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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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Highlights

- *H. italicum* stems/leaves and flowers tisanes have high phenolic content
- *H. italicum* stems/leaves and flowers tisanes were as antioxidant as green/red teas
- *H. italicum* had moderate *in vitro* antidiabetic properties and were not toxic

- Chlorogenic and quinic acids were the major phenolics in *H. italicum*
- *H. italicum* has potential to be explored as functional beverage

Abstract

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

mammalian!

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

1. Introduction

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

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

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

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

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

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

this work aimed to evaluate if this everlasting could be explored as a source of innovative food additives. For that purpose, infusions and decoctions were prepared from roots, vegetative aerial-organs and flowers from the everlasting and evaluated for *in vitro* antioxidant and anti-diabetic activities, and for polyphenolic profile. Additionally, a preliminary toxicological evaluation was made *in vitro* by determining samples toxicity against mammalian cells. Green and herbal red (rooibos) teas were used for comparison since they are the most consumed tea beverages worldwide and are sought for their strong antioxidant properties.

2. Materials and methods

2.1. Reagents

All chemicals used were of analytical grade. Reagents 1,1-diphenyl-2picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), sulphanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride (NED), ethylenediamine tetraacetic acid (EDTA), pyrocatechol violet, sodium nitrite, aluminium chloride, butylated hydroxytoluene (BHT), formic acid and ammonium formate were purchased from Sigma-Aldrich (Germany). Ultra-high performance liquid chromatography (UHPLC) grade acetonitrile was purchased from Biosolve (The Netherlands). Merck (Germany) supplied phosphoric acid and Folin-Ciocalteau (F-C) phenol reagent. Commercially available mixtures to calibrate the mass spectrometer, i.e., MSCAL5–1EA (caffeine, tetrapeptide ‘‘Met-Arg-Phe-Ala’’, Ultramark) for positive ion mode and MSCAL6–1EA (sodium dodecylsulfate, taurocholic acid sodium salt, Ultramark) for negative ion mode were purchased from Supelco (USA). Reference standards apigenin, catechin, epicatechin, epigallocatechin, epigallocatechin gallate, flavone, 4-hydroxybenzaldehyde, naringin, quercetin, rutin, uvaol, and caffeic, chlorogenic, coumaric, ferulic, gallic, gentisic, m-hydroxybenzoic, p-hydroxybenzoic, oleanolic, rosmarinic, salicylic

and syringic acids were purchased from Sigma-Aldrich (Germany); apigenin-7-O-glucoside (apigetrin), cyanidin-3-O-arabinoside, cyanidin-3-O-galactoside chloride (ideain chloride), cyanidin-3-O-glucoside chloride (kuromanin chloride), cyanidin-3-O-rutinoside chloride (keracyanin chloride), (+)-dihydrokaempferol ((+)-aromadendrin), galangin, kaempferol, kaempferol-3-O-glucoside (astragalin), luteolin, naringenin, quercetin-3-O-arabinoside (avicularin), quercetin-3-O-galactoside (hyperin), quercetin-3-O-glucoside (isoquercitrin), quercetin-3-O-rhamnoside (quercitrin), phloretin, phloretin-O-20-glucoside (phloridzin), and procyanidin B2 were purchased from Phytolab (Germany); hesperidin, hesperidin methyl chalcone, limonin, neohesperidin dihydrochalcone, protocatechuic acid, propyl gallate, and sinapinic, dihydrocaffeic, hydroferulic, ellagic, and quinic acids were obtained from Sigma–Aldrich (Belgium). Additional reagents / solvents were obtained from VWR International (Belgium).

2.2. Plant collection

Whole plants of *H. italicum* subsp. *picardii* were collected in the Ria Formosa area, a coastal lagoon in south Portugal, near Cabanas de Tavira (37°07'51.3"N 7°36'35.6"W) in June 2013. The taxonomical classification was performed by the botanist Dr. Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal). A voucher specimen is kept at the herbarium of the Marbiotech laboratory (voucher code MBH32). Plants were divided in roots, vegetative aerial-organs (stems and leaves) and flowers, oven dried for 3 days at 50°C, milled and stored at -20°C until use. Dried leaves of green tea plant (*C. sinensis*, produced in Azores, Portugal) and rooibos plant (*A. linearis*, produced in Cape Town, South Africa.) were bought in a regional supermarket, milled and stored at -20ºC.

2.3. Extracts preparation: *"cup-of-tea" infusions and decoctions*

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

2.4. Phytochemical composition of the extracts

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

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

2.4.2. Hydroxycinnamic acid derivatives (HAD) and flavonols content

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

flavonols using quercetin as standard. Results were expressed as standard equivalents per cup-of-tea (CAE and QE, respectively; mg/200mL).

2.4.3. Profile of moderately polar compounds by UHPLC

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

respectively, and capillary temperature at 350°C. The lowest calibration point that was included in the calibration curve was used to calculate the LOQs. The LOQs can be different for the same compound between extracts as the yields (mg dry extract/g plant value) differ per extract; these yields were used to calculate the LOQs. The concentration ranges described by De Paepe et al. [20] were also used during the present work. As quality control, a midrange calibration point was chosen as continuing calibration verification standard (CCV) in order to verify that the calibration of the analytical system was still acceptable. The frequency of CCV analysis was once every ten injections. Data were also recorded using data dependent fragmentation ($ddMS^2$) in positive and negative ionization mode (one analysis per mode) to obtain additional structural information (resolving power set at 17 500 FWHM, stepped collision energy 10, 30, 50 V, isolation window: 4 *m*/*z*). The PDA detector was set to scan from 190 to 800 nm during all analyses. Results regarding concentrations of identified compounds were calculated as µg/g dried plant material, i.e. µg/cup-of-tea, based on the extracts' yield.

2.5. Antioxidant activity

2.5.1. Determination of antioxidant activity by five radical-based assays

The radical scavenging activities (RSA) on DPPH, nitric oxide (NO), ABTS, superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet}$) radicals was evaluated as described previously [6,19] using respectively BHT, catechin (1 mg/mL) or ascorbic acid (10 mg/mL) as positive controls. Results were expressed as percentage of antioxidant activity in a cup-of-tea, relative to a control containing ultrapure water.

2.5.2. Determination of antioxidant activity by three metal-related methods

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

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

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

2.7. *Toxicological evaluation of the samples*

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

2.8. Statistical analysis

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

3. Results and Discussion

3.1. Phytochemical analysis

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

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

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

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

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

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

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

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

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

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

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

replace synthetic antioxidants [5].

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

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

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

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

3.3. Toxicological evaluation

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

4. Conclusion

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

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Figure 1. PDA chromatograms (280 + 330 nm) of the extracts from *H*. *italicum* subsp. *picardii* roots (A: infusion, B: decoction), vegetative aerial-organs (C: infusion, D: decoction) and flowers (E: infusion, F: decoction). Peak numbers refer to compounds listed in Tables 2 and 3.

Figure 2. Toxicity of infusions and decoctions (100 μg/mL extract dw) from *H*. *italicum* subsp. *picardii* organs and from green (*C*. *sinensis*) and red (*A*. *linearis*) teas on mammalian cell lines: A) N9, B) S17 and C) HepG2. Cells treated only with cell culture medium were used as controls; H_2O_2 was used as positive control for cell toxicity. Values represent the mean ± sd of at least three experiments performed in triplicate (n = 9). In each graph different letters mean significant differences ($p < 0.05$). RT: roots, VAO: vegetative aerial-organs, FL: flowers, *CS*: *Camelia sinensis*, *AL*: *Aspalathus linearis*.

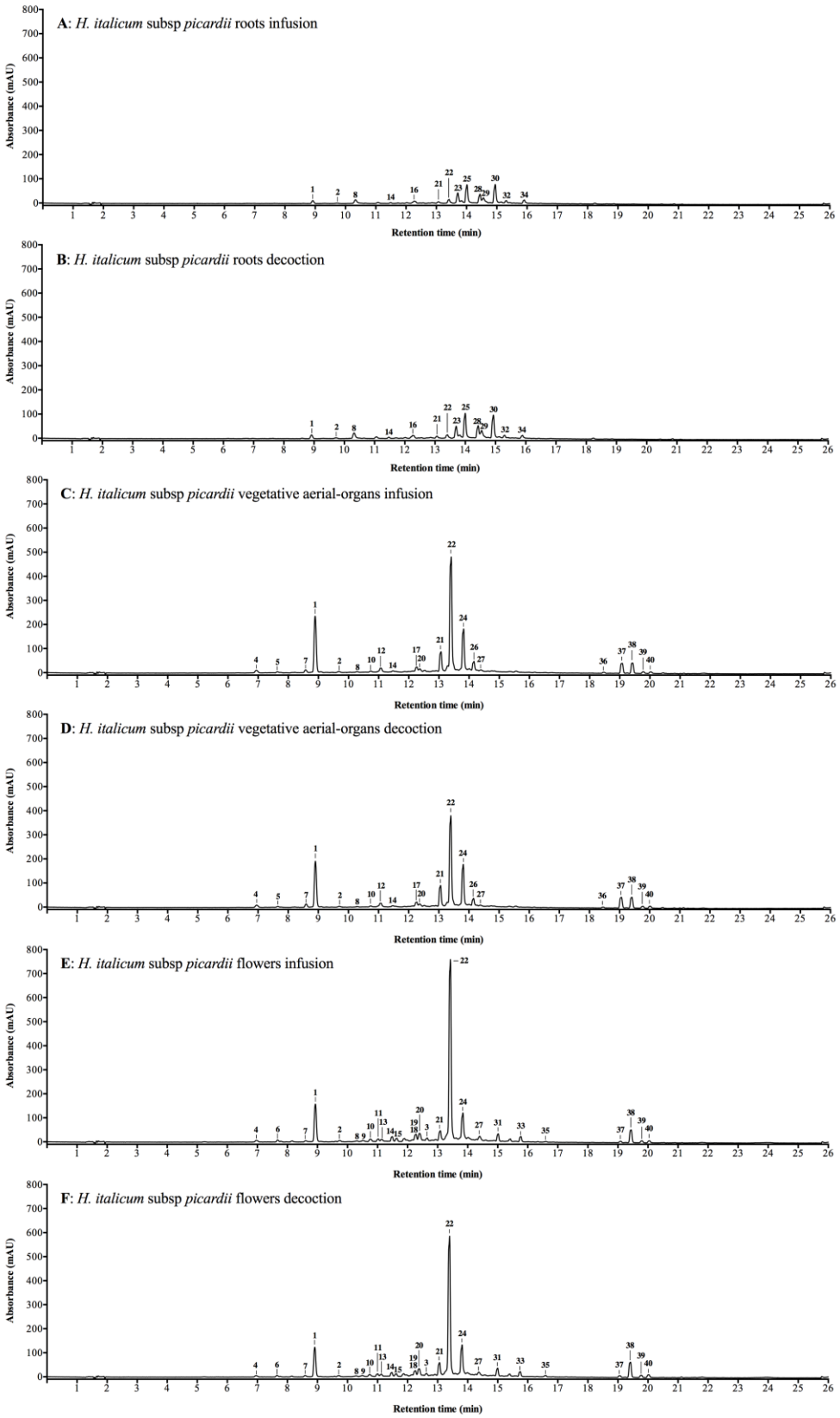

Figure 1

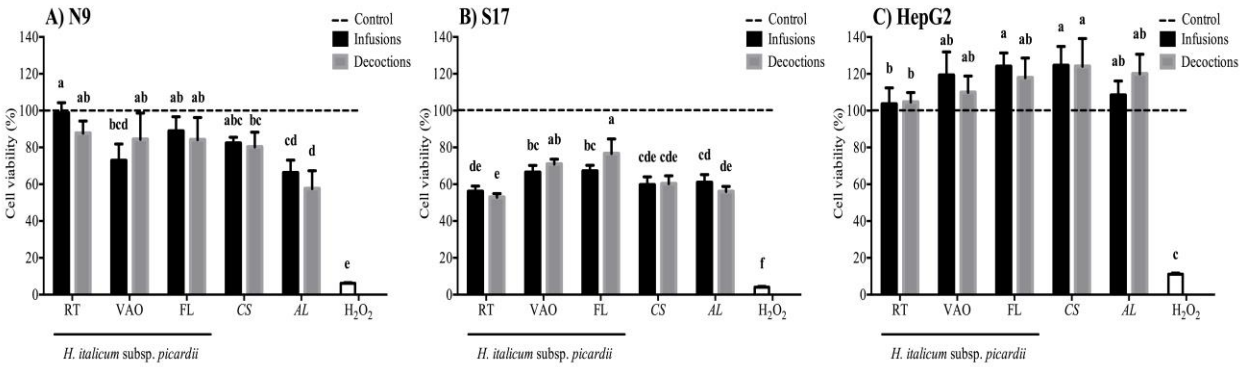

Figure 2

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		[b]RT	Roots		Vegetative aerial-organs		Flowers	
n°	Compound	(min)	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 n°	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).