

Nutrients, phytochemicals and bioactivity of wild Roman chamomile: a comparison between the herb and its preparations

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Abstract

Roman chamomile, *Chamaemelum nobile* L. (Asteraceae), has been used for medicinal applications, mainly through oral dosage forms (decoctions and infusions). Herein, the nutritional characterization of *C. nobile* was performed, and herbal material and its decoction and infusion were submitted to an analysis of phytochemicals and bioactivity evaluation. The antioxidant activity was determined by free radicals scavenging activity, reducing power and inhibition of lipid peroxidation, the antitumour potential was tested in human tumour cell lines (breast, lung, colon, cervical and hepatocellular carcinomas), and the hepatotoxicity was evaluated using a porcine liver primary cell culture. *C. nobile* proved to be an equilibrated valuable herb rich in carbohydrates and proteins, and poor in fat, providing tocopherols, carotenoids and essential fatty acids (C18:2n6 and C18:3n3). Moreover, the herb and its infusion are a source of phenolic compounds (flavonoids such as flavonols and flavones, phenolic acids and derivatives) and organic acids (oxalic, quinic, malic, citric and fumaric acids) that showed antioxidant and antitumour activities, without hepatotoxicity. The most abundant compounds in the plant extract and infusion were 5-*O*-caffeoylquinic acid and an apigenin derivative. These, as also other bioactive compounds are affected in *C. nobile* decoction, leading to a lower antioxidant potential and absence of antitumour potential. The plant bioactivity could be explored in the medicine, food, and cosmetic industries.

Keywords: *Chamaemelum nobile*; Roman chamomile; Nutrients; Phenolic compounds; Antioxidant activity; Antitumour potential

1. Introduction

Roman chamomile, *Chamaemelum nobile* L. (Asteraceae), is a perennial herb found in wild and cultivated habitats in western Europe, North America and northern Africa. Traditionally chamomile is considered to be an antiseptic, antibiotic, disinfectant, bactericidal, fungicidal and vermifuge. It has been used for centuries as anti-inflammatory, antioxidant, mild astringent, mild sedative, antispasmodic, antibacterial and healing medicine (Ma, Winsor, & Danaeshtalab, 2007). Oral dosage forms (decoctions and infusions) are used for the symptomatic treatment of gastrointestinal disorders and of the painful component of functional digestive symptoms. External applications of extracts and lotions are recommended as repellent, emollient, in the treatment of skin disorders and for eye irritation or discomfort of various etiologies. Furthermore, it is used as an analgesic in diseases of the oral cavity, oropharynx or both and as a mouthwash for oral hygiene (Srivastava, Shankar, & Gupta, 2010). Different classes of bioactive constituents are present in chamomile, including phenolic compounds (Carnat, Carnat, Fraisse, Ricoux, & Lamaison, 2004; Tschan, Konig, Wright & Stichert, 1996).

Phenolic compounds have the capacities to quench lipid peroxidation, prevent DNA oxidative damage, and scavenge reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals (Cao & Cao, 1999). Flavonoids are the most abundant antioxidants found in common diets (Mladěnka, Zatloukalová, Filipský, & Hrdina, 2010). The benefits of flavonoids on human health are very often ascribed to their potential ability to act diminishing free radical steady state concentration in biological systems providing antioxidant protection (Galleano, Verstraenten, Oteiza, & Fraga, 2010). Such ability could be possible considering that polyphenols have chemical

structures supporting the scavenging of free radicals and the chelation of redox-active metals. In parallel, it has been reported that certain flavonoids can provide benefits in pathological situations associated with high free radical production, (e.g. hypertension, cardiovascular and cancer diseases) (Galleano et al., 2010; Grassi et al., 2009; Schroeter et al., 2006). In fact, phenolic compounds, mainly flavonoids, proved to have the capacity of regulating proliferation and cell death pathways leading to cancer (López-Lázaro, 2002), through different mechanisms including cell growth and kinase activity inhibition, apoptosis induction, suppression of the secretion of matrix metalloproteinases and of tumour invasive behaviour, as also angiogenesis impairment (Kandaswami et al., 2005).

In the present work, the nutritional characterization (macronutrients, free sugars, fatty acids, tocopherols and carotenoids) of *C. nobile* was performed, and the herb and its decoction and infusion were submitted to a detailed analysis of phytochemicals (phenolic compounds and organic acids) and bioactivity evaluation. The antioxidant activity was determined by free radicals scavenging activity, reducing power and inhibition of lipid peroxidation, the antitumour potential was tested in human tumour cell lines (breast, lung, colon, cervical and hepatocellular carcinomas), and the hepatotoxicity was evaluated using a porcine liver primary cell culture.

2. Materials and methods

2.1 Sample

C. nobile was gathered during the flowering season (June-July 2010) from wild populations located in grasslands in Bragança (Trás-os-Montes, Northeastern Portugal), considering the local medicinal uses as well as healers and selected consumers criteria,

which are related to particular gathering sites, and requirements for safe herbal dosages forms, such as infusion and decoction.

Samples consist of pieces of about 8 cm, corresponding to terminal soft leafy stems and inflorescences with flowers fully open and functional, picked up in plants randomly selected in a meadow of about a hectare. The plant material was put together in a single sample for analysis. Voucher specimens are deposited in the Herbarium of the Escola Superior Agrária de Bragança (BRESA). The sample was lyophilized (FreeZone 4.5, Labconco, Kansas, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogenate sample.

2.2. Standards and reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, sugars (D-(-)-Fructose, D-(+)-Glucose, D-(+)-Sucrose, D-(+)-Trehalose), tocopherols (α -, β -, γ -, and δ -isoforms) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards. Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). The phenolic compound standards (apigenin-6-*C*-glucoside; apigenin-7-*O*-glucoside; caffeic acid; chlorogenic acid; *p*-coumaric acid; isorhamnetin-3-*O*-glucoside; kaempferol-3-*O*-rutinoside; luteolin-6-*C*-glucoside; luteolin-7-*O*-glucoside; myricetin-3-*O*-glucoside; protocatechuic acid; quercetin 3-*O*-glucoside and quercetin-3-*O*-rutinoside) were from Extrasynthese (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid),

penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (Saint Louis, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Characterization of plant nutrients

2.3.1. Crude composition. The sample was analysed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 1995). The crude protein content ($N \times 6.25$) was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 °C. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = $4 \times (\text{g protein} + \text{g carbohydrate}) + 9 \times (\text{g fat})$.

2.3.2. Sugars composition. Free sugars were determined by a High Performance Liquid Chromatography (HPLC) system consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000) and auto-sampler (AS-2057 Jasco), coupled to a refraction index detector (RI detector Knauer Smartline 2300) as previously described by the authors (Pereira, Barros, Martins, & Ferreira, 2012). Sugars identification was made by comparing the relative retention times of sample peaks with standards. Data were analyzed using Clarity 2.4 Software (DataApex). Quantification was based on the RI signal response of each standard, using the internal standard (IS, raffinose) method and by using calibration curves obtained

from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

2.3.3. Fatty acids composition. Fatty acids were determined after a transesterification procedure as described previously by the authors ([Pereira et al., 2012](#)), using a gas chromatographer (DANI 1000) equipped with a split/splitless injector and a flame ionization detector (GC-FID). Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7). The results were expressed in relative percentage of each fatty acid.

2.3.4. Tocopherols composition. Tocopherols were determined following a procedure previously optimized and described by the authors ([Pereira et al., 2012](#)). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight.

2.3.5. Carotenoids. β -carotene and lycopene were determined following a procedure previously described by [Nagata & Yamashita \(1992\)](#). A fine dried powder (500 mg) was vigorously shaken with 10 mL of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was

measured at 453, 505, 645 and 663 nm. Content of β -carotene and lycopene were calculated according to the following equations:

$$\beta\text{-carotene (mg/100 mL)} = 0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453};$$

$$\text{Lycopene (mg/100 mL)} = -0.0458 \times A_{663} + 0.204 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453};$$

and further expressed in mg per 100 g of dry weight (dw).

2.4. Analysis of phytochemicals in the herb and in its decoction and infusion

2.4.1. Plant extraction. For bioactivity assays, a methanolic extract was prepared from the lyophilized plant material. The sample (1 g) was extracted by stirring with 25 mL of methanol (25 °C at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 25 mL of methanol (25 °C at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 °C (rotary evaporator Büchi R-210) to dryness.

2.4.2. Decoction preparation. The sample (1 g) was added to 200 mL of distilled water, heated (heating plate, VELP scientific) and boiled for 5 min. The mixture was left to stand at room temperature for 5 min more, and then filtered under reduced pressure. The obtained decoction was frozen and lyophilized.

2.4.3. Infusion preparation. The sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. The obtained infusion was frozen and lyophilized.

2.4.4. Organic acids composition. Organic acids were determined following a procedure previously optimized and described by the authors ([Barros, Pereira, & Ferreira, 2012](#)).

Analysis was performed by ultra fast liquid chromatograph (UFLC) coupled to photodiode array detector (PDA), using a Shimadzu 20A series UFLC (Shimadzu Corporation). Detection was carried out in a PDA, using 215 nm and 245 as preferred wavelengths. The organic acids were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of lyophilized methanolic extract/decoction/infusion.

2.4.5. Phenolic compounds composition. Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, USA) as previously described by the authors ([Barros et al., 2012a](#)). Double online detection was carried out in the diode array detector (DAD) using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared from different standard compounds. The results were expressed in g per 100 g of lyophilized methanolic extract/decoction/infusion.

2.5. Evaluation of bioactivity

2.5.1 General. The lyophilized methanolic extract, decoction and infusion were redissolved in *i*) water and methanol, respectively (final concentration 2.5 mg/mL) for antioxidant activity evaluation, or *ii*) water for antitumour activity evaluation (final concentration 8 mg/mL). The final solutions were further diluted to different

concentrations to be submitted to distinct bioactivity evaluation *in vitro* assays. The results were expressed in *i*) EC₅₀ values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity, or *ii*) GI₅₀ values (sample concentration that inhibited 50% of the net cell growth) for antitumour activity. Trolox and ellipticine were used as standards in antioxidant and antitumour activity evaluation assays, respectively.

2.5.2. Antioxidant activity. DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{\text{DPPH}} - A_{\text{S}}) / A_{\text{DPPH}}] \times 100$, where A_{S} is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+} , measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β -carotene bleaching was evaluated through the β -carotene/linoleate assay; the neutralization of linoleate free radicals avoids β -carotene bleaching, which is measured by the formula: $(\beta\text{-carotene absorbance after 2h of assay} / \text{initial absorbance}) \times 100$. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: $[(A - B) / A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively (Pereira et al., 2012).

2.5.3. Antitumour activity

Five human tumour cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7, NCI-H460 and HCT-15) and 2 mM glutamine or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 °C, in a humidified air incubator containing 5% CO₂. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 µL) and incubated for 60 min at 4 °C. Plates were then washed with deionized water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 µL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with 10 mM Tris (200 µL) and the absorbance was measured at 540 nm in the microplate reader mentioned above.

2.5.4. Hepatotoxicity

A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in hank's balanced salt solution containing 100 U/mL penicillin, 100 µg/mL streptomycin and divided into 1×1 mm³ explants. Some of these explants were placed in 25 cm² tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and

incubated at 37 °C with a humidified atmosphere containing 5% CO₂. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10⁴ cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (Abreu et al., 2011).

2.6. Statistical analysis

All the assays were carried out in triplicate in three different samples, and the results are expressed as mean values±standard deviation (SD). The statistical differences represented by letters were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$. These treatments were carried out using SPSS v. 18.0 program.

3. Results and Discussion

3.1. Characterization of plant nutrients

The results of the nutritional characterization of *Chamaemelum nobile* are shown in **Table 1**. Carbohydrates were the most abundant macronutrients, followed by proteins. Ash and fat contents were low, and the energetic contribution was 389.88 kcal/100 g dw. The main sugar found in this plant material was fructose, followed by glucose and sucrose. Trehalose was found in lower amounts. Polyunsaturated fatty acids (PUFA) predominated over saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). The FA determined in higher percentages, were linoleic acid (C18:2n6), oleic acid (C18:1n9), α -linolenic acid (C18:3n3) and palmitic acid (C16:0). Regarding

tocopherols, only α - and γ -tocopherols were found in the studied plant material. β -Carotene and lycopene were also quantified in the studied sample.

As far as we know this is the first report on nutritional characterization of *C. nobile*, that proved to be an equilibrated valuable herb rich in carbohydrates and proteins, and poor in fat and calories. Moreover it provides tocopherols, carotenoids, essential fatty acids (C18:2n6 and C18:3n3), and ratios PUFA/SFA and n-6/n-3 fatty acids higher than 0.45 and lower than 4.0, respectively (Guil, Torija, Giménez, & Rodriguez, 1996), which are considered good nutritional ratios.

3.2. Analysis of phytochemicals in the herb and in its decoction and infusion

As *C. nobile* is mostly consumed as decoctions and infusions (aqueous extracts), a comparative study of phytochemicals present in the herb and in those preparations was performed.

Oxalic, quinic, malic, citric and fumaric acids were quantified in all the extracts of *C. nobile* (Table 2), malic acid being the most abundant organic acid. Infusion was the preparation with the highest content in organic acids (9.07 g/100 g dw), while the decoction showed the lowest amount (6.58 g/100 g dw). Some of the mentioned compounds have shown bioactive properties such as the capacity to chelate metal ions of citric acid by forming bonds between the metal and its carboxyl or hydroxyl groups. Citric acid is effective in retarding the oxidative deterioration of lipids in foods and is commonly added to vegetable oils after deodorization (Hraš, Halodin, Knez, & Bauman, 2000). Oxalic acid has a strong chelating ability with multivalent cations; nevertheless, it has been considered as an antinutrient due to the inhibitory effect on mineral bioavailability and to formative effect on calcium oxalate urinary stone (Kayashima & Katayama, 2002).

The main phenolic compounds found in *C. nobile* herbal material and in its decoction and infusion were flavonoids (flavonols and flavones), phenolic acids and derivatives (**Table 3 and 4**). In general, all the preparations revealed the same chromatographic profile, exemplified in **Figure 1A** for the herbal sample. Up to thirty-one phenolic compounds, including a phenolic acid, eleven hydroxycinnamic acid derivatives and nineteen flavonoids were detected in the *C. nobile* preparations (**Table 3**).

Peak 3 was identified as protocatechuic acid by comparison of its UV spectrum and retention time with a commercial standard. Six hydroxycinnamic acid derivatives (peaks 1, 2, 5, 13, 22 and 24) showed UV spectra with maximum wavelengths around 320-330 nm and yielded fragment ions at m/z 191 (deprotonated quinic acid) and 179 (deprotonated caffeic acid) in their MS² mass spectra, which together with their pseudo molecular ions [M-H]⁻ at m/z 353, 515 and 677, allowed their identification as quinic acid derivatives containing one, two or three caffeic acid moieties, respectively. Peak assignments of the different caffeoylquinic acid isomers were made using the recommended IUPAC numbering system (IUPAC, 1976) as also the hierarchical keys previously developed by Clifford, Johnston, Knight, & Kuhnert (2003) and Clifford, Knight, & Kuhnert (2005). The majority peak 5 ([M-H]⁻ at m/z 353) was positively identified as 5-*O*-caffeoylquinic acid by comparison with an authentic standard. Peak 1 ([M-H]⁻ at m/z 353) was identified as 3-*O*-caffeoylquinic acid, yielding the base peak at m/z 191 and the ion at m/z 179 with an intensity >50% base peak. Similar fragmentation pattern was reported by Clifford et al. (2003, 2005) as characteristic to distinguish 3-acylchlorogenic acids. Peaks 2, 22, 24 ([M-H]⁻ at m/z 515) could be assigned as dicaffeoylquinic acids. Peaks 22 and 24 were assigned to 3,4-*O*- and 3,5-*O*-dicaffeoylquinic acids, respectively, based on their elution order, fragmentation pattern and relative abundances (Clifford et al., 2003, 2005). MS² fragmentation of peak 22

yielded the formation of relatively intense signals corresponding “dehydrated” fragments at m/z 335 [caffeoylquinic acid $-H-H_2O$] $^-$ and m/z 173 [quinic acid- $H-H_2O$] $^-$, characteristic of 4-acyl-caffeoylquinic acids. Furthermore, according to Clifford et al. (2005), the intensity of signal at m/z 335 (16% of base peak) is more intense than in 4,5-*O*-dicaffeoylquinic (barely detectable, <5% of base peak). These observations allowed assigning peak 22 as 3,4-*O*-dicaffeoylquinic acid. The fragmentation pattern for 3,5-*O*-dicaffeoylquinic (peak 24) acid was similar to the one previously reported by Clifford et al. (2005). MS² base peak was at m/z 353, produced by the loss of one of the caffeoyl moieties [M- H -caffeoyl] $^-$, and subsequent fragmentation of this ion yielded the same fragments as a 5-caffeoylquinic acid at m/z 191, 179 and 135, although in this case with a comparatively more intense signal at m/z 179 [caffeic acid- H] $^-$ (<50% base peak).

Peak 2 showed the same pseudomolecular ion as peaks 22 and 24 but eluted much earlier than those dicaffeoylquinic acids and also than 5-*O*-caffeoylquinic acid. In addition to the fragments characteristic of a caffeoylquinic acid, the MS² spectrum of this compound also produced fragments at m/z 353 ([M- H -162] $^-$, loss of a hexose residue) and 341 ([M- H -174] $^-$, loss of a quinic acid moiety) and its “dehydrated” form at m/z 323. This fragmentation pattern pointed to a glycosylated chlorogenic acid, which could correspond to 1- or 5-caffeoylquinic-hexoside, according to the mass spectra characteristics and intensities of MS² fragment ions as reported by Clifford, Wu, Kirkpatrick, & Kuhnert (2007). The fact that 5-*O*-caffeoylquinic acid was the majority compound in the sample and 1-*O*-caffeoylquinic acid was not detected, permitted its tentative identification as 5-*O*-caffeoylquinic acid-hexoside, identity that was coherent with its early elution (greater polarity) compared with its parent aglycone.

Peak 13 was identified as tricaffeoylquinic acid according to its pseudomolecular ion [M- H] $^-$ at m/z 677 and diagnostic MS² fragments at m/z 515 (loss of the first caffeoyl),

m/z 353 (loss of the second caffeoyl), m/z 191 (loss of the third caffeoyl to give quinic acid ion), m/z 179 (caffeic acid ion). The signal observed at m/z 497 can be interpreted by the loss of a water molecule from the ion at m/z 515. According to the relative intensities of different tricaffeoylquinic acid isomers reported by [Lin & Harnly \(2008\)](#), this peak could be assigned as 1,3,5-*O*-tricaffeoylquinic acid.

Peaks 4, 9 and 10, with UV spectra similar to caffeic acid with λ_{\max} around 326 nm, were also assigned to caffeic acid derivatives. All of them presented an MS² fragment at m/z 179 ([caffeic acid- H]⁻). Peaks 4 and 9 also presented a fragment at m/z 307 (-162 mu, loss of a hexose moiety), and the formation of the ion at m/z 179 could be produced by the loss of 162 + 128 mu (loss of hexose + methyl-glutarate residues), so that they were tentatively identified as caffeoyl-hexoside-methylglutarate. Although they could not be fully identified, these compounds could be attributed as derived from the *cis* and *trans* isomers of caffeic acid. The MS² analysis of peak 10 yielded signals at m/z 427 ([M-H₂O]⁻) and m/z 265 ([M-18-162]⁻) pointing out to the presence of a hexose, although no further conclusions could be made about its definite identity.

Peaks 6 and 8 ([M-H]⁻ at m/z 453) presented a molecular weight 16 units lower than peaks 4 and 9 but a similar fragmentation pattern, indicating that they could be the corresponding coumaroyl derivatives; this assumption was also supported by the formation of the MS² fragment ion at m/z 163 ([coumaric acid-H]⁻). Thus, they were tentatively identified as two *p*-coumaroyl-hexoside-methylglutarate. Their later elution (lower polarity) compared with the caffeoyl counterparts (peaks 4 and 9) was also coherent with this identity; similarly, they could be speculated as the respective *cis* and *trans* isomers.

Flavones were the most abundant flavonoids present in the analysed samples (**Table 4**).

Peaks 7, 12, 15, 18, 26b, 27 and 29 were identified as apigenin derivatives, according to

their UV and mass spectra characteristics (**Figure 1B**). Peak 7 presented a pseudo molecular ion $[M-H]^-$ at m/z 593, releasing three MS^2 fragments ions at m/z 473 and 383, corresponding to loss of 120 and 90 amu, characteristic of *C*-hexosyl flavones, and at m/z 353 that might correspond to the apigenin aglycone bearing some sugar residues [apigenin + 83 mu] (Ferrerres, Silva, Andrade, Seabra, & Ferreira, 2003). The fact that no relevant fragment derived from the loss of a complete hexosyl residue (-162 mu) was detected, suggested that both sugars were *C*-attached, which allowed a tentative identification of the compound as apigenin 6-*C*-glucose-8-*C*-glucose. Peaks 15, 26b and 29 presented pseudo molecular ions $[M-H]^-$ at m/z 607 and 445 releasing a MS^2 fragment ion at m/z 269 ($[M-176-162]^-$ and $[M-176]^-$, corresponding to the loss of glucuronyl-hexoside and glucuronyl moieties, respectively), being tentatively identified as apigenin *O*-glucuronyl-hexoside (peak 15) and apigenin *O*-glucuronides (peaks 26b and 29).

Peaks 12, 18 and 27 presented pseudomolecular ions $[M-H]^-$ at m/z 621, 607 and 649, respectively, that release an MS^2 fragment at m/z 269 (apigenin). They all presented a similar fragmentation pattern, with a loss of 270 mu, and peaks 18 and 27 also have a fragment ion at $[M-338]^-$, that could correspond to glucuronyl-hexoside or feruloyl-hexoside $[M-176-162]$. Moreover, peak 27 presents a difference of 42 mu relatively to peak 18, that may be due to an acyl group. Peaks 12 and 18 also presented a difference of 14 mu that could correspond to a methyl group. However, the fragmentation patterns of these compounds did not allow us to conclude further about their chemical structure, but due to the UV spectra (**Figure 1B**) and the fragmentation mentioned above they were just associated to unknown apigenin derivatives.

Peaks 17, 19, 20, 21, 23 and 28 were identified as luteolin derivatives. Peaks 17, 20, 23 and 28 presented pseudomolecular ions $[M-H]^-$ at m/z 593, 461, 447 and 489 releasing a

common MS² fragment at m/z 285 ([M-308]⁻, [M-176]⁻, [M-162]⁻ and [M-42-162]⁻, associated to the loss of rutinoyl, glucuronoyl, hexosyl and acetylhexoside moieties, respectively). They were tentatively identified as luteolin *O*-rutinoside, luteolin *O*-glucuronide, luteolin *O*-hexoside (the retention time is different from luteolin 7-*O*-hexoside) and luteolin *O*-acetylhexoside. Peaks 19 and 21 showed pseudomolecular ions [M-H]⁻ at m/z 579 and 593, both releasing two MS² fragments at m/z 447 ([M-132]⁻ and [M-146]⁻ loss of pentosyl and rhamnosyl moieties, respectively) and 285 ([M-162]⁻, loss of a hexosyl moiety), being tentatively identified as luteolin *O*-pentosyl-hexoside and luteolin *O*-rhamnosyl-hexoside.

Flavonols (peaks 11, 14, 16, 25, 26a and 30) were also found in the studied samples (**Tables 3 and 4**). Peak 11 presented a pseudomolecular ion [M-H]⁻ at m/z 479, releasing an MS² fragment at m/z 317 ([M-H-162]⁻, loss of an hexosyl moiety), corresponding to myricitrin, which allowed a tentative identification of the compound as myricetin 3-*O*-glucoside, as confirmed by comparison with an authentic standard. Peak 16 showed a pseudomolecular ion [M-H]⁻ at m/z 579, releasing two MS² fragments at m/z 417 ([M-H-162]⁻, loss of a hexosyl moiety) and 285 (kaempferol; [M-H-162-132]⁻, loss of a further pentosyl moiety), being tentatively identified as kaempferol pentosyl-glucoside the two glycosyl residues are assumed to constitute a disaccharide taking into account that they are lost successively and not alternatively, with no fragment corresponding to a kaempferol-pentoside being released.

Peaks 14, 25 and 26a corresponded to quercetin derivatives. Peak 14 presented a pseudomolecular ion [M-H]⁻ at m/z 477, releasing an MS² fragment at m/z 301 ([M-H-176]⁻, loss of a glucuronoyl moiety); this compound was identified as quercetin 3-*O*-glucuronide, by comparison with a standard isolated in our laboratory (Dueñas et al., 2008). Peak 25 presented a pseudomolecular ion [M-H]⁻ at m/z 549, releasing a MS²

fragments at m/z 301 ($[M-H-162-86]^-$, loss of a malonylhexoside moiety). The UV/Vis spectra have long been used for structural analysis of flavonoids. The typical flavonoid spectrum consists of two maxima in the range 240–285 nm (Band II), and 300–550 nm (Band I), which is more specific and useful for obtaining information regarding identification. The position and relative intensities of these maxima yield information on the nature of the flavonoid and its hydroxylation and substitution patterns (Santos-Buelga, Garcia-Viguera, & Tomas-Barberan, 2003). It is known that the introduction of a glycoside on the hydroxyls at positions 7, 3' or 4' has no effect on wavelength maxima or the spectrum shape in relation to the aglycone. Thus, quercetin 7-*O*-glycosides would have λ_{\max} in Band I around 370 nm, while quercetin 3-*O*-glycosides are hypsochromically shifted to around 354 nm. Since peak 25 presented λ_{\max} at 370 nm it was tentatively identified as quercetin 7-*O*-malonylhexoside.

Peak 26a presented a pseudomolecular ion $[M-H]^-$ at m/z 505 releasing an MS^2 fragment at m/z 301 (quercetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety), and was tentatively identified as quercetin *O*-acetylhexoside. Peak 30 presented a pseudomolecular ion $[M-H]^-$ at m/z 519 releasing a MS^2 fragment at m/z 315 (isorhamnetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety), so that it was tentatively identified as isorhamnetin *O*-acetylhexoside.

The amounts of the phenolic compounds found varied among the different preparations and some compounds disappeared, mostly in decoctions (**Table 4**). Peak 26a, quercetin *O*-acetylhexoside that appeared in the plant material and infusion preparation, was not present in the decoction preparation, being detected at the same retention time another compound (peak 26b) associated to an apigenin glucuronide. Tschan, Konig, & Wright (1996) and Carnat, Carnat, Fraisse, Ricoux, & Lamaison (2004) reported in *Chamaemelum nobile* the presence of chamaemeloside (i.e., apigenin 7-glucoside-6"-

(3''-hydroxy-3''-methyl-glutarate), but this compound was not detected in the sample of roman camomile studied herein. [Carnat et al. \(2004\)](#) did not report in their study the presence in roman camomile of some flavonols described in the present work, such as the quercetin, kaempferol and isorhamnetin derivatives, but they only report flavones and phenolic acids also detected in this study.

3.3. Evaluation of bioactivity

The antioxidant properties were evaluated by four different tests as there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively: DPPH radical scavenging capacity, reducing power and inhibition of lipid peroxidation using β -carotene–linoleate model system in liposomes and TBARS assay in brain homogenates. As it can be observed in **Table 5**, herbal sample gave the highest β -carotene bleaching activity and lipid peroxidation inhibition (lowest EC_{50} values, **Table 5**) which can be related to its higher content in phenolic compounds (**Table 4**), while infusion showed the highest DPPH scavenging activity (**Table 5**) which may be related to their higher levels in organic acids (**Table 2**). Both samples showed similar reducing power (**Table 5**). The decoction presented the lowest antioxidant properties, probably due to its lower content in phenolic compounds and organic acids.

The effects of *C. nobile* extract, decoction and infusion on the growth of five human tumour cell lines (MCF-7, NCI-H460, HCT-15, HeLa and HepG2), represented as the concentrations that caused 50% of cell growth inhibition (GI_{50}), are also summarized in **Table 5**. The plant material extract was more potent than the infusion sample in all the tested cell lines, presenting GI_{50} values that ranged from 82.52 to 168.40 $\mu\text{g/mL}$ for the MCF-7 and HepG2 cells, respectively. Decoction preparation had no antitumour effects

at the maximal concentration used (400 µg/mL), which could indicate that these effects might be related to compounds (including phenolic compounds) that are not extracted or affected by the decoction. Nevertheless, none of the *C. nobile* preparation showed hepatotoxicity in the porcine liver primary cell culture (non-tumour cells; PLP2) (**Table 5**).

Overall, *C. nobile* is an equilibrated valuable species rich in carbohydrates and proteins, and poor in fat, providing tocopherols, carotenoids and essential fatty acids (C18:2n6 and C18:3n3). Moreover, the herb and its infusion are a source of phenolic compounds and organic acids that showed antioxidant and antitumour activities, without hepatotoxicity. Some bioactive compounds are affected by in *C. nobile* decoction, leading to a lower antioxidant potential and absence of antitumour potential. The plant bioactivity could be explored in the medicine, food, and cosmetic industries.

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Table 1. Nutritional characterization of *Chamaemelum nobile* (mean \pm SD).

Crude composition (g/100 g dw)		Free sugars (g/100g dw)	
Moisture (g/100g fw)	67.09 \pm 1.02	Fructose	3.37 \pm 0.24
Ash	6.43 \pm 0.05	Glucose	1.57 \pm 0.13
Proteins	26.63 \pm 1.92	Sucrose	1.08 \pm 0.08
Fat	3.12 \pm 0.33	Trehalose	0.60 \pm 0.02
Carbohydrates	63.83 \pm 1.66	Total sugars	6.62 \pm 0.31
Energy	389.88 \pm 1.32		
Fatty acids (relative %)		Tocopherols (mg/100 g dw)	
C16:0	17.89 \pm 0.16	α -Tocopherol	1.64 \pm 0.02
C18:0	3.36 \pm 0.03	β -Tocopherol	nd
C18:1n9	23.22 \pm 0.22	γ -Tocopherol	0.19 \pm 0.01
C18:2n6	28.89 \pm 0.33	δ -Tocopherol	nd
C18:3n3	18.22 \pm 0.11	Total tocopherols	1.83 \pm 0.01
SFA	27.67 \pm 0.19	Pigments (mg/100 g)	
MUFA	24.78 \pm 0.27	β -carotene	0.95 \pm 0.02
PUFA	47.56 \pm 0.46	Lycopene	0.02 \pm 0.01
PUFA/SFA	1.72 \pm 0.03		
n6/n3	1.56 \pm 0.01		

fw- fresh weight; dw – dry weight

Palmitic acid (C16:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); α -Linolenic acid (C18:3n3). Nineteen more fatty acids were also identified and quantified (total relative percentage 8.42%; data not shown).

SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

Table 2. Organic acids composition of *Chamaemelum nobile* (mean \pm SD).

Organic acid	Herb	Decoction	Infusion
Oxalic acid	2.02 \pm 0.06 ^a	1.74 \pm 0.21 ^b	1.99 \pm 0.13 ^{ba}
Quinic acid	1.74 \pm 0.13 ^b	1.40 \pm 0.04 ^b	2.56 \pm 0.17 ^a
Malic acid	3.02 \pm 0.07 ^a	2.21 \pm 0.19 ^b	3.06 \pm 0.05 ^a
Citric acid	1.33 \pm 0.01 ^a	1.23 \pm 0.16 ^a	1.46 \pm 0.24 ^a
Fumaric acid	0.02 \pm 0.00 ^a	0.01 \pm 0.00 ^b	0.01 \pm 0.00 ^b
Total (g/100g)	8.14 \pm 0.28 ^b	6.58 \pm 0.28 ^c	9.07 \pm 0.01 ^a

In each row different letters mean significant differences (p<0.05)

Table 3. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{\max}), pseudomolecular and MS² fragment ions (in brackets, relative abundances) and tentative identification of the phenolic compounds of *Chamaemelum nobile*.

Peak	Rt (min)	λ_{\max} (nm)	Molecular ion [M-H] ⁻ (m/z)	MS ² (m/z)	Tentative identification
1	5.11	326	353	191(100), 179(69), 161(7), 135(51)	3- <i>O</i> -Caffeoylquinic acid
2	5.65	320	515	353(11), 341(6), 323(100), 191(61), 179(6), 161(18), 135*	5- <i>O</i> -Caffeoylquinic acid-hexoside
3	6.15	262,294	153	109(100)	Protocatechuic acid
4	6.44	326	469	307(8), 179(100),161(37), 135(78)	Caffeoyl-hexoside-methylglutarate
5	7.79	326	353	191(100), 179(11), 173(9), 161(28), 135(8)	5- <i>O</i> -caffeoylquinic acid
6	9.62	312	453	291*, 163(100), 145(9), 119(51)	<i>p</i> -coumaroyl-hexoside-methylglutarate
7	10.48	332	593	593(100), 473(18), 383(6), 353(12)	Apigenin 6- <i>C</i> -glucose-8- <i>C</i> -glucose
8	11.05	316	453	163(100), 145(8),119(42)	<i>p</i> -coumaroyl-hexoside-methylglutarate
9	11.64	328	469	469(100), 307*, 179(6),161(19), 135(3)	Caffeoyl-hexoside-methylglutarate
10	16.19	320	445	445(100), 427(58), 265(15), 179(8)	Caffeic acid derivative
11	16.53	356	479	317(100)	Myricetin 3- <i>O</i> -glucoside
12	17.15	344	621	487(3), 351(100), 269(14)	Apigenin derivative
13	17.32	320	677	677(100), 515(28), 497(16), 353(65), 335(23), 191 (12), 179(8), 135(3)	1,3,5- <i>O</i> -tricafeoylquinic acid
14	18.26	356	477	301(100)	Quercetin 3- <i>O</i> -glucuronide
15	18.52	342	607	269(100)	Apigenin <i>O</i> -glucuronylhexoside
16	18.71	350	579	579(100), 417(26), 285(53)	Kaempferol <i>O</i> -pentosylhexoside
17	19.01	350	593	285(100)	Luteolin <i>O</i> -rutinoside
18	19.12	344	607	545(5), 337(27), 269(100)	Apigenin derivative
19	19.63	350	579	579(100), 447(4), 285(16)	Luteolin <i>O</i> -pentosylhexoside
20	19.70	350	461	285(100)	Luteolin <i>O</i> -glucuronide

21	20.06	344	593	593(100), 447(5), 285(17)	Luteolin <i>O</i> -rhamnosylhexoside
22	20.11	332	515	515(100), 353(54), 335(16), 299(3), 203(3), 191(16), 179(27), 173(44), 135(11)	3,4- <i>O</i> -DicaFFEoylquinic acid
23	21.14	350	447	285(100)	Luteolin <i>O</i> -hexoside
24	21.75	328	515	353(100), 335(6), 191(85), 179(42), 173(11), 135(16)	3,5- <i>O</i> -DicaFFEoylquinic acid
25	23.59	370	549	505(100), 301(74)	Quercetin 7- <i>O</i> -malonylhexoside
26a	24.26	318sh, 348	505	301(100)	Quercetin <i>O</i> -acetylhexoside
26b	24.23	338	445	269(100)	Apigenin <i>O</i> -glucuronide
27	24.52	338	649	605*, 587(5), 515(5), 427(5), 379(29), 361(3), 311(4), 269(100)	Apigenin derivative
28	25.05	332	489	285(100)	Luteolin <i>O</i> -acetylhexoside
29	25.27	334	445	269(100)	Apigenin <i>O</i> -glucuronide
30	27.18	318sh, 358	519	315(100)	Isorhamnetin <i>O</i> -acetylhexoside

*abundance \leq 2; 26b-only found in decoction sample.