihexoside	51
9	10.03	324	179	135(100)	Caffeic acid	52
10	12.63	288,326sh	537	493(100),359(33),313(5),295(3)	Caffeic acid trimer	52
11	13.67	330	439	359(10), 179(8),161(40),135(28)	Sulphated rosmarinic acid	52
12	13.75	260,332sh	413	311(100), 269(10)	Genistein derivate	51
13	14.27	278,324sh	537	493(67), 359(17), 313(31), 295(100),269(26),197(20),179(77)	Caffeic acid trimer	53
14	14.66	286,324sh	537	493(20), 339(100),285(10)	Salvianolic acid I	35
15	15.30	284,336sh	595	287(100)	Eriodictyol-*O*-deoxyhexosylhexoside	33
16	15.38	356	479	317(100)	Myricetin-3-*O*-glucoside	25
17	15.71	334	555	537(22),511(100),449(40),357(10),313(9),269(12)	Apigenin derivative	54
18	16.24	286,324sh	571	537(36),511(100),493(8),449(32)	Yunnaneic acid E	23
19	17.42	286,324sh	717	537(4), 519(100), 493(7), 359(18), 339(14)	Salvianolic acid B isomer 1	52
20	17.52	349	463	317(100)	Myricetin-*O*-rhamnoside	25
21	17.72	348	609	301(100)	Quercetin-3-*O*-rutinoside	29
22	18.16	348	593	285(100)	Luteolin-7-*O*-rutinoside	33
23	18.55	356	463	301(100)	Quercetin-3-*O*-glucoside	25, 29, 33;
24	18.65	348	461	285(100)	Luteolin-7-*O*-glucuronide	33
25	18.90	286,324sh	717	537(4),509(100),493(7),359(98),339(84),321(5),295(12),197(9),179(16)	Salvianolic acid B isomer 2	52

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26	18.94	356	463	301(100)	Quercetin-*O*-hexoside	25, 29;
27	19.49	284,328sh	719	539(17),521(15),359(100),197(22),179(26),161(81),135(7)	Sangerinic acid	7, 31
28	19.87	260,328sh	431	311(5),269(30)	Genistein-8-*C*-glucoside	51
29	20.01	284,338sh	717	537(21),519(54),493(21),339(24),321(27),313(9),295(100),277(18)	Salvianolic acid E	33
30	20.13	256,348sh	549	505(100),463(30),301(58)	Quercetin-*O*-malonylhexoside	55
31	21.05	260,336sh	461	446(35),341(5),299(88),283(29)	5,5'-Dihydroxy-3'-methoxy-isoflavone-7-*O*-β-glucoside	29
32	21.99	330	359	197(83),179(70),161(100),135(40)	Rosmarinic acid	23, 31-33
33	22.36	350	447	301(100)	Quercetin-*O*-rhamnoside	27
34	24.31	348	549	387(100)	Medioresinol-*O*-hexoside	56
35	24.65	256,322sh	505	459(3),297(100),282(22)	Methylbiochanin A/methylprunetin *O*-hexoside	29
36	25.05	350	461	285(100)	Luteolin-*O*-glucuronide	31, 33
37	25.49	288,326sh	537	493(57),359(13),313(27),295(100),269(27),197(19),179(78),135(45)	Lithospermic acid A	23, 31, 32
38	26.67	260,334sh	269	241(4),225(6),201(5),181(2),133(7)	Genistein	29
39	26.84	330	813	667(12),535(100),491(32),311(70), 293*, 179*	Salvianolic acid C derivative	52
40	27.04	341	431	285(100)	Kaempferol-*O*-rhamnoside	25, 57;
41	28.22	288,326sh	537	493(53),359(100),313(5),295(18),269(3),197(44),179(64)	Caffeic acid trimer	23, 31, 32, 58
42	28.33	347	489	447(37),301(100)	Acetylquercetin-*O*-rhamnoside isomer 1	27
43	29.49	324	493	359(48),313(5),295(4),197(15),179(19),161(100),135(4)	Salvianolic acid A	33
44	29.86	284,326sh	715	357(100),339(10),311(5),283(5)	Unknown	-
45	30.12	347	489	447(17),301(100)	Acetylquercetin-*O*-rhamnoside isomer 2	27
46	31.92	260,336sh	649	607(11),445(3),283(100)	Acetylbiochanin A *O*-hexoside-*O*-hexoside	29
47	32.37	342	473	285(100)	Acetylkaempterol-*O*-rhamnoside isomer 1	27
48	33.32	260,332sh	491	445(3),283(100)	Biochanin A *O*-hexoside	29
49	33.53	342	473	285(100)	Acetylkaempterol-*O*-rahmnoside isomer 2	50
50	33.62	288,320sh	715	535(100),491(37),311(92),293(4),179	Salvianolic acid C derivative	31, 32
51	35.85	262,332sh	283	268(100),239(7),224(5),195(2),135(2)	4-*O*-Methylgenistein (biochanin A)	29
52	36.44	262,334sh	283	268(100)	7-*O*-Methylgenistein (prunetin)	29

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Table 3. Phenolic compounds quantification (mg/g of extract) in *E. australis, M. officinalis, M. spicata, P. vulgaris*, *G. tridentata,* and mixtures 1, 2, and 3 infusions (mean±SD; mg/g extract).

Peak	Compounds	*E. australis*	*G. tridentata*	*M. officinalis*	*M. spicata*	*P. vulgaris*	Mixture 1	Mixture 2	Mixture 3
1	Dihydroquercetin 6-*C*-hesoxide	nd	45±1	nd	nd	nd	21.5±0.7	nd	nd
2	Caffeic acid hexoside	nd	nd	nd	nd	0.61±0.02	0.1541±0.0002*	0.101±0.004*	nd
3	4-*O*-Caffeoylquinic acid	nd	nd	nd	1.55±0.05	nd	nd	nd	0.22±0.01
4	Dihydroquercetin-6-C-hesoxide	nd	4.81±0.04	nd	nd	nd	2.0±0.1	nd	nd
5	*cis* 4-*p*-Coumaroylquinic acid	0.56±0.01	nd	nd	nd	nd	nd	nd	nd
6	(-)-Epicatechin	Traces	nd	nd	nd	nd	nd	nd	nd
7	*trans* 4-*p*-Coumaroylquinic acid	0.89±0.01	nd	nd	nd	nd	nd	nd	nd
8	Genistein-*O*-dihexoside	nd	1.43±0.02	nd	nd	nd	nd	nd	nd
9	Caffeic acid	nd	nd	0.44±0.01^{a}	0.65±0.005^{b}	0.6±0.2^{b}	0.40±0.01^{B}	0.104±0.003^{C}	0.445±0.001^{A}
10	Caffeic acid trimer	nd	nd	14.2±0.8	nd	nd	nd	8.6±0.4*	5.9±0.3*
11	Sulphated rosmarinic acid	nd	nd	6.2±0.3	nd	nd	nd	1.81±0.08	nd
12	Genistein derivate	nd	9.2±0.2	nd	nd	nd	3.601±0.001	nd	nd
13	Caffeic acid trimer	nd	nd	nd	nd	5.9±0.1	1.43±0.01*	nd	1.15±0.03*
14	Salvianolic acid I	nd	nd	nd	2.18±0.05	nd	nd	nd	nd
15	Eriodictyol-*O*-deoxyhexosylhexoside	nd	nd	nd	4.6±0.1	nd	0.99±0.03	nd	nd
16	Myricetin-3-*O*-glucoside	2.93±0.08^{a}	nd	nd	nd	nd	nd	nd	nd
17	Apigenin derivative	nd	nd	nd	nd	4.0±0.1	0.47±0.01^{C}	1.50±0.02^{A}	1.24±0.02^{B}
18	Yunnaneic acid E	nd	nd	nd	nd	3.0±0.1^{a}	nd	nd	nd
19	Salvianolic acid B isomer 1	nd	nd	11.2±0.6	nd	nd	nd	4.4±0.2*	3.4±0.2*
20	Myricetin-*O*-rhamnoside	1.12±0.03	nd	nd	nd	nd	nd	nd	nd
21	Quercetin-3-*O*-rutinoside	nd	0.98±0.05*	nd	nd	1.308±0.001*	0.69±0.03*	nd	1.37±0.04*
22	Luteolin-7-*O*-rutinoside	nd	nd	nd	9.4±0.5	nd	5.4±0.2*	nd	4.4±0.2*
23	Quercetin-3-*O*-glucoside	0.86±0.03^{c}	2.0±0.1^{b}	nd	2.7±0.1^{a}	nd	2.2±0.1	nd	nd
24	Luteolin-7-*O*-glucuronide	nd	nd	nd	13.52±0.01	nd	10.4±0.3*	nd	7.8±0.4*

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25	Salvianolic acid B isomer 2	nd	nd	nd	nd	3.01±0.09	nd	1.98±0.06*	0.78±0.01*
26	Quercetin-*O*-hexoside	0.9209±0.007*	2.21±0.02*	nd	nd	nd	1.01±0.02	nd	nd
27	Sangerinic acid	nd	nd	3.6±0.1^{b}	5.8±0.2^{a}	2.49±0.04^{c}	2.9±0.1^{C}	3.40±0.01^{B}	5.6±0.2^{A}
28	Genistein-8-*C*-glucoside	nd	4.4±0.2	nd	nd	nd	nd	nd	nd
29	Salvianolic acid E	nd	nd	nd	3.3±0.1	nd	1.77±0.03	nd	nd
30	Quercetin-*O*-malonylhexoside	nd	nd	nd	nd	1.375±0.005	nd	nd	nd
31	5,5'-Dihydroxy-3'-methoxy-isoflavone-7-*O*-β-glucoside	nd	2.219±0.004	nd	nd	nd	nd	nd	nd
32	Rosmarinic acid	nd	nd	53.9±0.4^{b}	89±2^{a}	53±2^{b}	40.3±0.9^{C}	52±2^{B}	65.0±0.4^{A}
33	Quercetin-*O*-rhamnoside	7.98±0.01	nd	nd	nd	nd	nd	nd	nd
34	Medioresinol-*O*-hexoside	nd	nd	nd	nq	nd	nd	nd	nd
35	Methylbiochanin A/methylprunetin *O*-hexoside	nd	7.7±0.1	nd	nd	nd	nd	nd	nd
36	Luteolin-*O*-glucuronide	nd	nd	1.5±0.1*	4.3±0.2*	nd	nd	2.3±0.1*	2.18±0.02*
37	Lithospermic acid A	nd	nd	3.7±0.2^{b}	11.8±0.5^{a}	1.65±0.06^{c}	3.52±0.03^{B}	2.74±0.02^{C}	5.5±0.2^{A}
38	Genistein	nd	3.73±0.05	nd	nd	nd	nd	nd	nd
39	Salvianolic acid C derivative	nd	nd	2.26±0.05	nd	nd	nd	0.451±0.004*	0.5529±0.0004*
40	Kaempferol-*O*-rhamnoside	3.0±0.1	nd	nd	nd	nd	nd	nd	nd
41	Caffeic acid trimer	nd	nd	14.6±0.7	nd	nd	nd	6.75±0.08*	5.9±0.2*
42	Acetylquercetin-*O*-rhamnoside isomer 1	1.71±0.04^{a}	nd	nd	nd	nd	nd	nd	nd
43	Salvianolic acid A	nd	nd	nd	11.7±0.2	nd	4.20±0.04*	nd	1.9±0.1*
44	Unknown	nd	nd	nd	nd	nq	nd	nq	nd
45	Acetylquercetin-*O*-rhamnoside isomer 2	12.7±0.1	nd	nd	nd	nd	nd	nd	nd
46	Acetylbiochanin A *O*-hexoside-*O*-hexoside	nd	0.862±0.01	nd	nd	nd	nd	nd	nd
47	Acetylkaempterol-*O*-rhamnoside isomer 1	1.28±0.03	nd	nd	nd	nd	nd	nd	nd
48	Biochanin A *O*-hexoside	nd	2.70±0.04	nd	nd	nd	1.7±0.2	nd	nd
49	Acetylkaempterol-*O*-rahmnoside isomer 2	0.89±0.03	nd	nd	nd	nd	nd	nd	nd
50	Salvianolic acid C derivative	nd	nd	nd	nd	2.9±0.1	0.93±0.03^{C}	1.44±0.07^{B}	1.8±0.1^{A}
51	4-*O*-Methylgenistein (biochanin A)	nd	1.08±0.03	nd	nd	nd	nd	nd	nd
52	7-*O*-Methylgenistein (prunetin)	nd	19.0±0.2	nd	nd	nd	15±1	nd	nd

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Total phenolic acids	1.45±0.02^{d}	nd	111±1^{b}	127±1^{a}	73±2^{c}	59.5±0.5^{C}	84±2^{B}	98.3±0.1^{A}
Total flavonoids	33.4±0.2^{c}	107±2^{a}	1.5±0.1e	35±1^{b}	6.7±0.1^{d}	68±1^{A}	3.8±0.1^{C}	17±1^{B}
Total phenolic compounds	34.8±0.2^{e}	107±2^{c}	112±1^{b}	164±2^{a}	80±1^{d}	128±1^{A}	88±2^{C}	115.4±0.4^{B}

nd: not detected. nq: not quantified. Mixture 1: 20% *P. vulgaris*, 40% *G. tridentata,* 40% *M. spicata;* Mixture 2: 30% *E. australis*, 30% *P. vulgaris*, 40% *M. officinalis*; Mixture 3: 30% *P. vulgaris*, 35% *M. officinalis*, 35% *M. spicata*. Calibration curves: taxifolin (y = 203766x – 208383, R^2=0.9999, peak 1 and 4); caffeic acid (y= 168823x – 161172, R^2=0.9939; peaks 2 and 9); chlorogenic acid (y = 168823x – 161172, R^2=0.9999; peak 3); *p*-coumaric acid (y = 301950x + 6966.7, R^2=0.9999; peaks 5 and 7); epicatechin (y = 10314x + 147331, R^2=0.9999; peak 6); naringenin (y=18433x+78903, R^2=0.9998; peaks 8, 12, 15, 28, 31, 35, 38, 46, 48, 51, and 52); quercetin-3-*O*-glucoside (y = 34843x – 160173, R^2=0.9998; peaks 16, 20, 21, 23 26, 30, 33, 40, 42, 45, 47, and 49); rosmarinic acid (y=191291x – 652903, R^2=0.9999; peaks 10, 11, 13, 14, 18, 19, 25, 27, 29, 32, 37, 39, 41, 43, and 50); and apigenina-7-*O*-glucoside (y = 10683x - 45794, R^2=0.9989; peaks 17, 22, 24, and 36). The statistical treatment was performed comparing the individual plants (small letters) and comparing the mixtures (capital letters), therefore in each row different letters mean statistically significant differences (p<0.05). *Mean statistical differences obtained by *t*-Student test.

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Table 4. Antioxidant, anti-inflammatory, and cytotoxic activity of *E. australis, M. officinalis, M. spicata, P. vulgaris*, *G. tridentata,* and mixtures 1, 2, and 3 infusions (mean±SD).

	E. australis	*M. officinalis*	*M. spicata*	*G. tridentata*	*P. vulgaris*	Mixture 1	Mixture 2	Mixture 3
Antioxidant activity (IC_{50}, µg/mL)								
TBARS	4.4± 0.2^{c}	$6.6±0.5^{b}$	$4.2±0.1^{c}$	$8.4±0.2^{a}$	2.56± 0.04^{d}	$3.79±0.08^{A}$	$3.1±0.1^{B}$	$3.33±0.03^{B}$
OxHLIA								
(Δt = 60 min)	$145±5^{a}$	$24.8±0.3^{c}$	$38.3±0.6^{b}$	$37.7±0.9^{b}$	n.a.	n.a.	n.a.	n.a.
(Δt = 120 min)	$230±11^{a}$	$46.6±0.4^{c}$	n.a.	$69±2^{b}$	n.a.	n.a.	n.a.	n.a.
Anti-inflammatory activity (IC_{50}, µg/mL)								
RAW 246.7	>400	>400	324±5	>400	>400	>400	$241±3^{*}$	$265±5^{*}$
Cytotoxic activity (GI_{50}, µg/mL)								
MCF-7	>400	>400	283±10	>400	>400	>400	>400	276±17
NCI-H460	>400	$290±9^{*}$	$322±15^{*}$	>400	>400	302±17	287±3	292±11
HeLa	>400	$241±4^{b}$	$251±6^{b}$	$242±10^{b}$	$359±10^{a}$	240±8	262±10	252±8
HepG2	$278±21^{b}$	$238±7^{b}$	$262±11^{b}$	$356±16^{a}$	>400	$255±15^{A}$	$166±10^{B}$	$249±6^{A}$
PLP2	>400	>400	>400	>400	>400	>400	>400	>400

Mixture 1: 20% *P. vulgaris*, 40% *G. tridentata,* 40% *M. spicata;* Mixture 2: 30% *E. australis*, 30% *P. vulgaris*, 40% *M. officinalis*; Mixture 3: 30% *P. vulgaris*, 35% *M. officinalis*, 35% *M. spicata*. EC_{50} values corresponded to the extract concentration that inhibits in 50% the oxidation and inflammatory process. Trolox (EC_{50} values): TBARS: 5.8 ± 0.6; OxHLIA (60 min): 19.6 ± 0.6., (120 min): 41.1 ± 0.8. Dexametasona (IC_{50} values): 16 ± 1. GI_{50} values correspond to the concentration that causes 50% inhibition of cell proliferation; n.d. - not detected; AGS - human gastric adenocarcinoma; MCF-7 - human breast adenocarcinoma; NCI-H460 - human lung carcinoma; HeLa - human cervix adenocarcinoma; HepG2 - hepatocellular carcinoma; PLP2 - primary culture of non-tumoral pig liver cells. Ellipticine (GI_{50} values). AGS: 2.59 ± 0.05; MCF-7: 1.21 ± 0.02; NCI-H460: 0.91 ± 0.11; HeLa: 1.03 ± 0.09; HepG2: 1.1 ± 0.09; PLP2: 2.29 ± 0.18. Results expressed in mean values ± standard deviation (SD). n.a: no activity. The statistical treatment was performed comparing the individual plants (small letters) and comparing the mixtures (capital letters), therefore in each row different letters mean statistically significant differences ($p<0.05$). 0.05). *Mean statistical differences obtained by t-Student test.

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Table 5. Antimicrobial activity of *E. australis, M. officinalis, M. spicata, P. vulgaris*, *G. tridentata,* and mixtures 1, 2, and 3 infusions.

	E. australis		***M. officinalis***		***M. spicata***		***G. tridentata***		***P. vulgaris***		**Mixture 1**		**Mixture 2**		**Mixture 3**		**Streptomycin**		**Ampicilin**	
	Antibacterial activity (mg/mL)																			
Bacteria	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative																				
Escherichia coli	1	2	1	2	0.5	1	0.5	1	1	2	1	2	1	2	4	8	0.20	0.30	0.40	0.50
Salmonela typhimurium	1	2	1	2	0.25	0.5	1	2	1	2	1	2	1	2	4	8	0.20	0.30	0.75	1.20
Gram-positive																				
Bacillus cereus	0.5	1	1	2	0.5	1	1	2	1	2	0.5	1	1	2	4	8	0.10	0.20	0.25	0.40
Listeria monocytogenes	0.5	1	1	2	0.5	1	1	2	1	2	1	2	2	4	4	8	0.20	0.30	0.40	0.50
	Antifungal activity (mg/mL)																			
																	Ketoconazole		**Bifonazole**	
Fungi	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus niger	4	8	1	2	1	2	8	>8	1	2	0.5	1	8	>8	2	4	0.20	0.50	0.15	0.20
Aspergillus versicolor	0.25	0.5	0.5	1	0.25	0.5	0.5	1	0.5	1	0.5	1	0.5	1	0.5	1	0.20	0.50	0.10	0.20
Penicillium funiculosum	0.5	1	0.5	1	0.5	1	0.5	1	0.5	1	0.25	0.5	1	2	0.5	1	0.20	0.50	0.20	0.25
Penicillium verrucosum	0.5	1	0.5	1	0.25	0.5	0.5	1	1	2	1	2	4	8	2	4	0.20	0.30	0.10	0.20

MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration; MFC – minimal fungal concentration; Mixture 1: 20% *P. vulgaris*, 40% *G. tridentata,* 40% *M. spicata;* Mixture 2: 30% *E. australis*, 30% *P. vulgaris*, 40% *M. officinalis*; Mixture 3: 30% *P. vulgaris*, 35% *M. officinalis*, 35% *M. spicata*.

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Challenges of traditional herbal teas: plant infusions and their mixtures with bioactive properties

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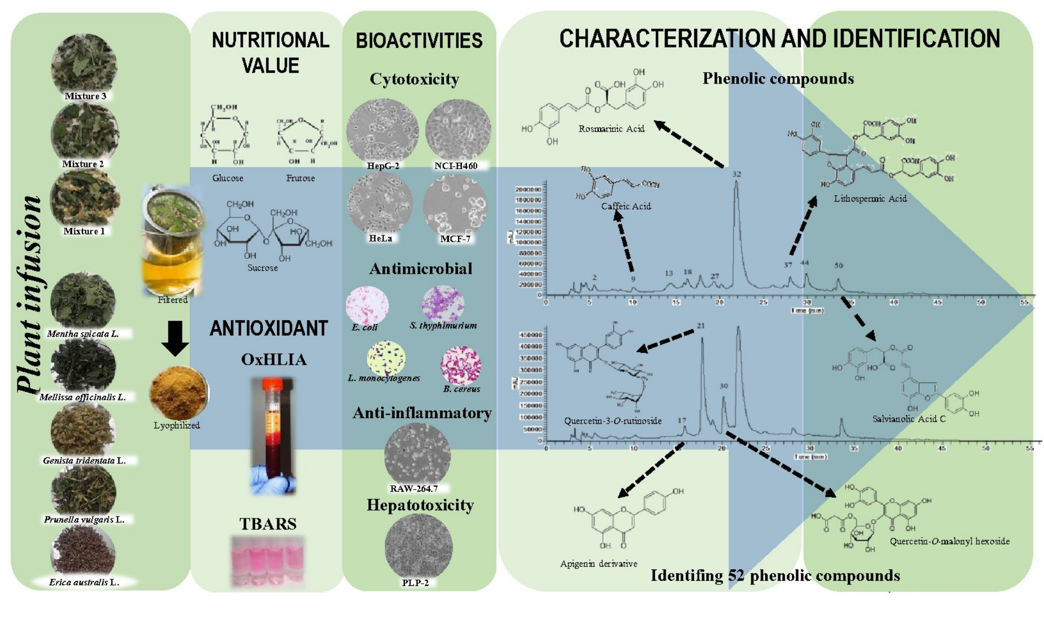

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