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Reference:
Pereira Catarina Guerreiro, Barreira Luísa, Bijttebier Sebastiaan, Pieters Luc, Neves Vanessa, Rodrigues Maria João, Rivas Ricardo, Varela João, Custódio Luísa.- Chemical profiling of infusions and decoctions of Helichrysum italicum subsp. picardii by UHPLC-PDA-MS and in vitro biological activities comparatively with green tea (Camellia sinensis) and rooibos tisane (Aspalathus linearis)
Journal of pharmaceutical and biomedical analysis - ISSN 0731-7085 - 145(2017), p. 593-603
Full text (Publisher's DOI): https://doi.org/10.1016/J.JPBA.2017.07.007
To cite this reference: https://hdl.handle.net/10067/1451180151162165141

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Chemical profiling of infusions and decoctions of *Helichrysum italicum* subsp. *picardii* by UHPLC-PDA-MS and *in vitro* biological activities comparatively with green tea (*Camellia sinensis*) and rooibos tisane (*Aspalathus linearis*)

Biochemical assets of *Helichrysum italicum picardii*

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1 **Abstract**

2 Several medicinal plants are currently used by the food industry as functional additives, for
3 example botanical extracts in herbal drinks. Moreover, the scientific community has recently
4 begun focusing on halophytes as sources of functional beverages. *Helichrysum italicum*
5 subsp. *picardii* (everlasting) is an aromatic halophyte common in southern Europe frequently
6 used as spice and in traditional medicine. In this context, this work explored for the first time
7 *H. italicum* subsp. *picardii* as a potential source of innovative herbal beverages with potential
8 health promoting properties. For that purpose, infusions and decoctions were prepared from
9 roots, vegetative aerial-organs (stems and leaves) and flowers and evaluated for *in vitro*
10 antioxidant and anti-diabetic activities. Samples were also assessed for toxicity in different
11 mammallian cell lines and chemically characterized by spectrophotometric methods and ultra-
12 high performance liquid chromatography–photodiode array–mass-spectrometry (UHPLC-
13 PDA-MS). Results were expressed relating to 'a cup-of-tea' and compared with those
14 obtained with green tea (*Camellia sinensis*) and rooibos tisane (*Aspalathus linearis*). Tisanes
15 from the everlasting's above-ground organs, particularly flowers, have high polyphenolic
16 content and several phenolics were identified; the main compounds were chlorogenic and
17 quinic acids, dicaffeoylquinic-acid isomers and gnaphaliin-A. The antioxidant activity of
18 beverages from the everlasting's above-ground organs matched or surpassed that of green tea
19 and rooibos. Its anti-diabetic activity was moderate and toxicity low. Overall, our results
20 suggest that the everlasting is a potential source of innovative and functional herbal
21 beverages.

22

23 **Keywords:** Herbal beverages, Functional beverages, Phenolics, Oxidative stress

24 **1. Introduction**

25 Tea is one of the most common beverages in the world. Pairing a pleasant taste to stimulating
26 effects and potential health benefits, this popular drink is a cocktail of biologically active
27 phytochemicals as, for example, catechins and gallocatechins. Herbal teas, or tisanes, are
28 infusions or decoctions of any plant material whereas real teas are prepared from the leaves of
29 the tea plant, *Camellia sinensis* (L.) Kuntze. [1]. The health benefits derived from the
30 consumption of real tea, particularly the green type, are well described and include cancer
31 prevention, reduction of cardiovascular risk, anti-diabetic and anti-obesity properties and / or
32 protection against oxidative damage and oxidative stress-related diseases [1,2]. As for herbal
33 teas, consumption benefits can be associated with the plants' medicinal properties. For
34 example, the popular herbal red tea, from the rooibos plant *Aspalathus linearis* (Burm.f.)
35 Dahlg., is marketed for its high antioxidant and anti-ageing potential [3].

36 Nowadays, a wide panoply of medicinal plants (e.g. *Aloe vera* and *Hibiscus* sp.) are
37 used by the food industry as sources of functional additives, such as botanical extracts in
38 herbal beverages, and are commercially available in local stores and supermarkets to be
39 consumed as tisanes for health-related purposes [4]. But medicinal extremophiles, halophytes
40 in particular, although representing an outstanding reservoir of bioactive compounds are still
41 quite unexploited [5]. Nevertheless, the scientific community has just recently begun focusing
42 on aqueous extracts from halophytes with potential to be functional beverages, like *Limonium*
43 *algarvense* Erben [6] and *Crithmum maritimum* L. [7]. Moreover, specialty stores have started
44 to sell some halophytes as functional herbal beverages, namely sea buckthorn (*Hippophae*
45 *rhamnoides*), and the gourmet food market has also turned its attention to halophyte products,
46 like *Salicornia* sp. [8,9].

47 *Helichrysum* plants (Asteraceae), commonly called "everlasting", have medicinal uses
48 reported since the first centuries, its decoctions being referred to as diuretic, or used to treat

49 urinary disorders, burns, venomous bites or hernias [10]. *Helichrysum italicum* (Roth) G. Don
50 plays an important role in the traditional medicine of Mediterranean countries and is often
51 used as spice due to its curry-like scent. Its subspecies *H. italicum* (Roth) G. Don subsp.
52 *picardii* (Boiss & Reuter) Franco is a facultative halophyte found in the southern Europe,
53 including Portugal [10,11]. Folk therapeutic uses of infusions and decoctions of the plant are
54 associated to analgesic properties and dermatologic, respiratory and digestive disorders with
55 inflammatory, allergic or infectious components [10,12]. Research concerning *H. italicum*
56 focuses mainly on organic extracts and *in vitro* studies indicate that this everlasting has
57 antimicrobial and anti-inflammatory properties, among others, and contains a wide
58 phytochemical profile that includes different classes of bioactive molecules from which the
59 most common are phenolic compounds and terpenes [10,12,13].

60 Herbal teas are a major source of dietary bioactive phytochemicals in our diet, including
61 phenolics with recognized antioxidant properties and with beneficial outcomes in certain
62 health challenges [4,14]. Oxidative stress is an underlying cause for several degenerative
63 diseases and the use of antioxidants can prevent or reduce the severity of oxidative stress-
64 related diseases [15]. Moreover, consumption of antioxidants from natural sources has
65 become a consumer-trend for health purposes, promoting the antioxidant market growth [14].
66 In this sense, medicinal plants like *H. italicum* subsp. *picardii* have a high commercial
67 potential to be explored not only in traditional medicine but also as herbal functional
68 beverages in the health foods category. A similar approach has already been reported for
69 different plants, including glycophytes such as *Lathyrus* species [16] and *Hymenocrater*
70 *bituminosus* L. [17] and halophytes, like *Chritmum maritimum* L. [7], *Limonium algarvense*
71 L. [6] and *Juncus* species [18].

72 To the best of our knowledge there is no information regarding the biological activities
73 or phenolic composition of infusions and decoctions of this everlasting species. Therefore,

74 this work aimed to evaluate if this everlasting could be explored as a source of innovative
75 food additives. For that purpose, infusions and decoctions were prepared from roots,
76 vegetative aerial-organs and flowers from the everlasting and evaluated for *in vitro*
77 antioxidant and anti-diabetic activities, and for polyphenolic profile. Additionally, a
78 preliminary toxicological evaluation was made *in vitro* by determining samples toxicity
79 against mammalian cells. Green and herbal red (rooibos) teas were used for comparison since
80 they are the most consumed tea beverages worldwide and are sought for their strong
81 antioxidant properties.
82
83 **2. Materials and methods**
84 *2.1. Reagents*
85 All chemicals used were of analytical grade. Reagents 1,1-diphenyl-2picrylhydrazyl (DPPH),
86 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), sulphanilamide, N-(1-
87 naphthyl) ethylenediamine dihydrochloride (NED), ethylenediamine tetraacetic acid (EDTA),
88 pyrocatechol violet, sodium nitrite, aluminium chloride, butylated hydroxytoluene (BHT),
89 formic acid and ammonium formate were purchased from Sigma-Aldrich (Germany). Ultra-
90 high performance liquid chromatography (UHPLC) grade acetonitrile was purchased from
91 Biosolve (The Netherlands). Merck (Germany) supplied phosphoric acid and Folin-
92 Ciocalteau (F-C) phenol reagent. Commercially available mixtures to calibrate the mass
93 spectrometer, i.e., MSCAL5–1EA (caffeine, tetrapeptide ‘‘Met-Arg-Phe-Ala’’, Ultramark) for
94 positive ion mode and MSCAL6–1EA (sodium dodecylsulfate, taurocholic acid sodium salt,
95 Ultramark) for negative ion mode were purchased from Supelco (USA). Reference standards
96 apigenin, catechin, epicatechin, epigallocatechin, epigallocatechin gallate, flavone, 4-
97 hydroxybenzaldehyde, naringin, quercetin, rutin, uvaol, and caffeic, chlorogenic, coumaric,
98 ferulic, gallic, gentisic, m-hydroxybenzoic, p-hydroxybenzoic, oleanolic, rosmarinic, salicylic

99 and syringic acids were purchased from Sigma-Aldrich (Germany); apigenin-7-O-glucoside
100 (apigetrin), cyanidin-3-O-arabinoside, cyanidin-3-O-galactoside chloride (ideain chloride),
101 cyanidin-3-O-glucoside chloride (kuromanin chloride), cyanidin-3-O-rutinoside chloride
102 (keracyanin chloride), (+)-dihydrokaempferol ((+)-aromadendrin), galangin, kaempferol,
103 kaempferol-3-O-glucoside (astragalin), luteolin, naringenin, quercetin-3-O-arabinoside
104 (avicularin), quercetin-3-O-galactoside (hyperin), quercetin-3-O-glucoside (isoquercitrin),
105 quercetin-3-O-rhamnoside (quercitrin), phloretin, phloretin-O-20-glucoside (phloridzin), and
106 procyanidin B2 were purchased from Phytolab (Germany); hesperidin, hesperidin methyl
107 chalcone, limonin, neohesperidin dihydrochalcone, protocatechuic acid, propyl gallate, and
108 sinapinic, dihydrocaffeic, hydroferulic, ellagic, and quinic acids were obtained from Sigma–
109 Aldrich (Belgium). Additional reagents / solvents were obtained from VWR International
110 (Belgium).
111
112 *2.2. Plant collection*
113 Whole plants of *H. italicum* subsp. *picardii* were collected in the Ria Formosa area, a coastal
114 lagoon in south Portugal, near Cabanas de Tavira (37°07'51.3"N 7°36'35.6"W) in June 2013.
115 The taxonomical classification was performed by the botanist Dr. Manuel J. Pinto (National
116 Museum of Natural History, University of Lisbon, Botanical Garden, Portugal). A voucher
117 specimen is kept at the herbarium of the Marbiotech laboratory (voucher code MBH32).
118 Plants were divided in roots, vegetative aerial-organs (stems and leaves) and flowers, oven
119 dried for 3 days at 50°C, milled and stored at -20°C until use. Dried leaves of green tea plant
120 (*C. sinensis*, produced in Azores, Portugal) and rooibos plant (*A. linearis*, produced in Cape
121 Town, South Africa.) were bought in a regional supermarket, milled and stored at -20ºC.
122
123 *2.3. Extracts preparation*: *"cup-of-tea" infusions and decoctions*

124 Extracts were prepared by homogenizing 1 g of the dried plant material in 200 mL of
125 ultrapure water to equal a "cup-of-tea". For infusions biomass was immersed in boiling water
126 for 5 min, for decoctions biomass was boiled in water for 5 min. Aqueous extracts were
127 filtered (Whatman nº 4) and stored at -20ºC until use. Independent extractions ($n \geq 3$) of the
128 different plant parts were made and extracts from the different extractions were tested for
129 their bioactivities and phytochemical (spectrophotometric) content. As no significant
130 differences were found among corresponding extracts from the different extractions, for the
131 LC-PDA-MS analysis aliquots of the extracts were freeze-dried and pooled accordingly, and
132 stored in a moist free environment at -20 °C protected from light.

133

134 *2.4. Phytochemical composition of the extracts*

135 *2.4.1. Total polyphenols (TPC), flavonoids (TFC) and condensed tannin (CTC) content*

136 TPC was determined by the F-C assay with absorbance measured at 725 nm using gallic acid
137 as a standard; results were expressed as milligrams of gallic acid equivalents per cup-of-tea
138 (mg GAE/200mL). TFC was estimated by the aluminium chloride colorimetric method;
139 absorbance was measured at 510 nm using rutin as standard and results were expressed as
140 rutin equivalents per cup-of-tea (mg RE/200mL). The CTC was assessed by the 4-
141 dimethylaminocinnamaldehyde (DMACA) method; absorbance was measured at 640 nm
142 using catechin as standard and results were expressed as mg of catechin equivalents per cup-
143 of-tea (mg CE/200mL). All methods are described in Rodrigues et al. [19].

144

145 *2.4.2. Hydroxycinnamic acid derivatives (HAD) and flavonols content*

146 HAD and flavonols were estimated as described by Rodrigues et al. [19]. Absorbance was
147 read at 320 nm to determine HAD using caffeic acid as standard, and at 360 nm to estimate

148 flavonols using quercetin as standard. Results were expressed as standard equivalents per cup-
149 of-tea (CAE and QE, respectively; mg/200mL).
150
151 *2.4.3. Profile of moderately polar compounds by UHPLC*
152 Standard stock solutions were prepared in UHPLC-grade methanol (1 mg/mL) and stored in
153 the dark, 4°C (standards are listed in section 2.1. Reagents). Dilutions were prepared in 60:40
154 (*v*:*v*) methanol:ammonium formate buffer (40 mM). Approximately 15 mg of freeze-dried *H.*
155 *italicum* subsp. *picardii* pooled extracts were dissolved in 20 mL 60:40 methanol:water +
156 ammonium formate (40 mM) followed by 10 min sonication (40 kHz, 100W). Samples were
157 centrifuged (3000 rpm), supernatants diluted 100x and stored together with undiluted extracts
158 at 4°C until analysis. Both undiluted and 100-fold diluted everlasting extracts were analysed
159 with a generic ultra-high performance liquid chromatography – photodiode array – mass
160 spectrometry (UHPLC-PDA-MS) method for moderately polar phytochemicals adapted from
161 De Paepe et al. [20]. For analysis, 5 µL of extract was injected with a CTC PAL™
162 autosampler (CTC Analytics, Zwingen, Switzerland) on a Waters Acquity UPLC BEH
163 SHIELD RP18 column (3.0 mm×150 mm, 1.7 µm; Waters, Milford, MA) and
164 thermostatically (40°C) eluted with an Accela™ quaternary solvent manager and a 'Hot
165 Pocket' column oven (Thermo Fisher Scientific, Bremen, Germany). The mobile phase
166 solvents consisted of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B),
167 and the gradient was set as follows (min/%A): 0.0/100, 9.91/74, 18.51/35, 18.76/0, 23.76/0,
168 23.88/100, 26.00/100. For detection, an MS (Q Exactive™; Thermo Fisher Scientific,
169 Bremen, Germany) was used with heated electrospray ionization (HESI). For quantitative
170 analysis, full scan data were acquired using polarity switching with a mass/charge (*m*/*z*) range
171 of 120-1800 and resolving power set at 70 000 at full width at half maximum (FWHM). Spray
172 voltage was set at ±2.5 kV, sheath gas and auxiliary gas at 47 and 15 (adimensional)

173 respectively, and capillary temperature at 350°C. The lowest calibration point that was
174 included in the calibration curve was used to calculate the LOQs. The LOQs can be different
175 for the same compound between extracts as the yields (mg dry extract/g plant value) differ per
176 extract; these yields were used to calculate the LOQs. The concentration ranges described by
177 De Paepe et al. [20] were also used during the present work. As quality control, a midrange
178 calibration point was chosen as continuing calibration verification standard (CCV) in order to
179 verify that the calibration of the analytical system was still acceptable. The frequency of CCV
180 analysis was once every ten injections. Data were also recorded using data dependent
181 fragmentation ($ddMS^2$) in positive and negative ionization mode (one analysis per mode) to
182 obtain additional structural information (resolving power set at 17 500 FWHM, stepped
183 collision energy 10, 30, 50 V, isolation window: 4 *m*/*z*). The PDA detector was set to scan
184 from 190 to 800 nm during all analyses. Results regarding concentrations of identified
185 compounds were calculated as µg/g dried plant material, i.e. µg/cup-of-tea, based on the
186 extracts' yield.
187
188 *2.5. Antioxidant activity*
189 *2.5.1. Determination of antioxidant activity by five radical-based assays*
190 The radical scavenging activities (RSA) on DPPH, nitric oxide (NO), ABTS, superoxide
191 ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) radicals was evaluated as described previously [6,19] using
192 respectively BHT, catechin (1 mg/mL) or ascorbic acid (10 mg/mL) as positive controls.
193 Results were expressed as percentage of antioxidant activity in a cup-of-tea, relative to a
194 control containing ultrapure water.
195
196 *2.5.2. Determination of antioxidant activity by three metal-related methods*

197 The ferric reducing antioxidant power (FRAP) of the extracts, i.e., their ability to reduce Fe^{3+},
198 along with the metal chelating activities on copper (CCA) and iron (ICA) were assayed as
199 described by Rodrigues et al. [19], using BHT and EDTA as positive controls (1 mg/mL).
200 Results were calculated and expressed as percentage of antioxidant activity in a cup-of-tea,
201 relative to a positive control for FRAP and to a negative control (ultrapure water) for CCA
202 and ICA.

203

204 *2.6* In vitro *anti-diabetic activity: inhibition of α-glucosidase*

205 The microbial α-glucosidase inhibitory activity was determined according to Kwon et al.
206 [21], using acarbose as a positive control (10 mg/mL). The enzyme was obtained from the
207 yeast *Saccharomyces cerevisiae*. Results were expressed as percentage of inhibitory activity
208 in a cup-of-tea, relative to a control (ultrapure water).

209

210 *2.7. Toxicological evaluation of the samples*

211 Cell culture was made as described by Rodrigues et al. [6]. Murine microglia cell line (N9
212 cells) was obtained from the Faculty of Pharmacy and Centre for Neurosciences and Cell
213 Biology (University of Coimbra, Portugal); human hepatocellular carcinoma cell line (HepG2
214 cells) was provided by Dr. Vera Marques, and murine bone marrow stromal cell line (S17
215 cells) by Dr. Nuno Santos (CBME, University of Algarve, Portugal). Toxicity of the samples
216 was evaluated following Rodrigues et al. [6]. Freeze-dried extracts were dissolved directly in
217 culture medium and applied at the concentration of 100 μg/mL for 72 h. Cells incubated with
218 culture medium alone were considered as negative control; hydrogen peroxide (H_2O_2) was
219 used as positive control for cell toxicity. Cell viability was determined by the MTT assay and
220 absorbance measured at 590 nm. Results were expressed in terms of cell viability (%).

221

222 *2.8. Statistical analysis*

223 Results were expressed as mean ± standard deviation (SD), and experiments were conducted
224 at least in triplicate. Significant differences ($p < 0.05$) were assessed by one-way analysis of
225 variance (ANOVA) using the Tukey pairwise multiple comparison test or Kruskal Wallis
226 one-way analysis of variance on ranks (Dunn's test) when parametricity of data did not
227 prevail. Statistical analyses were performed using XLStat2014® by Addinsoft (Spain).

228

229 **3. Results and Discussion**

230 *3.1. Phytochemical analysis*

231 The phenolic contents of the samples were assessed by spectrophotometric methods, namely
232 the total contents in polyphenols (TPC), flavonoids (TFC) and condensed tannins (TCT),
233 hydroxycinnamic acid derivatives (HAD) and flavonols, and are presented as mg per cup-of-
234 tea (mg/200mL) in Table 1 (further information pertained to the methods is presented in Table
235 S1, supplementary material). The green tea decoction and infusion had the highest TPC (107
236 and 91.7 mg/cup-of-tea, respectively), followed by the extracts from flowers and vegetative
237 aerial-organs (stems & leaves) of *H. italicum* subsp. *picardii*, which in turn were richer in
238 TPC than rooibos tisanes. The TFC was greatest in the flowers' decoction from *H. italicum*
239 subsp. *picardii* (119 mg/cup-of-tea) followed by its infusions and the vegetative aerial-organs
240 extracts, showing higher flavonoid content than the green and herbal red teas. The HAD and
241 flavonols showed a similar pattern to the TFC: highest values in the decoction from the
242 everlasting flowers (65.9 and 38.1 mg/cup-of-tea, respectively), and cups-of-tea from flowers
243 and stems & leaves having more of these compounds than green teas and rooibos tisanes.
244 Content of condensed tannins in *H. italicum* subsp. *picardii* teas was below the limit of
245 quantification (2.45 mg/cup-of-tea), which can be considered positive in terms of flavoring
246 from the consumer's perspective given the astringent taste these compounds are known for.

247 Overall, tisanes from *H. italicum* subsp. *picardii* above-ground organs, particularly decoctions
248 from flowers, can be considered of high polyphenolic content especially if compared to the
249 phenolic-rich *C. sinensis* and herbal *A. linearis* teas [22,23].

250 The phytochemical profile of infusions and decoctions from *H. italicum* subsp. *picardii*
251 organs was further analysed by a generic LC-PDA-MS method for moderately polar
252 phytochemicals, such as phenolic constituents. The analytical LC-PDA-MS methodology,
253 adapted from De Paepe et al. [20], was previously validated by the same authors [20] for the
254 quantitation of phenolic constituents in apple cultivars. The performance characteristics taken
255 into account for the validation of the measurement method were curve fit, range, sensitivity
256 (instrumental detection limit, instrumental quantification limit, method limits of detection and
257 quantification), precision (repeatability, intermediate precision) and trueness, as well as
258 specificity. The goal of the LC-PDA-MS analyses during this study is to explore the
259 phytochemical profile of infusions and decoctions from *H. italicum* subsp. *picardii* organs,
260 *i.e.* to (tentatively) identify unknown phytochemical constituents and to get an estimate of
261 their concentrations (when reference standards were available) and relative abundances. It is
262 out of the scope of the present work to perform a method validation for accurate quantitation
263 of phenolic constituents in *H. italicum* extracts. In Table 2 are the concentrations of phenolics
264 found in the everlasting extracts, using reference standards for quantification. However, in
265 natural products research analytical standards are often very expensive or not commercially
266 available. Therefore, when no standards were available, tentative identification of compounds
267 was accomplished based upon the available chromatographic and spectral information.
268 Orbitrap MS detectors can routinely generate mass spectra with a resolving power up to 140
269 000 FWHM and obtain mass accuracies within 1 – 2 ppm; this enables the calculation of the
270 most probable molecular formulae of the generated precursor and product ions [24]. The
271 higher the resolution and the mass accuracy, the more confident compound identification

272 becomes. This utility combined with the selectivity and sensitivity of current hyphenated
273 UHPLC-PDA-MS systems has paved the way towards generic phytochemical analysis [25].
274 During the current study, 96% and 89% of the mass deviations measured for the precursor
275 ions in HESI negative and HESI positive mode, respectively, were ≤ 2 ppm. During this
276 study, a hybrid quadrupole-orbital trap MS-analyser (Q Exactive, Thermo Fisher Scientific)
277 was used, which enables selective ion fragmentation. In a selective ion fragmentation
278 experiment, ions of a particular *m*/*z*-range are selected (precursor ions) with a quadrupole and
279 subsequently fragmented into product ions. This functionality contributes significantly to
280 compound identification by generating clean product ion spectra. Selective ion fragmentation
281 is particularly useful for associating product ions with precursor ions during coelution of
282 multiple compounds, as is often the case in complex plant extracts. Data-dependent
283 fragmentation was used to obtain clean product ion spectra of the detected analytes. Product
284 ions are substructures of the precursor ions, formed during fragmentation: structures were
285 assigned to unknown peaks only when both the *m*/*z*-values and molecular formulae/structures
286 of the precursor and product ions were in agreement. Additional information for dereplication
287 was often acquired from PDA spectra, in-house and commercial compound databases
288 (Dictionary of Natural Products [26], ChemSpider [27] and PubChem [28]) and peer reviewed
289 publications. An in-house database with chromatographic and spectral data of reference
290 standards and previously identified compounds was used to compare chromatographic
291 behaviour and product ion spectra of structurally similar compounds found during the current
292 study. These commercial databases allow to find known molecular structures for a most
293 probable molecular formula obtained from a precursor ion. As described by Sumner et al.
294 [29], the metabolomics community consensus is that the leading challenge of metabolomics is
295 the chemically accurate identification of large numbers of metabolites observed in various
296 non-targeted profiling experiments: accurate structure identification requires significant effort

297 which increases dramatically with the increased amount of detected metabolites per analysis.
298 The Chemical Analysis Working Group of the Metabolomics Standards Initiative proposed
299 four identification classes: 1 – confident identifications based upon a minimum of two
300 orthogonal data relative to an authentic standard; 2 – putatively annotated compounds (e.g.
301 without chemical reference standards, based upon physicochemical properties and/or spectral
302 similarity with public/commercial spectral libraries; 3 – putatively characterized compound
303 classes (e.g. based upon characteristic physicochemical properties of a chemical class of
304 compounds, or by spectral similarity to known compounds of a chemical class); 4 – unknown
305 compounds. The compounds identified with reference standards during the present study
306 belong to confidence class 1, while the compounds that were tentatively identified without
307 reference standards belong to classes 2 (reported in *H. italicum* previously) and 3 (not
308 reported in *H. italicum* before). Since different compounds tend to have different ionization
309 efficiencies during LC-MS analysis no absolute quantitative comparison can be made but
310 relative abundances per compound in-between samples can be calculated. Table 3 shows
311 relative abundances of the (tentatively) identified compounds in the everlasting extracts.
312 Diagnostic chromatographic and MS data used for compound identification plus literature
313 used for compound identity confirmation can be found in Table S2 (supplementary material).
314 Figure 1 represents the extracts' PDA-chromatograms at combined wavelengths (280-330
315 nm).

316 A wide versatility of predominantly phenolic constituents was (tentatively) identified in
317 *H. italicum* subsp. *picardii* extracts (Tables 2 and 3). Most phenolics were already described
318 in *Helichrysum* species except for salicylic acid that is, to the best of our knowledge, here
319 firstly described in the genus. For *H. italicum*, no reports were found detailing quinic,
320 protocatechuic, *p*-hydroxybenzoic and syringic acids (but quinic acid derivatives are
321 described in Mari et al. [30]), rutin, apigetrin and quercetin (its glycoside quercetin 3-*O*-

322 glucoside is reported in Mari et al. [30]), which are currently described for the first time in
323 this species. Additionally, Table S2 details the tentatively identified compounds that were
324 already reported in *H. italicum*.

325 According to Table 2, a higher diversity of compounds and with consistently higher
326 levels was found in tisanes from vegetative aerial-organs and flowers along with a similarity
327 in the composition of major phenolics in these aboveground organs. The main phenolics
328 detected were quinic and chlorogenic acids, higher in the vegetative aerial-organs (8.2 - 8.7
329 and 6.9 - 7.7 mg/cup-of-tea, respectively) than in the flower extracts (4.7 - 4.9 and 5.2 - 6.0
330 mg/cup-of-tea, respectively). Preferentially detected in flower's tisanes was astragalin (0.49 -
331 0.6 mg/cup-of-tea), hyperin and/or isoquercitrin (they are isomers and co-elute; 0.35 - 0.36
332 mg/cup-of-tea) and caffeic acid (0.14 - 0.15 mg/cup-of-tea), while syringic and oleanolic
333 acids were higher in vegetative aerial-organs extracts (0.16 - 0.17 and 0.11 mg/cup-of-tea,
334 respectively). Table 3 and Figure 1 also show the composition similarity of major constituents
335 and higher compound diversity of tisanes from vegetative aerial-organs and flowers, with the
336 main tentatively identified compounds being dicaffeoylquinic acid isomers and gnaphaliin A.
337 Note that the "maximum area detected" (Table 3) provides a semi-quantitative information of
338 compound abundance but it should not be interpreted as absolute quantitative comparison
339 since this is not possible based on areas obtained with LC-MS. Composition of root extracts
340 was very different from that of above-ground organs with less abundance and diversity of
341 compounds and, unfortunately, their most abundant compounds (peaks 25 and 30 in Figure 1)
342 shown in the PDA-chromatograms were not identified (Figure 1, Table 3). Moreover, some
343 major compounds already reported in *H. italicum* were not detected, such as naringenin-7-*O*-
344 glucoside [31], along with other phenolics compiled in Maksimovic et al. [13], possibly due
345 to the extraction solvent / methods or to the natural phytochemical variations in-between
346 plants, a variability that has been already reported for this species [10,32].

347 Secondary metabolites like phenolic compounds are implicated in the plant's response
348 to pressures such as predation, infection by pathogens and parasites or wounding, but content
349 in phenolics may also increase under abiotic stress conditions [33]. Extreme temperatures,
350 UV-radiation, salinity or drought are pronounced environmental challenges for halophyte /
351 extremophile plants that live and thrive under such harsh conditions [5,33]. Abiotic stress
352 enhances production and accumulation of reactive oxygen species (ROS) demanding a
353 powerful antioxidant system. As a result, those plants synthesize antioxidant compounds
354 including polyphenolics to counteract ROS and protect cellular structures and metabolic
355 functions from oxidative damage [5,34]. Therefore, the higher phenolic accumulation and
356 assortment in stems & leaves and flower extracts might suggest their protective role was in
357 play possibly against excessive UV-radiation, heat and predation in the above-ground organs.
358

359 *3.2. Biological activities:* in vitro *antioxidant and anti-diabetic properties*

360 ROS, such as the superoxide or hydroxyl radicals, are formed naturally in biological systems
361 but an imbalance between the antioxidant defenses and ROS production can result in
362 damaging oxidative stress. This involves damage to cellular macromolecules (like proteins,
363 lipids, DNA) and deregulation of cellular functions with implications in several degenerative
364 and pathological alterations (for example, aging, cancer, diabetes, and neurodegenerative
365 diseases) [15,35]. However, it is well documented that antioxidants effectively fight free
366 radicals and oxidative damage and thus they are able to reduce or prevent the severity of
367 different oxidative stress-related diseases [14,35]. Antioxidants are thus an essential group of
368 medicinal preventive molecules and are also used as food additives to prevent harmful
369 modifications of foods which are sensitive to oxidation [5]. In this context, there is a growing
370 economical and security interest on the identification of halophyte species with high
371 antioxidant content aiming at its use in the food industry and in preventive medicine to

372 replace synthetic antioxidants [5].

373 In this work, the antioxidant potential of a cup-of-tea from *H. italicum* subsp. *picardii* 374 organs was assessed in comparison to those of *C. sinensis* and *A. linearis* and results are 375 summarized on Table 4. Overall, the antioxidant activity of everlasting's vegetative aerial-376 organs and flowers herbal teas matched or even surpassed that of green teas and rooibos 377 beverages (Table 4). Comparing with *C. sinensis* teas, tisanes from *H. italicum* subsp. *picardii* 378 flowers and stems & leaves were more effective in scavenging DPPH, NO and $OH^{\bullet}$ radicals 379 and had similar RSA against ABTS. In relation to *A. linearis* herbal teas, the same 380 everlasting's tisanes matched its DPPH and ABTS scavenging capacity and surpassed its $OH^{\bullet}$ 381 RSA; from those tisanes, infusions along with roots decoctions were also more active against 382 the NO radical. Moreover, stems & leaves extracts, flowers infusions and roots decoctions 383 had higher $O_2^{\bullet-}$ scavenging activity than both green and herbal red teas. As for the metal-384 related activities, the capacity to reduce iron (FRAP) was similar between the everlasting's 385 vegetative aerial-organs tisanes and flowers' decoction and the commercial beverages, but 386 green tea was more active in chelating copper and iron. This was probably due to its higher 387 tannin contents since tannins are known metal chelating agents [36]. However, tisanes from 388 *H. italicum* subsp. *picardii* vegetative aerial-organs and flowers were more efficient in 389 chelating copper than rooibos extracts. High antioxidant activity has already been described in 390 *H. italicum* [12,37,38] but studies have seldom focused on aqueous extracts [10] and none 391 was found concerning "cup-of-tea" samples from the different anatomical organs. Our results 392 confirm the strong *in vitro* antioxidant capacity of *H. italicum* subsp. *picardii,* particularly 393 flowers and stems & leaves, and thus show that beverages made from this plant may be useful 394 in preventing oxidative-stress diseases much like the world renowned green tea is reported to 395 be [1,2].

396 The therapeutic benefits of herbal beverages are related to their high polyphenolic content [4].
397 Phenolics are recognized powerful antioxidants [5,35] and the everlasting's antioxidant
398 capacity seems to reflect its high phenolic content. In fact, flowers and vegetative aerial-
399 organs were consistently the extracts with higher amounts of TPC, TFC, HAD and flavonols
400 (Table 1) and, except for TPC, they were higher than those detected in the green and herbal
401 red teas. The amount and diversity of phenolics can contribute to the stronger antioxidant
402 activities in tisanes from flowers and stems & leaves: they were determined at higher amounts
403 in these organ's extracts and in greater variety than in roots (Tables 2 and 3). Moreover, the
404 levels of the main components detected in these organs namely quinic and chlorogenic acids,
405 which are reported antioxidant compounds [39,40], were more than 10-fold higher in the
406 above-ground organs. Additionally, some of the other phenolics can have also contributed
407 through addictive and / or synergistic effects. For example, the main tentatively identified
408 compounds dicaffeoylquinic acid isomers are also described as strong antioxidants [41].
409 Some of the phenolics identified in this everlasting's extracts are described in literature as
410 natural bioactive compounds, which can help explain the plant's medicinal uses. For example,
411 besides the above-mentioned antioxidant compounds, chlorogenic acid has anti-diabetic
412 properties [40], and gnaphaliin, pinocembrin, tiliroside and arzanol have anti-inflammatory
413 activity [10,13]. These and other bioactivities (antiviral, antimicrobial, cytotoxic) have been
414 confirmed in extracts or isolated compounds from other *H. italicum* subspecies [10,13] but, to
415 the best of our knowledge, the present study is the first reporting biological activities and
416 phenolic composition of infusions and decoctions from this everlasting subspecies.

417 The anti-diabetic potential was assessed through the inhibition of α-glucosidase; the
418 inhibition of such carbohydrate-hydrolyzing enzyme is a therapeutic strategy for the treatment
419 of diabetes mellitus type 2 (T2DM), delaying carbohydrate digestion and reducing
420 postprandial hyperglycemia [31,42]. *H. italicum* subsp. *picardii* tisanes had a moderate to low

421 activity particularly if compared to green tea (Table 5). A “cup-of-tea” from everlasting
422 flowers induced around 50% of α-glucosidase inhibition (infusion: 48.3%, decoction: 50.4%),
423 followed by tisanes from the vegetative aerial-organs (infusion: 41.2%, decoction: 45.7%),
424 while the roots’ enzyme inhibition was lowest (infusion: 22.9%, decoction: 31.0%). The
425 current α-glucosidase inhibitory capacity in everlasting’s tisanes was lower than reported by
426 Garza et al. [31] in *H. italicum* methanolic extracts but this difference can be ascribed to the
427 different extraction solvents / processes used and / or to a natural variability in secondary
428 metabolites’ content. Nevertheless, kaempferol 3-*O*-glucoside (astragalin), presently found at
429 noteworthy concentrations in flowers tisanes (0.49 - 0.60 mg/cup-of-tea, Table 2), has shown
430 *in vitro* and *in vivo* inhibitory effect on α-glucosidase [43]. Furthermore, chlorogenic acid,
431 one of the main compounds here determined in everlasting’s herbal teas, has claimed
432 hypoglycemic and hypolipidemic effects and can regulate glucose and lipid metabolic
433 disorders associated to the progression of diabetes and obesity, among others [40]. Our results
434 thus suggest that *H. italicum* subsp. *picardii* flowers’ tisanes can be useful in the control of
435 glucose levels, when used in combined anti-diabetic strategies, by inhibiting dietary
436 carbohydrate digestive enzymes. In fact, Garza et al. [31] also found this anti-diabetic
437 potential in methanolic extracts from *H. italicum*. Moreover, oxidative stress has been found
438 to mediate the effects of diabetes [42] and given the strong antioxidant potential of the
439 everlasting flowers’ tisanes, its consumption may also indirectly contribute to prevent or
440 attenuate the disease’s symptoms. As expected the commercial teas had a high *in vitro* anti-
441 diabetic potential: *C. sinensis* teas had 99% of α-glucosidase inhibition and *A. linearis* herbal
442 teas 72%. In fact, the consumption of both teas, but especially green tea, is associated with
443 anti-diabetic effects, either as prevention or to ameliorate symptoms associated with T2DM
444 [1,2,23].
445

446 *3.3. Toxicological evaluation*

447 To ascertain the safety of new products for human consumption, for example plant extracts or
448 herbal beverages, it is crucial to determine their toxicity. Preliminary toxicity screenings of
449 compounds or natural extracts are commonly assessed by *in vitro* methods such as
450 cytotoxicity towards different mammalian cell lines, providing fast and reliable results and
451 reducing *in vivo* testing [6,7,44,45]. In this sense, a preliminary toxicological evaluation of *H.*
452 *italicum* subsp. *picardii* tisanes was performed on three cell lines to assess cellular viability
453 after incubation with the extracts, alongside with *C. sinensis* and *A. linearis* beverages for
454 comparison. Results are summarized in Figure 2. The everlasting's extracts had low toxicity
455 with cell viability values similar or higher than those obtained for green and herbal red teas.
456 None of the extracts from the three plants were toxic for hepatocarcinoma (HepG2) cells. For
457 the microglia (N9) cell line, everlasting's tisanes toxicity was very low (>80% viability,
458 except for stems & leaves' infusion which was 73%), as was green tea, while rooibos extracts
459 exerted a moderate toxicity with cell viability between 58% and 66%. Stromal (S17) cells
460 were more sensitive to toxic effects but, nevertheless, everlasting roots' extracts were only
461 moderately toxic (53 - 56% viability) and not significantly different from the toxicity exerted
462 by commercial teas (56 - 61% viability), whereas everlasting's vegetative aerial-organs and
463 flowers had low toxicity (66 - 77% viability). Overall, these results are quite promising as
464 preliminary toxicological evaluation of the beverages under study, particularly if compared to
465 the ones obtained for the largely consumed green tea and rooibos tisanes, and suggest that
466 these aqueous extracts can be regarded as non-toxic beverages. *In vitro* toxicity studies of *H.*
467 *italicum* are scarce and include only essential oils and some organic extracts but nevertheless
468 they also indicate a favorable safe profile [10]. However, although *in vitro* cell culture
469 methods are generally accepted as a very effective method for safety testing [45], further
470 experiments on mammalian animal models should be pursued.

471

472 **4. Conclusion**

473 Our results indicate that infusions and decoctions made from *H. italicum* subsp. *picardii*
474 above-ground organs, particularly flowers, have a high and diverse polyphenolic content, with
475 similar or even higher antioxidant potential than the commercial green and herbal red teas,
476 showing moderate anti-diabetic potential and low toxicity in *in vitro* models. Altogether, our
477 data suggests that everlasting tisanes, especially those from flowers could be further explored
478 as potential health-promoting food additives to be used, for example, in innovative herbal
479 beverages.

480

481 **Acknowledgements**

482 This work received national funds through Foundation for Science and Technology (FCT,
483 Portugal) project CCMAR/Multi/04326/2013. Catarina Guerreiro Pereira and Maria João
484 Rodrigues acknowledges FCT for the PhD grants SFRH/BD/94407/2013 and
485 SFRH/BD/116604/2016, respectively. Sebastiaan Bijttebier thanks the Research Foundation -
486 Flanders (FWO) for a post-doc grant (12M8315N). Luísa Custódio was supported by FCT
487 Investigator Programme (IF/00049/2012).

488

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Table 1. Phenolic content[1] (mg/cup-of-tea) in infusions and decoctions from *H. italicum* subsp. *picardii* organs and from green (*C. sinensis*) and red (*A. linearis*) teas: total polyphenol content (TPC), total flavonoid content (TFC) condensed tannin content (CTC), hydroxycinnamic acid derivatives (HAD) and flavonols. In each column, different letters mean significant differences ($p < 0.05$).

Plant	Organ	Extract	TPC (mg GAE/200mL)	TFC (mg RE/200mL)	CTC (mg CE/200mL)	HAD (mg CAE/200mL)	Flavonols (mg QE/200mL)
H. italicum subsp. *picardii*	Roots	Infusion	13.9 ± 0.49^{i}	20.3 ± 1.66^{e}	0.0	13.1 ± 1.16^{e}	8.55 ± 1.13^{f}
		Decoction	20.5 ± 1.45^{h}	26.7 ± 2.77^{e}	0.0	17.4 ± 0.38^{d}	9.82 ± 0.30^{f}
	Vegetative aerial-organs	Infusion	62.0 ± 0.95^{e}	91.8 ± 9.67^{b}	<LQ	51.3 ± 2.86^{c}	24.8 ± 1.47^{c}
		Decoction	70.2 ± 4.35^{d}	89.0 ± 7.09^{b}	<LQ	56.3 ± 2.54^{b}	26.2 ± 1.18^{c}
	Flowers	Infusion	69.9 ± 3.88^{d}	101 ± 2.55^{b}	<LQ	58.3 ± 2.63^{b}	34.4 ± 1.62^{b}
		Decoction	76.5 ± 2.62^{c}	119 ± 15.6^{a}	0.0	65.9 ± 1.78^{a}	38.1 ± 1.10^{a}
C. sinensis		Infusion	91.7 ± 2.61^{b}	47.8 ± 2.23^{d}	67.3 ± 5.96^{b}	11.5 ± 1.08^{ef}	9.37 ± 0.83^{f}
		Decoction	107 ± 4.44^{a}	48.8 ± 7.58^{d}	73.7 ± 2.99^{a}	8.76 ± 1.07^{f}	6.12 ± 0.85^{g}
A. linearis		Infusion	43.1 ± 3.39^{g}	52.7 ± 5.41^{d}	11.8 ± 2.82^{c}	12.0 ± 0.75^{ef}	12.7 ± 0.60^{e}
		Decoction	51.3 ± 1.08^{f}	66.7 ± 2.81^{c}	13.2 ± 1.37^{c}	14.3 ± 0.25^{de}	15.5 ± 0.45^{d}

[1]Data represent the mean ± SD ($n \geq 6$). LQ (CTC) = 2.45 mg/200mL

GAE – Gallic acid equivalents; RE – Rutin equivalents; CE – Catechin equivalents; CAE – Caffeic acid equivalents; QE – Quercetin equivalents.

Table 2. Concentrations of compounds in infusions and decoctions from *H. italicum* subsp. *picardii* organs (µg/g dry biomass, i.e., µg/cup-of-tea), calculated with reference standards using LC-MS. Quantitation limits are presented as ≤ LOQs (µg/g dry biomass).

[a]Peak nº	Compound	[b]RT (min)	Roots		Vegetative aerial-organs		Flowers	
			Infusion	Decoction	Infusion	Decoction	Infusion	Decoction
	Quinic acid	1.56	300	510	8200	8700	4900	4700
	Protocatechuic acid	6.38	2.3	2.3	82	90	41	49
	p-Hydroxybenzoic acid	8.74	≤ 4	≤ 3	40	52	48	53
1	Chlorogenic acid	8.94	190	190	6900	7700	6000	5200
	Syringic acid	9.50	≤ 37	≤ 31	160	170	≤ 105	≤ 135
2	Caffeic acid	9.74	11	21	57	73	150	140
	Rutin	12.10	≤ 1.4	≤ 1.2	≤ 3	≤ 5	60	60
	Coumaric acid	12.20	≤ 0.5	≤ 0.4	6.2	6.9	4.5	6.6
	Ferulic acid	12.36	≤ 4	5.2	≤ 10	≤ 14	20	19
3	Hyperin and/or isoquercitrin	12.62	≤ 9	≤ 8	≤ 23	≤ 31	350	360
	Apigetrin	13.28	≤ 2	≤ 2	36	54	41	74
	Astragalin	13.35	≤ 2	≤ 2	29	45	490	600
	Salicylic acid	14.09	≤ 1.4	≤ 1.2	14	15	4.9	5.1
	Quercetin	16.44	≤ 4	≤ 3	≤ 10	≤ 14	12	≤ 15
	Kaempferol	18.13	≤ 2	≤ 2	4.9	6.8	36	45
	Galangin	20.10	≤ 2	≤ 2	34	59	41	79
	Oleanolic acid	23.24	≤ 10	13	≤ 23	110	≤ 28	40

[a] Corresponding peak number in the chromatograms on Fig.1.
[b] RT – retention times

Table 3. Relative abundances (%) of the tentatively identified compounds in infusions and decoctions from *H. italicum* subsp. *picardii* organs, analysed by LC-PDA-MS. Red = 0%, yellow = 50%, green = 100%; every percentage in between is a mixture of these colours.

[a]Peak nº	Tentative ID	[b]RT (min)	Roots		Vegetative aerial organs		Flowers		Maximum area detected
			Infusion	Decoction	Infusion	Decoction	Infusion	Decoction	
	Tryptophan	5.26	0.21	0.06	19.17	21.04	100.00	89.98	16 898 458
4	Caffeoylquinic acid isomer	7.01	1.41	2.16	83.32	100.00	62.86	66.15	163 433 756
	*NI ($C_{15}H_{28}O_{10}$)	7.02	NF	NF	20.61	22.33	100.00	92.97	16 451 750
5	Coumaric acid hexoside	7.65	0.27	0.34	88.39	100.00	5.58	6.68	91 587 672
6	Scopoletin hexoside	7.7	1.19	1.19	12.22	13.31	98.87	100.00	193 518 988
	Chlorogenic acid-3-*O*-glucoside	7.78	1.02	0.94	57.56	58.80	100.00	93.73	42 768 967
7	Caffeoylquinic acid isomer	8.63	1.68	3.30	63.77	100.00	27.71	43.43	177 435 679
	Phenylethyl primeveroside	9.67	0.06	0.11	100.00	91.51	8.18	9.13	40 285 455
8	*NI ($C_{24}H_{18}O_{14}$)	10.35	90.71	100.00	24.00	22.14	12.04	14.20	464 514 024
9	*NI ($C_{19}H_{20}O_{11}$)	10.53	4.28	5.62	16.92	19.29	100.00	98.41	47 646 805
10	Feruloylquinic acid	10.79	0.59	0.55	36.73	38.43	98.87	100.00	202 494 981
11	Myricetin glucoside or isomer	11	NF	NF	18.66	26.40	76.79	100.00	92 720 448
12	Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	11.1	0.01	0.01	77.20	100.00	4.18	5.81	157 605 397
13	$C_{15}H_{10}O_8$ coupled to 2hexoses, 1deoxyhexose, 1coumaric acid	11.15	NF	NF	0.05	0.37	75.48	100.00	27 088 733
14	*NI ($C_{34}H_{36}O_{19}$)	11.51	1.35	1.66	14.86	20.87	100.00	94.87	173 437 927
	Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{12}O_5$)	11.6	NF	0.04	86.98	100.00	8.21	10.38	14 262 052
15	*NI ($C_{39}H_{38}O_{23}$)	11.64	NF	NF	NF	0.11	100.00	99.82	66 806 043
	Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	12.17	0.62	0.63	17.98	27.02	69.54	100.00	25 803 936
16	$C_{14}H_{12}O_4$-*O*-hexoside	12.28	79.73	100.00	14.46	20.39	13.35	17.07	215 945 535
17	Isorhamnetin-*O*-hexoside	12.29	NF	NF	65.02	100.00	3.94	4.94	37 680 334
18	*NI ($C_{24}H_{22}O_{16}$)	12.29	0.19	0.21	12.31	14.10	100.00	89.62	197 543 105
19	*NI ($C_{24}H_{22}O_{17}$)	12.29	0.34	0.20	8.87	11.19	100.00	88.40	75 307 256
20	Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	12.44	NF	NF	15.13	24.40	75.96	100.00	246 188 987

21	Dicaffeoylquinic acid	13.1	1.20	1.58	66.49	100.00	36.56	61.17	774 049 004
	Isorhamnetin-*O*-hexoside	13.37	NF	NF	5.42	14.04	83.62	100.00	31 958 060
22	Dicaffeoylquinic acid	13.44	0.95	0.76	64.14	73.25	96.39	100.00	3 585 117 791
23	*NI ($C_{26}H_{30}O_{13}$)	13.74	100.00	97.09	5.64	4.89	1.12	1.10	901 518 717
24	Dicaffeoylquinic acid	13.85	1.17	1.28	71.37	100.00	55.80	78.69	1 636 538 799
	Flavonoid-*O*-hexoside (flavonoid aglycon: $C_{15}H_{10}O_7$)	13.92	0.25	0.29	3.60	5.80	100.00	99.59	49 122 107
25	*NI ($C_{33}H_{22}O_{17}$)	14.02	100.00	64.54	4.02	2.51	0.14	0.25	264 637 977
	Dicaffeoylquinic acid methyl ester	14.07	0.80	1.46	52.17	78.68	63.44	100.00	9 174 467
26	Methoxyoxalyl-dicaffeoylquinic acid	14.17	0.10	0.11	94.00	100.00	2.16	2.10	295 286 636
27	Dicaffeoylquinic acid methyl ester	14.42	0.14	0.17	26.04	26.87	100.00	99.84	101 372 663
28	*NI ($C_{33}H_{22}O_{17}$)	14.45	100.00	71.62	1.63	0.78	NF	NF	165 493 549
29	*NI ($C_{33}H_{22}O_{17}$)	14.57	100.00	66.92	1.88	0.91	NF	NF	144 606 985
	Helichrysin	14.77	NF	NF	80.65	100.00	2.79	4.15	3 404 695
	Dicaffeoylquinic acid methyl ester	14.82	0.90	1.03	59.96	73.11	70.60	100.00	25 279 181
30	*NI ($C_{37}H_{28}O_{19}$)	14.96	100.00	53.47	0.64	0.21	NF	NF	295 393 282
31	Quercetin coupled to coumaric acid and hexose	15.03	NF	NF	0.04	0.07	96.22	100.00	366 986 546
32	*NI ($C_{26}H_{32}O_{13}$)	15.34	100.00	86.95	3.00	3.16	0.26	0.17	178 374 605
33	Tiliroside (kaempferol-3-*O*-*p*-coumaroylglucopyranoside)	15.79	0.01	0.00	0.52	0.90	89.74	100.00	536 636 121
34	*NI ($C_{29}H_{34}O_{16}$)	15.94	100.00	70.98	3.05	2.91	0.29	0.17	334 978 768
	Isomer of naringenin	16.52	NF	NF	89.35	100.00	24.84	31.56	63 885 073
35	Methoxyluteolin	16.58	NF	NF	25.79	35.96	83.25	100.00	29 212 020
	Isomer of naringenin	16.76	0.01	0.01	83.14	100.00	26.41	32.50	178 428 280
	Apigenin	17.79	4.82	3.05	62.44	89.81	73.34	100.00	2 029 743
	Isorhamnetin	17.91	0.89	0.51	66.05	93.53	78.95	100.00	2 525 227
36	Methoxyflavonoid ($C_{16}H_{14}O_5$)	18.5	0.01	0.02	85.11	100.00	7.48	10.98	161 014 058
	4-Hydroxy-3-(3-methyl-2-butenyl) acetophenone	18.66	NF	NF	24.27	20.73	100.00	98.39	5 640 296
37	Pinocembrin	19.08	0.02	0.02	70.77	100.00	16.17	25.25	5 192 433 767
38	Gnaphaliin A	19.43	0.09	0.12	47.20	63.41	60.78	100.00	2 251 428 101
39	Gnaphaliin B	19.81	0.05	0.07	49.77	72.41	56.30	100.00	207 717 204
40	Methoxyflavonoid ($C_{16}H_{12}O_5$)	20.06	0.08	0.10	41.55	64.99	58.76	100.00	1 646 873 985

Helipyrone	20.92	NF	NF	16.37	8.97	100.00	88.27	39 453 111
Arzanol	21.66	NF	NF	NF	NF	45.77	100.00	35 054 309
Methylarzanol	22.03	NF	NF	NF	NF	27.85	100.00	14 237 273

*NI – compound not identified

NF – not found

[a] Corresponding peak number in the chromatograms on Fig.1.

[b] RT – retention times

Table 4. Antioxidant activity of infusions and decoctions from *H. italicum* subsp. *picardii* organs and from green (*C. sinensis*) and red (*A. linearis*) teas (% activity in a cup-of-tea): radical scavenging on DPPH, ABTS, NO, $O_2^{\bullet-}$ and $OH^{\bullet}$ radicals, metal chelating activities on copper (CCA) and iron (ICA), and ferric reducing antioxidant power (FRAP). In each column different letters mean significant differences (p<0.05).

Plant / compound			**Antioxidant activity (%)**							
	Organ	**Extract**	**DPPH**	**NO**	**ABTS**	**$O_2^{\bullet-}$**	**$OH^{\bullet}$**	**FRAP**	**CCA**	**ICA**
H. italicum subsp. *picardii*	Roots	Infusion	74.4 ± 3.56^{d}	62.6 ± 3.26^{bc}	57.6 ± 6.60^{c}	75.1 ± 2.07^{d}	26.0 ± 0.35^{e}	89.2 ± 3.66^{bc}	27.6 ± 2.98^{g}	12.8 ± 2.17^{e}
		Decoction	81.0 ± 0.76^{bc}	64.3 ± 1.91^{b}	66.5 ± 11.1^{b}	87.9 ± 0.81^{b}	19.1 ± 3.53^{f}	76.8 ± 1.45^{d}	37.3 ± 2.70^{f}	17.8 ± 3.24^{de}
	Vegetative aerial-organs	Infusion	82.8 ± 0.38^{ab}	65.3 ± 2.99^{b}	93.1 ± 0.69^{a}	89.7 ± 0.64^{ab}	68.3 ± 1.59^{c}	94.0 ± 6.68^{ab}	63.3 ± 3.46^{de}	25.5 ± 2.21^{c}
		Decoction	83.4 ± 0.65^{ab}	57.8 ± 1.91^{d}	93.1 ± 0.43^{a}	88.8 ± 0.15^{b}	65.8 ± 0.85^{c}	99.7 ± 0.50^{a}	60.8 ± 2.15^{e}	27.5 ± 5.48^{c}
	Flowers	Infusion	85.7 ± 0.29^{a}	63.6 ± 1.89^{b}	93.3 ± 0.38^{a}	91.6 ± 0.13^{a}	77.5 ± 1.18^{b}	82.9 ± 7.20^{cd}	72.2 ± 5.19^{c}	24.1 ± 3.80^{cd}
		Decoction	85.7 ± 0.41^{a}	61.5 ± 1.64^{bcd}	92.7 ± 0.34^{a}	82.6 ± 0.33^{c}	71.2 ± 1.70^{c}	99.8 ± 0.53^{a}	67.8 ± 5.30^{cd}	14.7 ± 3.51^{e}
C. sinensis		Infusion	76.8 ± 3.78^{d}	52.1 ± 0.56^{e}	93.3 ± 0.45^{a}	84.4 ± 1.13^{c}	52.8 ± 1.48^{d}	100 ± 0.00^{a}	80.6 ± 1.73^{b}	41.1 ± 2.87^{b}
		Decoction	77.1 ± 3.79^{cd}	51.5 ± 2.57^{e}	93.1 ± 0.46^{a}	82.1 ± 1.90^{c}	49.1 ± 1.36^{d}	100 ± 0.00^{a}	81.1 ± 0.42^{b}	43.9 ± 4.82^{b}
A. linearis		Infusion	84.6 ± 0.41^{ab}	58.6 ± 0.69^{d}	92.7 ± 0.85^{a}	84.4 ± 0.24^{c}	18.8 ± 2.40^{f}	98.1 ± 2.00^{a}	26.6 ± 3.52^{g}	26.2 ± 1.70^{c}
		Decoction	84.6 ± 0.51^{ab}	59.5 ± 1.29^{cd}	92.9 ± 0.63^{a}	84.9 ± 0.15^{c}	25.6 ± 1.40^{e}	100 ± 0.00^{a}	28.6 ± 3.73^{g}	41.8 ± 4.29^{b}
BHT*			81.7 ± 1.65^{ab}		93.4 ± 0.26^{a}			-		
Ascorbic acid*				90.6 ± 1.35^{a}						
Catechin*						75.2 ± 2.83^{d}	84.4 ± 9.31^{a}			
EDTA*									94.6 ± 0.36^{a}	99.7 ± 0.15^{a}

*Positive controls tested at 1 mg/mL (BHT, catechin and EDTA) or 10 mg/mL (ascorbic acid).

Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9).

Table 5. Inhibitory activity on microbial α-glucosidase of infusions and decoctions from *H. italicum* subsp. *picardii* organs and from green (*C. sinensis*) and red (*A. linearis*) teas (% activity in a cup-of-tea). In each column different letters mean significant differences (p<0.05).

Plant/ compound	Extract	Organ	Inhibitory activity (%)
H. italicum subsp. *picardii*	Roots	Infusion	22.9 ± 5.6[g]
		Decoction	31.0 ± 4.7[f]
	Vegetative aerial-organs	Infusion	41.2 ± 3.5[e]
		Decoction	45.7 ± 3.9[de]
	Flowers	Infusion	48.3 ± 5.7[d]
		Decoction	50.4 ± 3.1[d]
C. sinensis		Infusion	98.8 ± 0.3[a]
		Decoction	99.9 ± 0.3[a]
A. linearis		Infusion	72.3 ± 3.3[c]
		Decoction	72.7 ± 4.2[c]
Acarbose*			88.3 ± 0.5[b]

*Positive control at 10 mg/mL.

Values represent the mean ± SD of at least three experiments performed in triplicate (n = 9).