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1 # *Melissa officinalis* L. decoctions as functional beverages: a bioactive
2 approach and chemical characterization

3

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21 **Abstract**

22 Lemon balm (*Melissa officinalis* L.) is a member of the *Lamiaceae* family with a long
23 story of human consumption. It has been consumed for decades, directly in food and as
24 decoction or infusion for its medicinal purposes. In this manuscript, a detailed chemical
25 characterization of the decoction of this plant is described, encompassing antimicrobial,
26 antioxidant and antitumor activities. Rosmarinic acid and lithospermic acid A were the
27 most abundant phenolic compounds. Quinic acid, fructose, glucose and γ-tocopherol
28 were the most abundant within their group of molecules. *M. officinalis* decoctions were
29 active against a wide range of microorganisms, being *Pseudomonas aeruginosa* and
30 *Salmonella thyphimurium*, and *Penicillium funiculosum* the most sensitive bacteria and
31 fungi, respectively. It was also observed growth inhibition of different human tumor cell
32 lines (mainly MCF-7 and HepG2), as also high free radicals scavenging activity and
33 reducing power. This manuscript highlights some beneficial effects of these functional
34 beverages.

35

36 **Keywords:** *Melissa officinalis*; Decoctions; Chemical characterization; Functional
37 beverage

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39 **1. Introduction**

40 Medicinal herbs are used for their benefits towards health and are also incorporated into
41 cosmetics, food, pharmaceuticals and other products designed for human consumption.
42 Essential oils, polyphenols, terpenes, alkaloids, steroids and other compounds of the
43 secondary metabolism of plants are examples of the many compounds present in the
44 Plant kingdom that have been used for different purposes. The use of these products is
45 limited since they require methods of extraction and purification, but they have gained
46 considerable interest by researchers and consumers for the advantages in comparison to
47 synthesized molecules[1].

48 *Melissa officinalis* L., commonly known as lemon balm, is a member of the *Lamiaceae*
49 family that has been used for centuries for its outstanding traditional medicinal effects
50 on health and against illnesses. It is credited for its antibacterial, antifungal, and anti-
51 inflammatory effects. Furthermore, it acts as an expectorant, relieves digestion,
52 headaches and rheumatism[2, 3]. Recently, and apart from some empiric claims of its
53 applicability, other effects have been discovered for this plant, namely against
54 neurodegenerative diseases, as an antitumor, antiproliferative, anticholinesterase,
55 antioxidant and anti-Alzheimer[2, 4]. Further uses have also been explored, namely as food
56 ingredient for stabilization and preservation[5]. The beneficial effects found in extracts of
57 *M. officinalis* could in part be attributed to its phenolic compounds, namely the caffeic
58 acid dimer rosmarinic acid, and other caffeic acid derivatives[3].

59 The most consumed drinks in the world are "teas". They are consumed as functional
60 beverages, thanks to the beneficial properties of the plants used in them. The two most
61 important methods of preparation of "teas" are infusions and decoctions, with the latter
62 being the most common and more effective for a better extraction of larger molecules

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63 like tannins and other hard extracting ones[6]. Some studies, comparing infusions and
64 decoctions have proven that the decoctions have better extracting capacity and this can
65 be translated in higher levels of phenolic compounds and, therefore, increased
66 bioactivity[7,8].
67 Given the outstanding effects of lemon balm on human health and its potential as a
68 functional beverage, in the present work its decoctions have been chemically
69 characterized (phenolic compounds, organic acids, sugars and tocopherols) and
70 evaluated regarding antimicrobial, antioxidant and antitumor *in vitro* effects.
71

72 **2. Materials and Methods**

73 *2.1. Samples and decoctions preparation*

74 *Melissa officinalis* L. dry leaves were provided by the company Pragmático Aroma Lda.
75 ("Mais Ervas") based in Trás-os-Montes, Portugal. After confirmation of the
76 taxonomical identification, the samples were submitted to a decoction extraction, in
77 which 1 g of the plants was added to 200 mL of cold distilled water. After heating, it
78 was left to boil for 5 min, and stood at room temperature for 5 more minutes. After
79 filtration through a Whatman Nº4 filter paper, the obtained decoctions were frozen and
80 lyophilized and all the assays were performed using these samples. The extractions and
81 all the assays were carried out in triplicate and the results were expressed as mean
82 values ± standard deviations (SD).
83

84 *2.2. Standards and reagents*

85 Acetonitrile, n-hexane, ethyl acetate (HPLC grade) and sulphuric acid were acquired
86 from Fisher Scientific (Lisbon, Portugal). Formic acid was purchased from Panreac
87 (Barcelona, Spain). Sugar standards (D(−)-fructose, D(+)- glucose anhydrous and D(+)-

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88 sucrose), organic acid standards (malic, shikimic, oxalic and quinic acids), tocopherol
89 standards (α, β, γ, and δ isoforms), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-
90 carboxylic acid), acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue,
91 trichloroacetic acid (TCA), phosphate buffered saline (PBS), and Tris buffer were
92 acquired from Sigma Chemical Co. (Saint Louis, MO, USA). Dimethyl sulfoxide
93 (DMSO) was obtained from Merck (KGaA, Germany). Phenolic compound standards
94 (caffeic and rosmarinic acid) were purchased from Extrasynthese (Genay, France). 2,2-
95 Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA,
96 USA). Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS),
97 trypsin-EDTA (ethylenediaminetetraacetic acid), nonessential amino acids solution (2
98 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively),
99 RPMI-1640, and DMEM media were acquired from HyClone (Logan, UT, USA).
100 Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of
101 Immunology and Virology, Torlak (Belgrade, Serbia). Racemic tocol (50 mg/mL) was
102 purchased from Matreya (Pleasant Gap, PA, USA). All other chemicals were obtained
103 from official scientific retailers. Water was treated by means of a Milli-Q water
104 purification system (TGI Pure Water Systems, Greenville, SC, USA).

105 *2.3. Chemical characterization*

106 *2.3.1 Phenolic compounds.* Phenolic compounds were determined by HPLC (Hewlett-
107 Packard 1100, Agilent Technologies, Santa Clara, CA, USA) as previously described by
108 Barros et al.[9]. Double online detection was carried out in a DAD using 280 nm as
109 preferred wavelength and in a mass spectrometer connected to the HPLC system via the
110 DAD cell outlet. Mass spectrometric detection was performed by means of an API 3200
111 (Applied Biosystems, Darmstadt, Germany) triple quadrupole-ion trap analyser
112 equipped with an ESI source. Spectra were recorded in negative ion mode between *m/z*

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113 100 and 1700. The phenolic compounds were identified by comparing their retention
114 time, UV-vis and mass spectra with those obtained from standard solutions, when
115 available. Otherwise, compounds were tentatively identified comparing the obtained
116 information with available data in our compound library or reported in the literature.
117 For quantitative analysis, a 5-level calibration curve was obtained by plotting the areas
118 of the peaks recorded at 280 nm against known concentrations (1-100 mg/mL) of
119 phenolic standards, i.e., caffeic acid ($y=359x+488.4$; $R^2=0.999$) and rosmarinic acid
120 ($y=312.2x-424.06$; $R^2=0.999$). Compound quantification was performed through the
121 calibration curve of the phenolic standard of the same group. The results were expressed
122 in mg per g of lyophilized decoctions.
123
124 *2.3.2. Organic acids.* Organic acids were determined following a procedure previously
125 described by Pinela et al.[10]. Analyses were performed on a Shimadzu 20A series ultra-
126 fast liquid chromatograph (UFLC, Shimadzu Cooperation, Kyoto, Japan) coupled to
127 diode array detector (DAD, Shimadzu), using 215 nm and 245 nm (for ascorbic acid) as
128 the preferred wavelengths. Separation was achieved on a SphereClone (Phenomenex,
129 Torrance, CA, USA) reverse phase C18 column (5 μm, 250 mm × 4.6 mm i.d)
130 thermostated at 35 °C. Analytes were eluted with 3.6 mM sulphuric acid at a flow-rate
131 of 0.8 mL/min. The organic acids found were quantified by comparison of peak areas
132 recorded at 215 nm with calibration curves obtained from commercial standards of each
133 compound: oxalic acid ($y = 9\times10^6 x + 377946$; $R^2 = 0.994$); quinic acid ($y = 612327x +$
134 16563; $R^2 = 1$); malic acid ($y = 863548x + 55591$; $R^2 = 0.999$); shikimic acid ($y =$
135 $8\times10^7x + 55079$; $R^2 = 0.999$); citric acid ($y = 1\times10^6x + 16276$; $R^2 = 1$); succinic acid (y
136 $= 603298x + 4994.1$; $R^2 = 1$); fumaric acid ($y = 148083x + 96092$; $R^2 = 1$). The results
137 were expressed in mg per g of lyophilized decoctions.

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138

139 *2.3.3. Free Sugars.* Free sugars were determined by HPLC coupled to a refraction index
140 (RI) detector as described by Pinela et al.[10]. The equipment consisted of a pump
141 (Knauer, Smartline System 1000, Berlin, Germany), a degasser (Smart line Manager
142 5000), an autosampler (AS-2057 Jasco, Easton, MD, USA), and a RI detector (Knauer
143 Smartline 2300). The chromatographic separation was achieved with a Eurospher 100-5
144 NH_2 column (5 µm, 250 mm × 4.6 mm i.d., Knauer) operating at 35 ºC (7971 R Grace
145 oven). The mobile phase was acetonitrile/deionized water, 70:30 (*v/v*) at a flow rate of 1
146 mL/min. The identification was achieved by comparing the relative retention times of
147 sample peaks with standards. Quantification was made by the internal standard method,
148 and the results are expressed in mg per g of lyophilized decoctions.

149

150 *2.3.4. Tocopherols.* Tocopherols were determined following a procedure previously
151 described by Guimarães et al.[11]. The HPLC system described above for sugar analysis
152 was connected to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA)
153 programmed for excitation at 290 nm and emission at 330 nm. The chromatographic
154 separation was achieved with a Polyamide II normal-phase column (5 µm, 250 mm ×
155 4.6 mm i.d., YMC Waters), operating at 35 °C. The mobile phase used was a mixture of
156 n-hexane and ethyl acetate (70:30, *v/v*) at a flow rate of 1 mL/min. The compounds were
157 identified by chromatographic comparisons to authentic standards. Quantification was
158 based on the fluorescence signal response, using the internal standard method.
159 Tocopherols content in the samples were expressed in µg per g of lyophilized
160 decoctions.

161

162 *2.4. Evaluation of bioactive properties*

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163 *2.4.1. General.* Lyophilized decoctions were redissolved in water (5 mg/mL for
164 antimicrobial and antioxidant activity evaluation, or 8 mg/mL for antitumor activity and
165 hepatotoxicity evaluation). The final solutions were further diluted to different
166 concentrations to be submitted to the distinct *in vitro* assays.
167
168 *2.4.2. Antibacterial activity.* The methodology previously described by Petrović et al.[12]
169 and Vieira et al.[13] was followed. The following Gram-negative bacteria were used:
170 *Escherichia coli* (ATCC (American type culture collection) 35210), *Pseudomonas*
171 *aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter*
172 *cloacae* (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC
173 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and
174 *Listeria monocytogenes* (NCTC (National collection of type cultures) 7973). These
175 microorganisms were obtained from the Mycological laboratory, Department of Plant
176 Physiology, Institute for biological research "Siniša Stanković" at the University of
177 Belgrade in Serbia. Fresh overnight culture of bacteria was adjusted with a
178 spectrophotometer to a concentration of 1×10^5 CFU/mL. The requested colony forming
179 units (CFU)/mL corresponded to a bacterial suspension determined in a
180 spectrophotometer at 625 nm. Dilutions of the inocula were cultured on solid medium to
181 verify the absence of contamination and to check the validity of the inoculum. The
182 sample solutions were pipetted into the wells containing 100 µL of Tryptic Soy Broth
183 (TSB), with 10 µL of inoculum being added to all the wells. The microplates were
184 incubated for 24 h at 37 °C. The MIC (minimal inhibitory concentration) of the samples
185 was determined by adding 40 µL of iodonitrotetrazolium chloride (INT) (0.2 mg/mL)
186 and incubation at 37 °C for 30 min. The lowest concentration that produced a significant
187 inhibition (around 50%) of the growth of the bacteria in comparison with the positive

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188 control was identified as the MIC. The MICs obtained from the susceptibility testing of
189 various bacteria to tested samples were also determined by a colorimetric microbial
190 viability assay based on reduction of INT color and compared with positive control for
191 each bacterial strain. MBC (minimal bactericidal concentration) was determined by
192 serial sub-cultivation of 10 μL into microplates containing 100 μL of TSB. The lowest
193 concentration that showed no growth after this sub-culturing was regarded as the MBC.
194 Streptomycin and ampicillin were used as positive controls, while 5% dimethyl
195 sulfoxide (DMSO) was used as negative control. The results of MIC and MBC were
196 expressed in mg per mL of the aqueous solutions of the lyophilized decoctions.
197
198 *2.4.3. Antifungal activity.* The methodology previously described by Petrović et al.[12]
199 and Vieira et al.[13] was followed. The following microfungi were used: *Aspergillus*
200 *fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor*
201 (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM (Culture
202 Collection, Centre for Cellular and Molecular Research, Institute of Molecular and
203 Cellular Biosciences, The University of Tokyo, Japan) 5061), *Penicillium funiculosum*
204 (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum*
205 *var. cyclopium* (food isolate). These organisms were also obtained from the
206 Mycological Laboratory, Department of Plant Physiology, Institute for Biological
207 Research "Siniša Stanković" at the University of Belgrade in Serbia. The micromycetes
208 were maintained on malt agar (MA) and the cultures were stored at 4 °C and sub-
209 cultured once a month. The fungal spores were washed from the surface of agar plates
210 with sterile 0.85% saline containing 0.1% Tween 80 (*v/v*). The spore suspension was
211 adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final
212 volume of 100 μL/well. The inocula were stored at 4 °C for further use. Dilutions of the

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213 inocula were cultured on solid MA to verify the absence of contamination and to check
214 the validity of the inoculum. The MICs determination was performed by a serial
215 dilution technique using 96-well microplates. The sample solutions were added to broth
216 malt medium with the fungal inoculum. The microplates were incubated for 72 h at 28
217 °C[14]. The lowest concentrations without visible growth (using a binocular microscope)
218 were defined as the MIC. The minimum fungicidal concentrations (MFC) were
219 determined by serial sub-cultivation of 2 μL in microtiter plates containing 100 μL of
220 malt broth per well and further incubation for 72 h at 28 °C. The lowest concentration
221 with no visible growth was defined as the MFC, indicating 99.5% killing of the original
222 inoculum. 5% DMSO was used as a negative control, while bifonazole and
223 ketoconazole were used as positive controls. The results of MIC and MFC were
224 expressed as mg per mL of the aqueous solutions of the lyophilized decoctions.
225
226 *2.4.4. Antioxidant activity.* DPPH radical-scavenging activity was evaluated using an
227 ELX800 microplate Reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA) and
228 calculated as a percentage of DPPH discolouration after 1 hour of incubation with the
229 antioxidant extract, using the formula: $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the
230 absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance
231 of the DPPH solution. Reducing power was evaluated by the capacity to reduce Fe^{3+}
232 into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned
233 above. Inhibition of ß-carotene bleaching was evaluated through the ß-
234 carotene/linoleate assay; the neutralization of linoleate free radicals avoids ß-carotene
235 bleaching, which is measured by the formula: (ß-carotene absorbance after 2h of
236 assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*)
237 brain homogenates was evaluated by the decrease in thiobarbituric acid reactive

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238 substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid
239 (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was
240 calculated using the following formula: $[(A-B)/A] \times 100\%$, where A and B were the
241 absorbance of the control and the sample solution, respectively[9]. Trolox was used as
242 positive control. The results were expressed as EC_{50} values (sample concentration
243 providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay)
244 in μg per mL of the aqueous solutions of the lyophilized decoctions.
245

246 *2.4.5. Antitumor activity and hepatotoxicity.* Four human tumor cell lines were tested:
247 MCF7 (breast adenocarcinoma), NCI-H460 (non-small cell lung carcinoma), HeLa
248 (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely
249 maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-
250 inactivated FBS and 2 mM glutamine at 37 ºC, in a humidified air incubator containing
251 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for
252 MCF7 and NCI-H460 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates.
253 For hepatotoxicity evaluation, a cell culture was prepared from a fresh porcine liver
254 obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver
255 tissues were rinsed in hank's balanced salt solution containing 100 U/mL penicillin, 100
256 μg/mL streptomycin and divided into 1×1 mm^3 explants. Some of these explants were
257 placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine
258 serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL
259 streptomycin and incubated at 37 ºC with a humidified atmosphere containing 5% CO_2.
260 The medium was changed every two days. Cultivation of the cells was continued with
261 direct monitoring every two to three days using a phase contrast microscope. Before
262 confluence was reached, cells were subcultured and plated in 96-well plates at a density

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263 of 1.0×10^{4} cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL
264 penicillin and 100 μg/mL streptomycin[15].
265 Sulphorhodamine B assay was performed according to a procedure previously described
266 by Guimarães et al.[11]. Ellipticine was used as positive control. The results were
267 expressed as GI_{50} values (sample concentration that inhibited 50% of the net cell
268 growth) in μg per mL of the aqueous solutions of the lyophilized decoctions.
269

270 **3. Results and Discussion**

271 *3.1. Chemical characterization*

272 **Figure 1** shows a chromatogram with **t**he phenolic profile of *M. officinalis* decoctions
273 recorded at 280 nm**.** Twenty compounds could be detected and identified, mostly
274 corresponding to caffeic acid derivatives. Peak characteristics and tentative
275 identifications based on their UV and mass spectra comparing with the available
276 literature[16-22] are presented in **Table 1**. Although there are studies about the phenolic
277 profile in aqueous or alcoholic extracts of *M.* officinalis[3, 23-30], as far as we know, this is
278 the first report regarding phenolic characterization in decoctions of this plant. In a
279 previous study[3] the phenolic characterization of infusions obtained from different
280 samples of *M. officinalis* (cultivated, *in vitro* cultured, commercial granulate and bag)
281 had been already performed, being the profiles similar to the one observed in the
282 decoctions studied herein.
283 Compound 1 was identified as 3-(3,4-dihydroxyphenyl)-lactic acid, based on its
284 fragmentation pattern[17], and previous identification in *M. officinalis* infusions[3].
285 Compounds 5 ($[M-H]^-$ at *m/z* 179) and 15 ($[M-H]^-$ at *m/z* 359) were positively identified
286 as caffeic and rosmarinic acids according to their retention time, mass and UV-vis
287 characteristics by comparison with commercial standards. Compound 14, with similar

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288 characteristics to compound 15, was tentatively identified as the *cis* isomer of
289 rosmarinic acid. Hydroxycinnamoyl *cis* derivatives would be expected to elute before
290 the corresponding trans ones, as observed after UV irradiation (366 nm, 24 h) in our
291 laboratory. Compounds 6 ($[M-H]^-$ at *m/z* 439) and 12 ($[M-H]^-$ at *m/z* 521) yielded a
292 fragment at *m/z* 359 (rosmarinic acid) from the loss of 80 mu (sulphate moiety) and 162
293 mu (hexoside moiety), respectively, which allowed their tentative identification as
294 sulphated and hexoside derivatives of rosmarinic acid, as previously reported in *M.*
295 *officinalis* infusions[3]. Similarly, compound 3 ($[M-H]^-$ at *m/z* 341) was assigned as a
296 caffeic acid hexoside. Compounds 2 ($[M-H]^-$ at *m/z* 311), 4 ($[M-H]^-$ at *m/z* 325) and 10
297 ($[M–H]^-$ at *m/z* 473) were tentatively identified as hydroxycinnamoyl tartaric esters,
298 namely caftaric (caffeoyltartaric acid), fertaric (feruloyltartaric acid) and cichoric
299 (dicaffeoyltartaric acid) acids, respectively, based on their mass spectra[20]. Compound 8
300 ($[M–H]^-$ at *m/z* 357) presented a fragmentation pattern and UV-vis spectra that allowed
301 its identification as the caffeic acid dimer prolithospermic acid[19, 20].

302 Compounds 7, 9, 16, 18 and 19 were associated to caffeic acid trimers.

303 Compound 7 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 571 releasing various
304 fragments from the combination of successive losses of 44 mu (CO_2) and 197 mu
305 (dihydroxyphenyl-lactic acid). These characteristics are similar to the ones described for
306 yunnaneic acid E[31], identity that was tentatively associated to this compound.
307 Compounds 9 and 18 showed the same pseudomolecular ion ($[M-H]^-$ at *m/z* 537), which
308 may match the structure of salvianolic acids H/I and lithospermic acid A. They were
309 tentatively assigned as lithospermic acid A isomers duet to the absence of a fragment
310 *m/z* 339, as reported in the literature[17, 20, 22]. Compound 18 was assigned as lithospermic
311 acid A that was expected to elute later than rosmarinic acid[17, 18, 20]. Similar behaviour
312 was observed in the infusions of *M. officinalis*[3]. Compounds 16 and 19 presented a

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313 pseudomolecular ion $[M-H]^-$ at *m/z* 493, which together with their characteristic
314 fragment ions at *m/z* 313, 295 and 197[20, 22] and UV spectra allowed assigning them as
315 salvianolic acid A isomers. Compound 16 was tentatively identified as salvianolic acid
316 A that was expected to elute earlier than lithospermic acid A, as previously reported[3],
317 whereas compound 19 was associated to a salvianolic acid A isomer.

318 Compound 13 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 719 releasing a main MS^2
319 fragment at *m/z* 359 ($[M-2H]^{2-}$, rosmarinic acid) as well as other fragments
320 characteristic of caffeic acid oligomers, which allowed its identification as sagerinic
321 acid, a rosmarinic acid dimer that was also previously identified in *M. officinalis*
322 infusions[3].

323 No definite structure could be proposed for compounds 11 ($[M-H]^-$ at *m/z* 553), 17 ([M-
324 H]$^-$ at *m/z* 829) and 20 ($[M-H]^-$ at *m/z* 715), although their fragmentation pattern allowed
325 assigning them as caffeic acid oligomers; furthermore, the presence of a common MS^2
326 fragment at *m/z* 491 that may be attributed to salvianolic acid C, might suggest that are
327 derivatives of that compound. Compounds with similar characteristics as 17 and 20
328 were previously detected[3, 30] in samples of *M. officinalis*, although no structure was
329 proposed, either.

330 Quantification of the detected phenolic compounds expressed as rosmarinic or caffeic
331 acid equivalents is shown in **Table 2.** Rosmarinic acid was the most abundant phenolic
332 compound present in the decoctions; many authors have also reported this compound as
333 the main phenolic compound in this species[3, 24, 26, 28-30]. Luteolin derivatives have also
334 been reported as relevant phenolics in *M. officinalis*[3, 23, 25, 28, 30], but this type of
335 derivatives (with low hydrophilicity) was absent in the decoction of *M. officinalis*.
336 Rosmarinic acid has been reported as displaying high antioxidant and antibacterial
337 activity[32].

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338 In terms of organic acids, the decoctions of *M. officinalis* presented oxalic, quinic,
339 malic, shikimic, citric, succinic and fumaric acids. The higher quantities were found for
340 quinic acid and the lower ones for fumaric acid as detailed in **Table 3**. When compared
341 to decoctions of other plant species, namely *Matricaria recutita* L., quinic acid proved
342 to be higher for *M. officinalis*[33]; furthermore, lemon balm decoctions contained higher
343 contents in all organic acids when compared to *Juglans regia* L. leaves, although
344 ascorbic acid was found in the latter species[34]. Quinic acid is a normal constituent of our
345 diet, capable of conversion to tryptophan and nicotinamide via the GI tract microflora,
346 thus providing an *in situ* physiological source of these essential metabolic ingredients to
347 humans. Pero, Lund, & Leanderson[35] conducted a clinical trial that confirmed the
348 efficacy of quinic acid as an antioxidant, and extends its mode of action to include a
349 basic nutritional benefit due to the enhanced metabolism of both tryptophan and
350 nicotinamide, which are simultaneously induced by oral exposure to quinic acid.
351 The only free sugars detected in the decoctions of *M. officinalis* were fructose, glucose
352 (the most abundant ones) and trehalose as depicted in **Table 3**. When compared to other
353 decocted samples, like wild *Tuberaria lignosa* (Sweet) Samp.[10], *J. regia*[34] and *Castanea*
354 *sativa* Mill.[7], *M. officinalis* proved to have a much lower content of sugars.
355 Tocopherols, which are isoforms of vitamin E, the most important lipophilic vitamin for
356 the human metabolism, were also found in the decoctions (**Table 3**). Only α- and γ-
357 isoforms were found, with the latter being the most abundant one. As expected, the
358 quantity of γ-tocopherol found in these decoctions was lower than the one reported in a
359 previous study with garden cultivated, *in vitro* cultured, commercial bag and granulated
360 *M. officinalis* plants (results expressed in dry weight and after extraction with non-polar
361 solvents); this is certainly related with the lower extractability of these compounds
362 using water[36].

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363 *3.2. Bioactive properties*

364 Regarding the decoctions antibacterial activity (**Table 4**), the most sensitive bacteria
365 were *Pseudomonas aeruginosa* and *Salmonella typhimurium*, and for the last case even
366 better than the positive controls. The decoctions gave the same MBC as ampicillin
367 against *Staphylococcus aureus*, and the same MIC as streptomycin against *P.*
368 *aeruginosa*. In terms of antifungal activity, *M. officinalis* decoctions displayed higher
369 activity, with *Penicillium funiculosum* being more sensitive to the decoction than the
370 two positive controls (bifonazole and ketoconazole). The behaviour of the decoctions
371 against *Aspergillus versicolor*, *A. ochraceus*, *Trichoderma viride* and *P. ochrochloron*
372 was better than the positive control ketoconazole (lower MICs and MFCs).

373 The antioxidant activity of the decoctions was determined by DPPH scavenging
374 activity, reducing power, β-carotene bleaching inhibition and neutralization of
375 thiobarbituric reactive species. DPPH scavenging activity and reducing power of the
376 decoctions were higher than their lipid peroxidation inhibition capacity (**Table 5**).
377 These differences could be explained by the underlying different mechanisms involved
378 in each type of antioxidant reaction and the interaction between radical and antioxidant
379 species[37]. To get a better overview of the antioxidant power of the studied decoctions,
380 for the DPPH and reducing power assays, they yielded higher activity (lower EC_{50}
381 values) than methanolic extracts of *M. officinalis*[36] and *Chenopodium ambrosioides* L.[9],
382 infusions of *Cynara scolymus* L., *C. ambrosioides*[9], *Silybum marianum* (L.) Gaertn, and
383 *Cochlospermum angolensis* Welw.[38], decoctions of *Chamaemelum nobile* L.[11], *J.*
384 *regia*[34], *M. recutita*[33], *Ginkgo biloba* L.[39], *Origanum vulgare* L.[2], *C. sativa* flowers[7] and
385 *Taraxacum sect.* Ruderalia[40].

386 The antitumor activity was evaluated against human tumor cell lines, namely against
387 breast (MCF-7), non-small lung (NCI-H460), cervical (HeLa) and hepatocellular

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388 carcinoma (HepG2) (**Table 5**). The best inhibition was detected for MCF-7 cell line and
389 the least affected cell line was the NCI-H460. Although not outstandingly low when
390 compared to the positive control (ellipticine), the decoctions did not show any
391 hepatotoxicity (tested against porcine liver cells primary culture PLP2), while ellipticine
392 had very strong toxic effects. Comparing their antitumor activity to other decocted and
393 infused plant samples, *M. officinalis* proved to be better against HepG2 cell line than
394 hepatoprotective plants such as *S. marianum* and *C. angolensis*[38], *C. nobile*[11], *C.*
395 *ambrosioides*[9], *J. regia*[34] and *M. recutita*[33]. Furthermore, the four latter species had
396 higher GI_{50} for all the assayed cell lines, when compared to *M. officinalis*.
397

398 **4. Conclusions**

399 *M. officinalis* decoctions proved to be a very good source of rosmarinic acid and
400 lithospermic acid A, quinic acid and γ-tocopherol. Rosmarinic acid might be one of the
401 responsible compounds for the various bioactive properties attributed to lemon balm
402 including antibacterial and antioxidant effects. The antimicrobial activity of the
403 decoctions was observed against a variety of bacteria and fungi, supporting the claims
404 as an antiseptic, especially against *P. aeruginosa*, *S. thyphimurium* and P. *funiculosum*.
405 The antioxidant activity was also outstanding, due to the very low EC_{50} values obtained
406 for DPPH scavenging activity and reducing power, placing the decoctions of this
407 medicinal plant as one of the most powerful antioxidants assayed, supporting their
408 consumption as a functional beverage against oxidative stress. Finally, the activity
409 against specific tumor cell lines proved to be quite satisfactory, yielding lower GI_{50}
410 values, especially against HepG2 cell lines, when compared to some hepatoprotective
411 plants. No hepatotoxicity was observed for the studied decoctions, which favours their
412 consumption as functional beverages, given their pleasant taste.

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413 **Conflict of Interest**

414 The authors state no conflict of interest regarding this manuscript.

415

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422

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Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification of phenolic compounds in *M. officinalis* decoctions.

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	4.57	280	197	179(92),135(100)	3-(3,4-dihydroxyphenyl)-lactic acid
2	4.91	330	311	179(100),149(98),135(31)	Caftaric acid
3	6.91	320	341	179(100),149(7),135(31)	Caffeic acid hexoside
4	8.13	324	325	193(100),149(11),145(25),134(43)	Fertaric acid
5	11.19	324	179	135(100)	Caffeic acid
6	12.01	330	439	359(12),179(10),161(46),135(22)	Sulphated rosmarinic acid
7	12.53	270	571	527(14),483(61),439(52),329(23),259(22),241(49),197(100),179(77),135(98)	Yunnaneic acid E
8	13.05	270,310sh	357	313(30),269(100),203(56),159(61),109(50)	Prolithospermic acid
9	13.85	276,324sh	537	493(57),359(13),313(27),295(100),269(27),197(19),179(78),135(45)	Lithospermic acid A isomer
10	14.95	328	473	311(19),293(19),179(75),149(100),135(28)	Chicoric acid
11	17.54	266,336sh	553	491(9),359(3),311(5),197(3),179(21),161(12),135(100)	Salvianolic acid C derivative
12	18.88	322	521	359(100),197(16),179(32),161(72),135(16)	Rosmarinic acid hexoside
13	21.17	284,328sh	719	539(17),521(15),359(100),197(22),179(26),161(81),135(7)	Sagerinic acid
14	23.32	328	359	197(35),179(34),161(100),135(15)	*cis* Rosmarinic acid
15	24.03	330	359	197(83),179(70),161(100),135(40)	*trans* Rosmarinic acid
16	27.60	324	493	359(78),313(8),295(52),269(7),197(33),179(44)	Salvianolic acid A
17	28.42	328	829	667(86),535(100),491(21),311(39),293(15),179(10)	Salvianolic acid C derivative
18	30.11	288,326sh	537	493(53),359(100),313(5),295(18),269(3),197(44),179(64)	Lithospermic acid A
19	31.32	320	493	359(100),313(5),295(6),269(4),197(14),179(34)	Salvianolic acid A isomer
20	35.17	288,320sh	715	535(100),491(38),311(69),293(4),179(5),135(20)	Salvianolic acid C derivative

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Table 2. Phenolic compounds quantification in *M. officinalis* decoctions.

Phenolic compound	**Content (mg/g lyophilized decoction)**
3-(3,4-dihydroxyphenyl)-lactic acid	6.5 ± 0.2
Caftaric acid	5.7 ± 0.1
Caffeic acid hexoside	1.1 ± 0.1
Fertaric acid	0.7 ± 0.1
Caffeic acid	2.2 ± 0.1
Sulphated rosmarinic acid	1.6 ± 0.2
Yunnaneic acid E	1.4 ± 0.1
Prolithospermic acid	2.2 ± 0.1
Lithospermic acid A isomer	62.5 ± 0.3
Chicoric acid	3.8 ± 0.1
Salvianolic acid C derivative	4.1 ± 0.2
Rosmarinic acid hexoside	4.9 ± 0.4
Sagerinic acid	7.9 ± 0.3
cis Rosmarinic acid	5.1 ± 0.2
trans Rosmarinic acid	83 ± 1
Salvianolic acid A	6.2 ± 0.2
Salvianolic acid C derivative	7.8 ± 0.2
Lithospermic acid A	10.9 ± 0.3
Salvianolic acid A isomer	2.1 ± 0.1
Salvianolic acid C derivative	2.84 ± 0.02
Total phenolic compounds	223 ± 1

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Table 3. Quantification of organic acids, free sugars and tocopherols in *M. officinalis* decoctions.

Organic acids	**Content (mg/g lyophilized decoction)**
Oxalic	11.8 ± 0.1
Quinic	96.9 ± 0.2
Malic	18.8 ± 0.1
Shikinic	1.80 ± 0.01
Citric	24.1 ± 0.1
Succinic	26 ± 1
Fumaric	0.032 ± 0.001
Total	179 ± 1
Sugars	**Content (mg/g lyophilized decoction)**
Fructose	49 ± 4
Glucose	47 ± 1
Trehalose	19.8 ± 0.2
Total	116 ± 5
Tocopherols	**Content (μg/g lyophilized decoction)**
α-tcopherol	0.44 ± 0.02
γ-tocopherol	1.43 ± 0.05
Total	1.87 ± 0.03

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Table 4. Minimum inhibitory (MIC) and bactericidal (MBC) or fungicidal (MFC) concentrations of *M. officinalis* lyophilized decoctions, expressed in mg/mL.

Bacteria species	***M. officinalis* decoction** MIC MBC	**Streptomycin** MIC MBC	**Ampicillin** MIC MBC
Staphylococcus aureus	0.30 0.40	0.04 0.10	0.25 0.40
Bacillus cereus	0.40 0.75	0.10 0.20	0.25 0.40
Micrococcus flavus	0.75 1.50	0.20 0.30	0.25 0.40
Listeria monocytogenes	1.00 1.50	0.20 0.30	0.40 0.50
Pseudomonas aeruginosa	0.20 0.40	0.20 0.30	0.75 1.20
Escherichia coli	0.50 0.75	0.20 0.30	0.40 0.50
Enterobacter cloacae	0.50 0.75	0.20 0.30	0.25 0.50
Salmonella typhimurium	0.20 0.40	0.25 0.50	0.40 0.75

Fungi species	***M. officinalis* decoction** MIC MFC	**Bifonazole** MIC MFC	**Ketoconazole** MIC MFC
Aspergillus fumigatus	0.40 0.75	0.15 0.20	0.20 0.50
Aspergillus versicolor	0.20 0.40	0.10 0.20	0.20 0.50
Aspergillus ochraceus	0.40 0.75	0.15 0.20	1.50 2.00
Aspergillus niger	0.40 0.75	0.15 0.20	0.20 0.50
Trichoderma viride	0.20 0.40	0.15 0.20	1.00 1.00
Penicillium funiculosum	0.10 0.20	0.20 0.25	0.20 0.50
Penicillium ochrochloron	0.40 0.75	0.20 0.25	2.50 3.50
Penicillium verrucosum	0.75 1.50	0.10 0.20	0.20 0.30

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Table 5. Antioxidant, antitumor and hepatotoxic activities of *M. officinalis* lyophilized decoctions.

Antioxidant activity	**EC_{50} values (µg/mL)**
DPPH scavenging activity	49 ± 5
Reducing power	35 ± 1
β-carotene bleaching inhibition	6073 ± 348
TBARS inhibition	271 ± 2
Antitumor activity	**GI_{50} values (µg/mL)**
MCF-7 (breast carcinoma)	51 ± 4
NCI-H460 (non-small cell lung carcinoma)	258 ± 17
HeLa (cervical carcinoma)	155 ± 10
HepG2 (hepatocellular carcinoma)	67 ± 2
Hepatotoxicity	**GI_{50} values (µg/mL)**
PLP2	>400

The antioxidant activity was expressed as EC_{50} values, what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC_{50} values: 41 µg/mL (reducing power), 42 µg/mL (DPPH scavenging activity), 18 µg/mL (β-carotene bleaching inhibition) and 23 µg/mL (TBARS inhibition). GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. Ellipticine GI_{50} values: 1.21 µg/mL (MCF-7), 1.03 µg/mL (NCI-H460), 0.91 µg/mL (HeLa), 1.10 µg/mL (HepG2) and 2.29 (PLP2).

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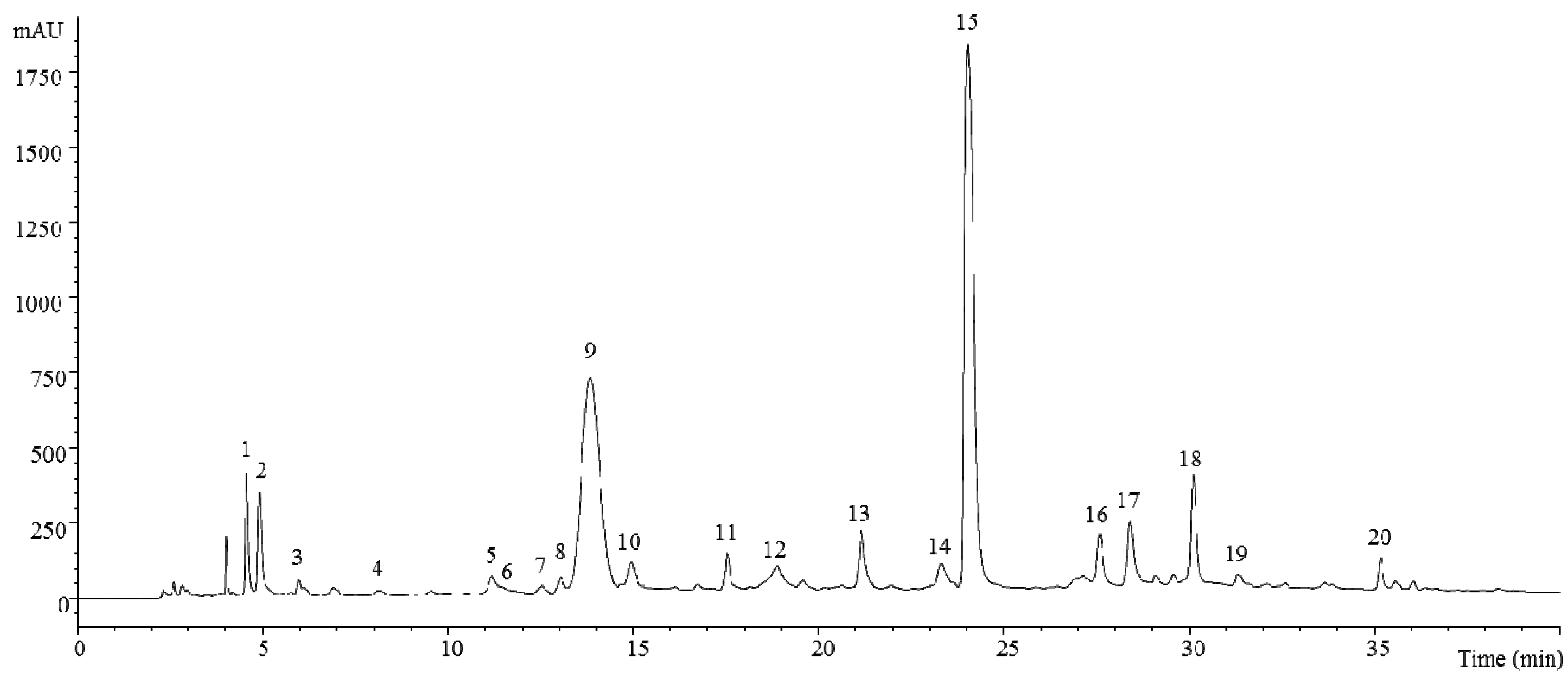

Figure 1. Phenolic profile of *M. officinalis* decoction, recorded at 280 nm.

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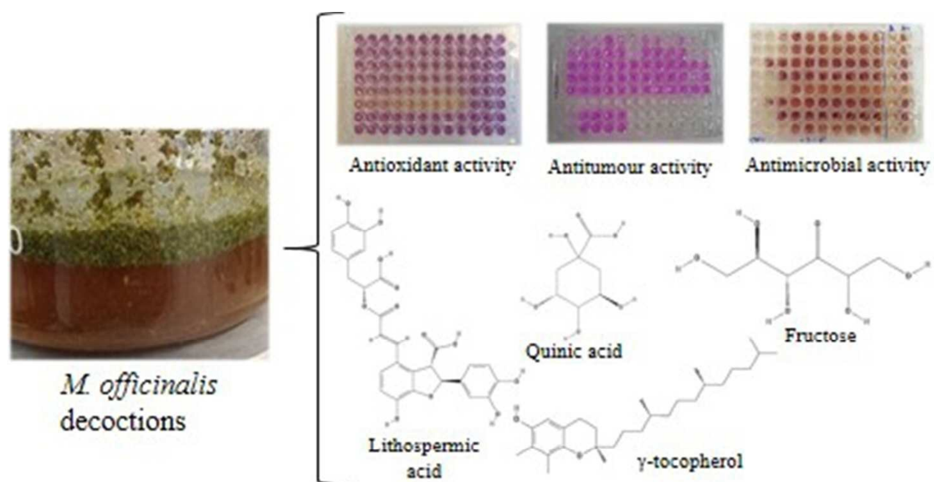

Graphical abstract
80x46mm (150 x 150 DPI)

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