Chemical composition of wild and commercial *Achillea millefolium* L. and bioactivity of the methanolic extract, infusion and decoction

Maria Inês Dias[a,b], Lillian Barros[a,c], Montserrat Dueñas[c], Eliana Pereira[a], Ana Maria Carvalho[a], Rita C. Alves[b], M. Beatriz P.P. Oliveira[b], Celestino Santos-Buelga[c], Isabel C.F.R. Ferreira[a,*]

[a]*Mountain Research Centre (CIMO), ESA, Polytechnic Institute of Bragança, Campus de Santa Apolónia, 1172, 5301-855 Bragança, Portugal.*

[b]*REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia da Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal.*

[c]*GIP-USAL, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain.*

*Corresponding author. Tel.+351 273 303219; fax +351 273 325405.

E-mail address: iferreira@ipb.pt (I.C.F.R. Ferreira)

Abstract

Medicinal plants used in folk medicine are being increasingly studied and used on pharmaceutical, food and nutraceutical fields. Herein, wild and commercial samples of *Achillea millefolium* L. (yarrow) were chemically characterized with respect to their macronutrients, free sugars, organic acids, fatty acids and tocopherols. Furthermore, *in vitro* antioxidant properties (free radicals scavenging activity, reducing power and lipid peroxidation inhibition) and antitumour potential (against breast, lung, cervical and hepatocellular carcinoma cell lines) of their methanolic extract, infusion and decoction (the most consumed forms) was evaluated and compared to the corresponding phenolic profile obtained by high performance liquid chromatography and mass spectrometry. Data obtained showed that the chemical profiles of wild and commercial samples, and also their methanolic extract, infusion and decoction were similar, varying only in the quantities found. Commercial yarrow have higher content of fat and saturated fatty acids, proteins, ash, energy value, sugars and flavonoids, while the wild sample revealed higher levels of carbohydrates, organic acids, unsaturated fatty acids, tocopherols and phenolic acids. The heterogeneity among the antioxidant and antitumour results of the samples and some low correlations with total phenolic compounds indicates that specific compounds, rather than the totality of them, are involved in the bioactive properties of samples.

Keywords: Achillea millefolium L.; Wild/commercial; Chemical composition; Bioactive properties; Phytochemicals

1. Introduction

In a society increasingly concerned with health and nutrition, medicinal plants emerge as alternative to synthetic products, used not only in traditional medicine but also in a number of food and pharmaceutical products, due to their nutritional properties and bioactivity (Phillipson, 2007). *Achillea millefolium* L., commonly known as yarrow, belongs to Asteraceae family and it is very common in mountain meadows, pathways, crop fields and homegardens. Its infusion or alcohol extract is widely used in Europe as a remedy to treat digestive problems, diabetes, hepato-biliary diseases and amenorrhea, and also consumed for its antitumour, antimicrobial, anti-inflammatory and antioxidant properties, among others (Baretta et al., 2012; Candan et al., 2010; Carvalho, 2010, Cavalcanti et al., 2006; Dall'Acquaa, Bolegob, Cignarellab, Gaionb, & Innocentia, 2011; Jonsdottir, Omarsdottird, Vikingssona, Hardardottirc, & Freysdottir, 2011; Potrich et al., 2010; Trumbeckaite et al., 2011). The decoction is used for digestive and intestinal disorders, but it is also used externally for skin and mucosa inflammations (Rauchensteiner, Nejati & Saukel, 2004).

Antioxidant properties of *A. millefolium* have previously been reported in hydroalcoholic, methanolic and aqueous extracts, as also in the essential oil (Candan et al., 2010; Kintzios, Papageorgiou, Yiakoumettis, Baričevič, & Kušar, 2010; Trumbeckaite et al., 2011; Vitalini et al., 2011), but not in the infusion or decoction, the most consumed form. Cytotoxicity against human tumour cell lines was also only evaluated with the ethanolic extract (Ghavami, Sardari, & Shokrgozar, 2010) and was related to the presence of sesquiterpene lactones and flavonols (Csupor-Löffler et al., 2009). Antioxidant molecules such as tocopherols and ascorbic acid were quantified in *A. millefolium* and found to be present in considerable amounts (Chanishvili, Badridze, Rapava, & Janukashvili, 2007). Flavonoids, apigenin and quercetin, and the phenolic

acid, caffeoylquinic acid, were reported as the major phenolic compounds present in yarrow plant (Benedek, Gjoncaj, Saukel, & Kopp, 2007; Benetis, Radušienė, & Janulis, 2008; Radušienė, 2011; Vitalini et al., 2011). The above mentioned compounds have the capacity to function as reducing agents, hydrogen donators or singlet oxygen quenchers against reactive species involved in oxidative stress, the main cause for cell death (Carocho & Ferreira, 2013).

The main objective of the present work was to compare chemical composition of wild and commercial *A. millefolium* regarding macronutrients, free sugars, organic acids, fatty acids and tocopherols. Furthermore, *in vitro* antioxidant properties (free radicals scavenging activity, reducing power and lipid peroxidation inhibition) and antitumour potential (against breast, lung, cervical and hepatocellular carcinoma cell lines) of their methanolic extract, infusion and decoction (the most consumed forms) were evaluated and compared to the corresponding phenolic profile.

2. Materials and methods

2.1 Samples

The wild yarrow (inflorescences and upper leaves) was collected in Cova de Lua, Bragança, Portugal from 50 plants growing in two different grasslands of about one hectare. The gathered material was mixed, made into a unique sample and further lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). A voucher specimen was deposited at the Herbarium of the Escola Superior Agrária de Bragança (BRESA). The commercial yarrow was purchased from a local company, Ervital from Castro Daire, Portugal, which produces Mediterranean herbs using organic principles and methods. Each sample was 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). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) were purchased from Sigma (St. Louis, MO, USA), as well as other individual fatty acid isomers, L-ascorbic acid, tocopherol, sugar and organic acid standards. Phenolic standards were from Extrasynthèse (Genay, France). Racemic tocol (50 mg/mL), was purchased from Matreya (Pleasant Gap, PA, USA). 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, UT, USA). Acetic acid, ellipticine, sulphorhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (Saint Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Chemical composition of wild and commercial samples

2.3.1. Macronutrients. The samples were analysed for proteins, fat, carbohydrates and ash using the AOAC (1995) procedures. The crude protein content (N×6.25) of the samples 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 ^{o}C. Total

carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein) + 3.75 × (g carbohydrate) + 9 × (g fat).

2.3.2. Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), after an extraction procedure previously described (Guimarães et al., 2013a) using melezitose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD, USA) and an RI detector (Knauer Smartline 2300, Berlin, Germany). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer, Berlin, Germany) operating at 30 ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight.

2.3.3. Organic acids. Organic acids were determined following a procedure previously described (Pereira, Barros, Carvalho, & Ferreira, 2013). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) thermostatted at 35 ºC. The elution was performed with sulphuric acid (3.6 mM) using a flow rate of 0.8 mL/min. Detection was carried out in a PDA, using 215 and 245 nm (for ascorbic acid) as preferred

wavelengths. The organic acids found 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 dry weight.

2.3.4. Fatty acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column as described previously (Guimarães et al., 2013a). The analysis was carried out with a DANI model GC 1000 instrument (Contone, Switzerland), equipped with a split/splitless injector, a flame ionization detector (FID at 260 ºC) and a Macherey–Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm d_f).. The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC, 20 ºC/ min ramp to 180 ºC, 3 ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. 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 the CSW 1.7 Software (DataApex 1.7) and expressed in g/100 g fat.

2.3.5. Tocopherols. Tocopherols were determined following a previously described procedure (Guimarães et al., 2013a). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco, Easton, MD, USA) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 mm × 4.6 mm i.d.) normal-phase column from YMC Waters (Dinslaken, Germany) operating at 30 ºC. The mobile phase

used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, and the injection volume was 20 µL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on calibration curves obtained from commercial standards of each compound using the internal standard (IS) methodology; racemic tocol was used as IS. The results were expressed in mg per 100 g of dry weight.

2.4. Bioactivity and phenolic profile of the methanolic extract, infusion and decoction

2.4.1. Samples preparation. The methanolic extract was obtained from the lyophilized wild and commercial plant material. Each sample (1 g) was extracted twice by stirring with 30 mL of methanol (25 ºC at 150 rpm) for 1 h and subsequently filtered through a Whatman No. 4 paper. The combined methanolic extracts were evaporated at 40 ºC (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness.

For infusion preparation the lyophilized plant material (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. For decoction preparation the lyophilized plant material (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 for 5 min and then filtered under reduced pressure. The obtained infusions and decoctions were frozen and lyophilized.

Methanolic extracts and lyophilized infusions and decoctions were redissolved in *i)* methanol and water, respectively (final concentration 2.5 mg/mL) for antioxidant activity evaluation, *ii)* water (final concentration 8 mg/mL) for antitumour potential evaluation; and *iii)* water:methanol (80:20, *v/v*) and water, respectively (final concentration 1 mg/mL) for phenolic compounds identification and quantification. The final solutions were further diluted to different concentrations to be submitted to distinct

bioactivity evaluation in *in vitro* assays. The results were expressed in *i)* EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity, or *ii)* GI_{50} values (sample concentration that inhibited 50% of the net cell growth) for antitumour potential. Trolox and ellipticine were used as positive controls in antioxidant and antitumour activity evaluation assays, respectively.

2.4.2. Antioxidant activity. DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}] \times 100$, 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+} to Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 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] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Guimarães et al., 2013b).

2.4.3. Antitumour potential and cytotoxicity in non-tumour liver primary cells. 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 and 2 mM glutamine (MCF-7, NCI-H460 and HCT-15) 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 ºC, in a humidified air incubator containing 5% CO_2. 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. Sulphorhodamine B assay was performed according to a procedure previously described by the authors (Guimarães et al., 2013b).

For hepatotoxicity evaluation, a cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, according to an established procedure (Guimarães et al., 2013b); it was designed as PLP2. 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 1.0×10^4 cells/well, and commercial in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin.

2.4.4. Phenolic profile. Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) as previously described by the authors (Rodrigues et al., 2012). Double online detection was carried out in the diode array detector (DAD) using 280 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, mass spectra, retention times, and comparison with authentic standards when available. For quantitative analysis, a 5-level calibration curve was obtained by injection of known concentrations (2.5-100 μg/mL) of different standards compounds: apigenin-6-*C*-glucoside (y=246.05x-309.66; R^2=0.9994); apigenin-7-*O*-glucoside (y=159.62x+70.50; R^2=0.999); caffeic acid (y=611.9x-4.5733; R^2=0.999); 5-*O*-caffeoylquinic acid (y=313.03x-58.20; R^2=0.999); kaempferol-3-*O*-glucoside (y=288.55x-4.05; R^2=1); kaempferol-3-*O*-rutinoside (y=239.16x-10.587; R^2=1); luteolin-6-*C*-glucoside (y=508.54x-152.82; R^2=0.997); luteolin-7-*O*-glucoside (y=80.829x-21.291; R^2=0.999); quercetin-3-*O*-glucoside (y=253.52x-11.615; R^2=0.999) and quercetin-3-*O*-rutinoside (y=281.98x-0.3459; R^2=1). The results were expressed in mg per g of methanolic extract and lyophilized infusion or decoction.

2.5. Statistical analysis

For wild and commercial plant material, three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program.

3. Results and Discussion

3.1 Chemical composition of wild and commercial samples

The chemical composition of wild and commercial *A. millefolium* in macronutrients, free sugars and organic acids is presented in **Table 1**.

Carbohydrates, followed by proteins, were the major macronutrients in both samples. The commercial sample revealed higher contents of all the macronutrients except in carbohydrates which were higher in the wild yarrow. Fructose, glucose, sucrose and trehalose were found in both samples, while raffinose was only detected in the wild sample. Commercial sample also showed the highest levels of individual and total free sugars. Wild sample presented the highest content in total organic acids, mainly oxalic, quinic, and citric; succinic acid was not detected in the commercial sample and fumaric acid was only found in traces (**Table 1**).

Up to twenty-nine fatty acids were identified on wild and commercial *A. millefolium* (**Table 2)**. In both samples linoleic acid (C18:2n-6, PUFA) was the major fatty acid, followed by palmitic acid (C16:0, SFA) in the case of commercial sample, and oleic acid (C18:1n-9, PUFA) in the case of wild sample. The wild sample gave higher levels of PUFA (with the major contribution of linoleic acid) and MUFA (mainly due to oleic acid), while the commercial sample showed the highest levels of SFA (with the important contribution of palmitic acid).

Although both samples presented similar tocopherol profile (α-, β-, and γ- isoforms), wild yarrow presented higher levels of total tocopherols (**Table 2**), γ-tocopherol being the most abundant isoform. δ-Tocopherol was not found in the samples. Chanishvili et al. 2007 previously reported the presence of tocopherols in *A. millefolium* samples from Georgia, but without quantification of the individual isoforms.

3.2 Bioactivity of the methanolic extract, infusion and decoction

Antioxidant properties of the methanolic extract and of the most consumed forms of *A. millefolium*, infusion and decoction, were evaluated and the results are shown in **Table 3**. In general, commercial yarrow presented lower EC_{50} values (higher antioxidant

activity). In both cases (wild and commercial samples), decoctions showed the highest DPPH scavenging activity, β-carotene bleaching inhibition and TBARS inhibition, while infusions presented the highest reducing power.

The samples herein studied gave lower DPPH scavenging activity than water and methanolic extracts of *A. millefolium* from Slovenia and Lithuania (Kintzios et al., 2010; Trumbeckaite et al., 2011). They also showed lower DPPH scavenging activity but higher lipid peroxidation inhibition than methanolic extracts of *A. millefolium* from Turkey (45.60 and 892.67 μg/mL, respectively; Candan et al., 2010). These variations cab be either due to intrinsic factors, mainly genetics or to extrinsic factors, such as storage, type of soil, agronomic practices, climatic factors and technological treatments (Ghasemnezhad, Sherafati, & Payvast, 2011).

The effects of the methanolic extracts, infusions and decoctions on different human tumour cell lines (MCF-7, NCI-H460, HCT-15, HeLa and HepG2) were also evaluated (**Table 3**). The infusion of wild yarrow showed the highest potential against breast (MCF-7; in this case the methanolic extract gave statistically similar results) and hepatocellular (HepG2) carcinoma cell lines, while the methanolic extract of commercial yarrow was most potent against lung (NCI-H460), colon (HCT-15) and cervical (HeLa) carcinoma cell lines. Although the samples present some toxicity for non-tumour liver primary cells (PLP2), the GI_{50} values obtained for tumour cell lines (HepG2) were always lower than the hepatotoxic GI_{50} concentration, suggesting that the samples could be used for antitumour proposes, at the GI_{50} concentration, without toxic effects for non-tumour cells. The results reported for MCF-7 cell line, mainly in the case of decoction and infusion of the commercial sample, are consistent with the ones obtained with ethanolic extracts of *A. millefolium* from Iran (GI_{50}=64.078 μg/mL) (Ghavami et al., 2010). The antiproliferative activity against HeLa and MCF-7 tumour

cell lines of sesquiterpene lactones and flavonols isolated from *A. millefolium* samples from Hungary was also studied by Csupor-Löffler et al. (2009) and correlated to the activity of alcoholic and aqueous extracts of the plant.

3.3. Phenolic profile of the methanolic extract, infusion and decoction

The HPLC phenolic profile of a wild sample of *A. millefolium* recorded at 280 and 370 nm is shown in **Figure 1**, and peak characteristics and identification are presented in **Table 4**. Twenty-eight compounds were detected, eight of which were phenolic acid derivatives (hydroxycinnamic acid derivatives). Among them, seven compounds (peaks 1, 3, 4, 16, 19, 20 and 22) were caffeoylquinic acid derivatives identified according to their UV spectra and pseudomolecular ions. 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 >70% base peak, characteristic of 3-acylchlorogenic acids as reported by Clifford, Johnston, Knight, & Kuhnert (2003) and Clifford, Knight, & Kuhnert (2005).

Peak 3 was easily distinguished from the other two isomers by its base peak at *m/z* 173 $[\text{quinic acid-H-H}_2\text{O}]^-$, accompanied by a secondary fragment ion at *m/z* 179 with approximately 88% abundance of base peak, which allowed identifying it as 4-*O*-caffeoylquinic acid according to the fragmentation pattern described by Clifford et al. (2003, 2005). Peak 4 was identified as 5-*O*-caffeoylquinic acid by comparison of its UV spectrum (λ_{max} 326 nm) and retention time with a commercial standard.

Peaks 16, 19, 20 and 22 ($[M-H]^-$ at *m/z* 515) corresponded to dicaffeoylquinic acids and were assigned to 3,4-*O*-, 3,5-*O*- and 4,5-*O*- dicaffeoylquinic acids, respectively, based on their elution order and fragmentation patterns (Clliford et al., 2003; Clliford et al., 2005). MS^2 fragmentation of peak 16 yielded signals corresponding to "dehydrated"

fragment ions 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 (34% of base peak), greater than in the other dicaffeoylquinic acids, would allow assigning compound 16 as 3,4-*O*-dicaffeoylquinic acid. The fragmentation pattern of peaks 19 and 20 was similar to the one previously reported by Clifford et al. (2005) for 3,5-*O*-dicaffeoylquinic acid. MS^2 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 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]$^-$ (~70% base peak). These peaks 19 and 20 were identified as *cis* and *trans* 3,5-*O*-dicaffeoylquinic acid, respectively, based on the elution order described in a previous study (Barros, Dueñas, Carvalho, Ferreira, & Santos-Buelga, 2012). Compound 22 was assigned to 4,5-*O*-dicaffeoylquinic acid according to its fragmentation, identical to the one previously reported by Clifford et al. (2005). Contrary to 3,4-*O*-dicaffeoylquinic acid (peak 16), in this case the signal at *m/z* 335 was barely detectable (3% of base peak). The intense signal at *m/z* 173, characteristic of an isomer substituted at position 4, would indicate that whereas 3,4-*O*-dicaffeoylquinic acid initially loses the caffeoyl moiety at position 3, the 4,5-*O*-dicaffeoylquinic acid first loses that at position 5. Peak 2 ([M-H]$^-$ at *m/z* 341) was assigned as a caffeic acid hexoside based on the ion at *m/z* 179 (-162 u; hexosyl residue; [caffeic acid-H]$^-$) and UV spectrum (λ_{max} 326 nm).

Flavones were also found in the studied samples, most of them associated to apigenin derivatives (nine compounds) according to their UV spectra (λ_{max} around 330-340 nm) and MS^2 fragmentation pattern (**Table 4**).

Apigenin 7-*O*-glucoside (peak 23) was positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standard. Peaks 5-7 presented pseudomolecular ions $[M-H]^-$ at *m/z* 593 or 563, releasing MS^2 fragment ions corresponding to loss of 90 and 120 u (*m/z* at 473 and 443), characteristic of *C*-hexosyl flavones, and at *m/z* 383 and 353 that might correspond to the apigenin aglycone plus residues of the sugars that remained linked to it (apigenin + 113 u) and (apigenin + 83 u), respectively (Ferreres, Silva, Andrade, Seabra, & Ferreira, 2003). The fact that no relevant fragments derived from the loss of complete hexosyl (-162 u) or pentosyl residues (-132 u) were detected suggested that sugars were *C*-attached, which allowed an identification of these compounds as apigenin-*C*-hexoside-*C*-hexoside (Peak 5) and apigenin-*C*-hexoside-*C*-pentoside (peaks 6 and 7).

Peaks 14 and 21 (also pseudomolecular ions at $[M-H]^-$ at *m/z* 593 and 563) could be assigned to an apigenin dihexoside and an apigenin *O*-pentosyl-hexoside, respectively, based on the loss of two hexosyl moieties (162+162 u) in the first case, and of pentosyl and hexosyl residues (132+162 u) in the second one, to yield the aglycone (*m/z* at 269, apigenin). The fact that the two moieties were lost simultaneously suggested that they might constitute a disaccharide *O*-linked to the aglycone.

Peaks 26, 27 and 28, all of them with a pseudomolecular ion $[M-H]^-$ at *m/z* 473 releasing a unique MS^2 fragment at *m/z* 269 (apigenin; $[M-H-42-162]^-$), were identified as apigenin *O*-acetylhexosides according to their mass, 42 u greater than apigenin-hexoside. The observation of three peaks with the same characteristics could be explained by the location of the acetylhexoside moiety on different positions of the aglycone and/or the substitution of the acetyl residue on different positions of the hexose. The positive identification of apigenin 7-*O*-glucoside in the samples would point to one or all of these compounds could be derived from it.

Peaks 8 and 24 were assigned to luteolin derivatives. Peak 8 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 447 giving place to three MS^2 fragment ions, a major one at *m/z* 357 $[M-H-90]^-$, and other two at *m/z* 327 $[M-H-120]^-$ and at *m/z* 297 $[M-H-30]^-$. The fragmentation pattern was characteristic of *C*-glycosylated flavones at C-6/C-8, and the relative abundance of fragments pointed out to sugar substitution at C-6 according to the fragmentation patterns described by Ferreres, Silva, Andrade, Seabra, & Ferreira (2003), Ferreres, Llorach, & Gil-Izquierdo (2004) and Ferreres, Gil- Izquierdo, Andrade, Valentao, & Tomás-Barberán (2007) The peak was identified as luteolin-6-*C*-glucoside, which was further confirmed by comparison to a standard. Peak 24 ($[M-H]^-$ at 489 *m/z*) released a unique MS^2 fragment at *m/z* 285 (luteolin; $[M-H-42-162]^-$) which allowed its identification as luteolin *O*-acetylhexoside.

The remaining phenolic compounds corresponded to flavonols derivatives, most of them derived from quercetin (λ_{max} around 350 nm and an MS^2 fragment at *m/z* 301) (**Table 4**). Quercetin 3-*O*-rutinoside (peak 13) was positively identified according to its retention, mass and UV-vis characteristics by comparison with a commercial standard. Peak 10 ($[M-H]^-$ at *m/z* 463) was assigned to a quercetin hexoside, although the position and nature of the hexosyl moiety could not be identified, because its retention time did not correspond to any of the standards available (quercetin 3-*O*-glucoside, Rt = 20.05 min.). Peak 9 ($[M-H]^-$ *m/z* at 595) was assigned to a quercetin derivative bearing pentosyl and hexosyl residues, based on the loss of 294 u (132+162 u) to yield the aglycone (*m/z* at 301, quercetin). The fact that the two moieties were lost simultaneously suggested that they could constitute a disaccharide *O*-linked to the aglycone. Peaks 17 and 18 ($[M-H]^-$ at *m/z* 505) should correspond to quercetin *O*-acetylhexosides according to their pseudomolecular ion and MS^2 fragment released at *m/z* 301 (quercetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety).

Peak 11 ($[M-H]^-$ at *m/z* 695) released a majority MS^2 fragment at *m/z* 651 ($[M-H-44]^-$) interpreted as the loss of CO_2, coherent with the existence of a non-substituted carboxyl. The observation of other fragments at *m/z* 609 ($[M-H-86]^-$) and 447 ($[M-H-86-162]^-$) further support that supposition as they can be interpreted by the loss malonyl and malonylhexosyl residues, respectively. Finally, the fragment at *m/z* 301 ($[M-H-86-162-146])^-$; quercetin) would be explained by further loss of a rhamnosyl residue. The observation of fragments derived from the alternative loss of the malonylhexosyl and the rhamnosyl moieties could suggest that they were located at different positions on the aglycone; however, it might also be rationalised as a quercetin malonylhexosyl-rhamnoside where the two sugars were constituting a disaccharide, in which case the fragment at *m/z* 447 should be explained by structural rearrangement following the loss of the internal malonylhexosyl residue and further linkage of the terminal rhamnose to the aglycone, as observed by (Ma, Li, Van den Heuvel, & Claeys, 1997). In that case, the presence in the samples of quercetin 3-*O*-rutinoside might point to peak 11 as quercetin 3-*O*-malonylrutinoside.

Peak 12 ($[M-H]^-$ at *m/z* 579) was identified as a kaempferol derivative bearing pentosyl and hexosyl residues, owing to the loss of 132+162 u to yield a fragment ion at *m/z* at 285 (kaempferol). The observation that no fragment from the loss of the pentosyl residue was observed pointed to the two sugars were constituting a disaccharide, and the minority fragment ion detected at *m/z* 417 (-162 u, hexosyl residue) suggests that the hexose was the terminal moiety of the disaccharide. Thus, the peak was identified as a kaempferol *O*-pentosyl-hexoside.

Finally, peaks 15 and 25 presented pseudomolecular ions $[M-H]^-$ at *m/z* 477 and 519, which were coherent with an isorhamnetin *O*-hexoside and an isorhamnetin *O*-

acetylhexoside, as indicated by the respective losses of 162 u and 162+42 u yielding a unique MS^2 fragment ion at *m/z* 315 (isorhamnetin).

Phenolic acids were the major phenolic compounds present in both wild and commercial samples (**Table 5**), being caffeoylquinic and dicaffeoylquinic acids derivatives the most abundant ones; c*is* and *trans* 3,5-*O*-dicaffeoylquinic acids (peaks 20 and 21) were the compounds found in the highest amounts. Benedek et al. (2007) and Vitalini et al. (2011) also reported 3,5-*O*-dicaffeoylquinic acid as being the main dicaffeoylquinic acid in *A. millefolium* from Austria and Italy, respectively. Those authors also described a similar phenolic profile to the one obtain herein, although with some differences in the flavonoids identified, being apigenin 7-*O*-glucoside, luteolin 7-*O*-glucoside and rutin the main flavonoids reported by them. In our samples luteolin *O*-acetylhexoside and apigenin *O*-acetylhexoside (peaks 24 and 27) were the most abundant flavonoids in both wild and commercial samples. In fact, the presence of acetyl derivatives seems a characteristic of the flavonoid composition in these samples. In this study, besides the mentioned majority flavones, flavonols such as quercetin, kaempferol and isorhamnetin glycosides derivatives were also found, as also *C*-glycosides linkage of apigenin and luteolin, which were not previously reported for this sample. In *A. millefolium* sample from Lithuania, Benetis et al. (2008) described the presence of some similar compounds but they did not identify all the compounds present; the authors identified and quantified only eight phenolic compounds.

Regarding contents of total phenolic compounds and phenolic families, different results were obtained depending on the origin of the sample (wild or commercial) and the type of preparation (**Table 5**). Thus, whereas the methanolic extract of wild *A. millefolium*

presented higher amount of total phenolic compounds than the commercial sample, the opposite was found in the case of the decoction; infusion yielded more similar amounts of total phenolics in both samples. In all cases phenolic acid derivatives were more abundant than flavonoids, but the contents of these latter were greater in the commercial sample. Benedek et al. (2007) expressed the results in relative percentages, which difficults the comparison with our study; moreover, they reported the presence of 15 compounds whilst 28 are described herein. Vitalini et al. (2011) did not present any type of quantification for samples of *A. millefolium* from Italy, presenting a profile with 10 different compounds. Benetis et al. (2008) performed the identification and quantification of 8 phenolic compounds, which presented similar values to the ones obtained in our samples.

Overall, commercial yarrow gave higher content of fat (and SFA), proteins, ash, energetic value, total sugars (including fructose, glucose, sucrose and trehalose) and flavonoids (mainly luteolin *O*-acetylhexoside and apigenin *O*-acetylhexoside), while the wild sample revealed higher levels of carbohydrates, organic acids (including malic, oxalic and quinic acids), unsaturated fatty acids, tocopherols (γ-, α- and β-isoforms) and phenolic acids (mainly *cis* and *trans* 3,5-*O*-dicaffeoylquinic acids). In general, commercial yarrow also gave higher antioxidant activity. The decoctions of both samples showed higher free radicals scavenging activity and lipid peroxidation inhibition, while the infusions gave higher reducing power. The methanolic extract of the commercial sample revealed higher antitumour potential against non-small lung, colon and cervical carcinoma cell lines, while the infusion of the wild yarrow gave higher antitumour potential against hepatocellular and breast carcinoma cell lines; for the latter cell line, the methanolic extract showed statistically similar results. The

opposite was observed for phenolic compounds concentrations: the methanolic extract of the wild sample revealed the highest levels, while for commercial sample the infusion gave the highest concentration. The heterogeneity among the bioactivity results of the samples and some low correlations with total phenolic acids, flavonoids and phenolic compounds (data not shown) suggested that specific compounds, rather than the totality of them, might be involved in different bioactive properties of samples; the bioactivity could also be related to interactions between specific compounds present in each sample. Moreover, as the most bioactive compounds may be present in lower amounts, further studies should be conducted in order to identify the specific compounds responsible for distinct bioactivities in the samples.

As far as we know, there are no reports of the comparison of different extracts of *A. millefolium*, being this a groundbreaking study on the nutraceutical composition, bioactivity and phenolic profile of wild and commercial yarrow. This study also showed that the chemical qualitative profiles of wild and commercial samples, as also their preparations (i.e., methanolic extract, infusion and decoction) are, in general, similar, varying only in the quantities found. Data obtained are clear evidence that traditional medicinal plants can be used not only in household products but also in pharmaceutical and food industry as a source of new and safer bioactive compounds.

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Table 1. Chemical composition of wild and commercial *Achillea millefolium* L. in macronutrients, free sugars and organic acids.

	Wild sample	Commercial sample
Fat (g/100 g dw)	5.20 ± 0.13[b]	8.03 ± 0.00[a]
Proteins (g/100 g dw)	12.53 ± 0.85[b]	19.53 ± 0.05[a]
Ash (g/100 g dw)	6.43 ± 0.11[b]	8.54 ± 0.88[a]
Carbohydrates (g/100 g dw)	75.84 ± 0.76[a]	63.90 ± 0.86[b]
Energy (kcal/100 g dw)	400.28 ± 0.21[b]	405.99 ± 3.52[a]
Fructose	1.11 ± 0.02[b]	1.31 ± 0.06[a]
Glucose	0.66 ± 0.04[b]	1.43 ± 0.08[a]
Sucrose	0.80 ± 0.03[a]	0.95 ± 0.11[a]
Trehalose	0.42 ± 0.04[b]	1.18 ± 0.17[a]
Raffinose	0.15 ± 0.00	nd
Total sugars (g/100 g dw)	3.14 ± 0.08[b]	4.86 ± 0.29[a]
Oxalic acid	1.08 ± 0.06[a]	0.92 ± 0.01[b]
Quinic acid	0.69 ± 0.03[b]	1.50 ± 0.08[a]
Malic acid	1.64 ± 0.04[a]	0.77 ± 0.13[b]
Shikimic acid	0.02 ± 0.00[a]	0.02 ± 0.00[a]
Citric acid	0.83 ± 0.03[b]	1.25 ± 0.13[a]
Succinic acid	0.27 ± 0.03	nd
Fumaric acid	0.03 ± 0.00	tr
Total organic acids (g/100g dw)	4.55 ± 0.10[a]	4.46 ± 0.19[b]

nd- not detected; dw- dry weight. In each row different letters mean significant differences (p<0.05).

Table 2. Chemical composition of wild and commercial *Achillea millefolium* L. in fatty acids and tocopherols.

	Wild sample	Commercial sample
C6:0	0.72 ± 0.07	0.26 ± 0.03
C8:0	0.05 ± 0.01	0.36 ± 0.04
C10:0	0.20 ± 0.02	4.25 ± 0.37
C11:0	0.05 ± 0.01	0.68 ± 0.01
C12:0	0.09 ± 0.01	0.53 ± 0.06
C13:0	0.02 ± 0.00	0.22 ± 0.02
C14:0	0.05 ± 0.01	1.39 ± 0.12
C14:1	0.03 ± 0.00	0.27 ± 0.09
C15:0	0.07 ± 0.00	0.44 ± 0.02
C15:1	0.09 ± 0.01	0.45 ± 0.04
C16:0	15.54 ± 0.18	20.70 ± 0.17
C16:1	0.06 ± 0.00	1.46 ± 0.06
C17:0	0.26 ± 0.00	0.79 ± 0.02
C18:0	2.85 ± 0.01	6.49 ± 0.07
C18:1n-9	28.23 ± 0.11	9.79 ± 0.00
C18:2n-6	47.16 ± 0.12	26.22 ± 0.10
C18:3n-6	0.10 ± 0.00	3.66 ± 0.03
C18:3n-3	0.23 ± 0.02	11.36 ± 0.70
C20:0	0.72 ± 0.01	1.22 ± 0.04
C20:1	0.30 ± 0.00	0.49 ± 0.03
C20:2	0.08 ± 0.04	0.44 ± 0.32
C20:3n-6	nd	0.20 ± 0.01
C20:4n-6	0.17 ± 0.02	0.46 ± 0.02
C20:3n-3+C21:0	0.47 ± 0.01	0.30 ± 0.00
C20:5n-3	0.96 ± 0.00	0.67 ± 0.17
C22:0	0.79 ± 0.04	2.18 ± 0.15
C22:1n-9	0.04 ± 0.01	0.17 ± 0.15
C23:0	0.14 ± 0.01	0.50 ± 0.02
C24:0	0.55 ± 0.06	4.04 ± 0.06
SFA (g/100 g fat)	22.09 ± 0.22[b]	44.06 ± 0.74[a]
MUFA (g/100 g fat)	28.75 ± 0.09[a]	12.64 ± 0.07[b]
PUFA (g/100 g fat)	49.16 ± 0.12[a]	43.30 ± 0.67[b]
α-tocopherol	0.95 ± 0.21[a]	0.87 ± 0.14[a]
β-tocopherol	4.63 ± 0.30[a]	1.81 ± 0.16[b]
γ-tocopherol	13.04 ± 1.38[a]	12.49 ± 1.21[a]
Total tocopherols (mg/100 g dw)	18.62 ± 1.89[a]	15.16 ± 1.51[b]

nd- not detected; dw- dry weight Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Undecylic acid (C11:0); Lauric acid (C12:0); Tridecanoic acid (C13:0); Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); *cis*-10-Pentadecenoic acid (C15:1); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n-9c); Linoleic acid (C18:2n-6c); α-Linolenic acid (C18:3n-3); γ-Linolenic acid (C18:3n-6); Arachidic acid (C20:0); *cis*-11-Eicosenoic acid (C20:1); *cis*-11,14-Eicosadienoic acid (C20:2); Arachidonic acid methyl ester (C20:3n-6); Arachidonic acid methyl ester (C20:4n-6); *cis*-11,14,17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n-3+C21:0); Eicosapentaenoic acid (C20:5n-3); Behenic acid (C22:0); Erucic acid (C22:1n-9); Tricosanoic acid (C23:0); Lignoceric acid (C24:0). SFA – saturated fatty acids; MUFA –

monounsaturated fatty acids; PUFA – polyunsaturated fatty acids. In each row different letters mean significant differences between species ($p<0.05$).

Table 3. Bioactivity of the methanolic extract, infusion and decoction of wild and commercial *Achillea millefolium* L..

	Wild sample			Commercial sample			Positive control*
	Methanolic extract	Infusion	Decoction	Methanolic extract	Infusion	Decoction	
Antioxidant activity							
DPPH scavenging activity (EC_{50}, mg/mL)	0.50 ± 0.01^{a}	0.40 ± 0.01^{b}	0.25 ± 0.01^{d}	0.37 ± 0.01^{c}	0.22 ± 0.00^{e}	0.20 ± 0.01^{f}	0.04 ± 0.00
Reducing power (EC_{50}, mg/mL)	0.25 ± 0.01^{b}	0.12 ± 0.00^{e}	0.45 ± 0.00^{a}	0.18 ± 0.01^{d}	0.13 ± 0.00^{e}	0.23 ± 0.00^{c}	0.03 ± 0.00
β-carotene bleaching inhibition (EC_{50}, mg/mL)	2.08 ± 0.04^{a}	0.59 ± 0.30^{b}	0.18 ± 0.03^{c}	0.30 ± 0.21^{c}	0.53 ± 0.06^{b}	0.22 ± 0.00^{c}	0.003 ± 0.00
TBARS inhibition (EC_{50}, mg/mL)	0.81 ± 0.09^{a}	0.45 ± 0.14^{b}	0.04 ± 0.01^{d}	0.26 ± 0.02^{c}	0.07 ± 0.01^{d}	0.08 ± 0.01^{d}	0.004 ± 0.00
Antitumour potential							
MCF-7 (breast carcinoma) (GI_{50}, µg/mL)	17.11 ± 1.05^{c}	14.98 ± 1.68^{c}	64.15 ± 1.75^{a}	48.30 ± 6.07^{b}	64.90 ± 0.79^{a}	64.22 ± 1.02^{a}	0.91 ± 0.04
NCI-H460 (non-small cell lung cancer) (GI_{50}, µg/mL)	54.24 ± 0.46^{a}	29.17 ± 4.12^{b}	56.24 ± 3.09^{a}	24.64 ± 0.80^{b}	56.26 ± 1.15^{a}	55.71 ± 0.04^{a}	1.42 ± 0.00
HCT-15 (colon carcinoma) (GI_{50}, µg/mL)	18.88 ± 0.77^{bc}	15.24 ± 2.10^{c}	22.67 ± 3.82^{ab}	13.90 ± 0.75^{c}	26.23 ± 2.26^{a}	24.27 ± 0.16^{ab}	1.91 ± 0.06
HeLa (cervical carcinoma) (GI_{50}, µg/mL)	39.02 ± 2.90^{b}	20.73 ± 1.16^{c}	52.06 ± 3.87^{a}	19.68 ± 0.47^{c}	47.31 ± 4.84^{ab}	40.96 ± 6.07^{b}	1.14 ± 0.21
HepG2 (hepatocellular carcinoma) (GI_{50}, µg/mL)	47.14 ± 1.85^{b}	37.60 ± 0.86^{b}	61.26 ± 3.77^{a}	41.12 ± 0.54^{b}	67.46 ± 4.47^{a}	66.13 ± 7.10^{a}	3.22 ± 0.67
Hepatotoxicity PLP2 (GI_{50}, µg/mL)	58.14 ± 1.05^{e}	57.08 ± 0.97^{e}	314.41 ± 0.24^{a}	250.42 ± 3.30^{c}	118.95 ± 0.29^{d}	288.82 ± 6.30^{b}	2.06 ± 0.03

*Trolox and ellipticine for antioxidant and antitumour activity assays, respectively. EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p<0.05$).

Table 4. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and concentration of phenolic acids and flavonoids in *Achillea millefolium* L..

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Identification
1	5.24	326	353	191(100),179(70),173(5),135(53)	3-*O*-Caffeoylquinic acid
2	6.51	326	341	179(100)	Caffeic acid hexoside
3	7.30	328	353	191(50),179(88),173(100),135(70)	4-*O*-Caffeoylquinic acid
4	8.08	326	353	191(100),179(11),173(8),135(5)	5-*O*-Caffeoylquinic acid
5	11.37	330	593	473(19),383(12),353(27)	Apigenin *C*-hexoside-*C*-hexoside
6	15.12	332	563	473(9),443(11),383(20),353(21)	Apigenin *C*-hexoside-C-pentoside
7	15.44	342	563	473(10),443(20),383(15),353(27)	Apigenin *C*-glucose-*C*-pentoside
8	16.36	350	447	357(83),327(88),297(30),285(16)	Luteolin 6-*C*-glucoside
9	17.37	356	595	301(100)	Quercetin *O*-pentosyl-hexoside
10	17.66	344	463	301(100)	Quercetin *O*-hexoside
11	18.17	334	695	651(100),609(3),447(16),301(17)	Quercetin *O*-malonylhexosyl-rhamnoside
12	19.47	350	579	417(7),285(49)	Kaempferol *O*-pentosyl-hexoside
13	19.61	352	609	301(100)	Quercetin 3-*O*-rutinoside
14	20.45	340	593	269(100)	Apigenin *O*-dihexoside
15	20.64	336	477	315(100)	Isorhamnetin *O*-hexoside
16	21.01	328	515	353(71),335(34),299(3),255(4),203(8),191(41),179(70),173(93),161(15),135(32)	3,4-*O*-dicaffeoylquinic acid
17	21.37	346	505	301(100)	Quercetin *O*-acetylhexoside
18	22.35	352	505	301(100)	Quercetin *O*-acetylhexoside
19	22.64	328	515	353(96),335(4),191(100),179(70),173(8),161(14),135(22)	*cis* 3,5-*O*-dicaffeoylquinic acid
20	22.88	330	515	353(96),335(10),191(100),179(68),173(7),161(15),135(15)	*trans* 3,5-*O*-dicaffeoylquinic acid
21	23.46	344	563	269(100)	Apigenin *O*-pentosyl-hexoside

22	25.41	328	515	353(17),335(3),299(5),255(3),203(15),191(49),179(57),173(79),161(14),135(17)	4,5-*O*-dicaffeoylquinic acid
23	25.53	332	431	269(100)	Apigenin 7-*O*-glucoside
24	26.21	350	489	285(100)	Luteolin *O*-acetylhexoside
25	28.25	362	519	315(100)	Isorhamnetin *O*-acetylhexoside
26	29.22	338	473	269(100)	Apigenin *O*-acetylhexoside
27	30.34	336	473	269(100)	Apigenin *O*-acetylhexoside
28	31.20	340	473	269(100)	Apigenin *O*-acetylhexoside

Table 5. Phenolic compounds quantification in the methanolic extract (mg/g extract), infusion (mg/g infusion) and decoction (mg/g decoction) of wild and commercial *Achillea millefolium* L..

	Wild sample			Commercial sample		
	Methanolic extract	**Infusion**	**Decoction**	**Methanolic extract**	**Infusion**	**Decoction**
Extraction yield (%)	20.39 ± 0.91	21.50 ± 1.02	13.31 ± 0.52	21.32 ± 1.10	22.72 ± 0.48	12.64 ± 0.27
1	0.86 ± 0.04	0.96 ± 0.05	1.22 ± 0.04	0.96 ± 0.07	1.28 ± 0.12	0.89 ± 0.05
2	0.28 ± 0.01	0.21 ± 0.03	0.09 ± 0.01	0.16 ± 0.00	0.21 ± 0.03	0.57 ± 0.03
3	1.01 ± 0.10	1.00 ± 0.00	0.65 ± 0.01	0.31 ± 0.04	0.65 ± 0.04	0.67 ± 0.03
4	24.20 ± 0.18	24.58 ± 0.30	12.76 ± 0.12	13.99 ± 0.64	19.34 ± 0.85	15.24 ± 0.38
5	0.52 ± 0.01	0.76 ± 0.06	0.56 ± 0.05	1.73 ± 0.14	2.28 ± 0.16	2.31 ± 0.10
6	0.75 ± 0.10	0.73 ± 0.02	0.43 ± 0.00	1.18 ± 0.11	1.68 ± 0.13	1.90 ± 0.13
7	0.26 ± 0.02	0.22 ± 0.02	0.17 ± 0.00	0.27 ± 0.01	0.34 ± 0.02	0.42 ± 0.03
8	0.12 ± 0.00	0.12 ± 0.00	0.08 ± 0.00	0.28 ± 0.01	0.32 ± 0.02	0.43 ± 0.05
9	0.15 ± 0.00	0.10 ± 0.00	0.08 ± 0.00	0.35 ± 0.04	0.34 ± 0.01	0.72 ± 0.05
10	2.71 ± 0.12	1.15 ± 0.01	0.29 ± 0.00	0.16 ± 0.04	0.21 ± 0.03	0.18 ± 0.01
11	0.44 ± 0.02	0.19 ± 0.01	0.12 ± 0.01	0.28 ± 0.03	0.22 ± 0.01	0.31 ± 0.03
12	0.29 ± 0.02	0.32 ± 0.01	0.31 ± 0.02	0.49 ± 0.00	0.63 ± 0.04	0.63 ± 0.01
13	0.94 ± 0.03	0.79 ± 0.01	0.10 ± 0.00	0.64 ± 0.02	0.79 ± 0.06	0.86 ± 0.02
14	0.61 ± 0.06	0.98 ± 0.02	0.51 ± 0.06	1.13 ± 0.11	1.52 ± 0.14	1.54 ± 0.05
15	0.34 ± 0.04	0.68 ± 0.04	0.28 ± 0.02	0.29 ± 0.03	0.42 ± 0.07	0.50 ± 0.01
16	5.45 ± 0.19	5.69 ± 0.20	1.60 ± 0.09	4.41 ± 0.27	5.34 ± 0.55	6.16 ± 0.04
17	1.61 ± 0.05	1.88 ± 0.02	0.87 ± 0.06	nd	nd	nd
18	0.76 ± 0.10	0.68 ± 0.09	0.23 ± 0.03	0.55 ± 0.06	0.77 ± 0.08	0.18 ± 0.03
19	35.73 ± 0.44	28.05 ± 0.16	7.40 ± 0.29	25.30 ± 0.24	28.45 ± 2.41	27.83 ± 0.03

20	26.02 ± 0.05	19.96 ± 0.53	6.85 ± 0.05	10.50 ± 0.24	13.46 ± 0.87	11.98 ± 0.28
21	1.13 ± 0.13	1.06 ± 0.01	0.39 ± 0.04	0.88 ± 0.07	0.82 ± 0.32	0.71 ± 0.00
22	10.24 ± 0.02	8.87 ± 0.22	1.94 ± 0.03	10.75 ± 0.67	12.17 ± 0.31	13.53 ± 0.37
23	1.43 ± 0.01	1.20 ± 0.18	0.57 ± 0.00	2.65 ± 0.06	2.56 ± 0.35	2.58 ± 0.10
24	6.21 ± 0.59	5.32 ± 0.05	2.80 ± 0.09	6.49 ± 0.10	7.22 ± 0.04	6.80 ± 0.44
25	0.15 ± 0.01	0.16 ± 0.00	0.24 ± 0.01	0.10 ± 0.00	0.08 ± 0.01	0.07 ± 0.00
26	0.36 ± 0.02	0.35 ± 0.01	0.15 ± 0.01	0.64 ± 0.08	0.75 ± 0.08	0.68 ± 0.03
27	5.45 ± 0.35	5.89 ± 0.04	2.72 ± 0.07	9.85 ± 0.45	12.12 ± 1.04	9.47 ± 0.29
28	0.35 ± 0.00	0.37 ± 0.00	0.25 ± 0.04	0.69 ± 0.06	0.71 ± 0.07	0.79 ± 0.02
TPA	**103.80 ± 0.45^{a}**	**89.32 ± 0.12^{b}**	**32.52 ± 0.52^{e}**	**66.39 ± 2.18^{d}**	**80.91 ± 5.19^{c}**	**76.88 ± 0.39^{c}**
TF	**24.56 ± 0.36^{d}**	**22.96 ± 0.10^{d}**	**11.14 ± 0.05^{e}**	**28.63 ± 1.01^{c}**	**33.78 ± 1.98^{a}**	**31.09 ± 0.47^{b}**
TP	**128.36 ± 0.0^{a}**	**112.28 ± 0.22^{bc}**	**43.66 ± 0.57^{e}**	**95.02 ± 3.19^{d}**	**114.69 ± 7.17^{b}**	**107.97 ± 0.86^{c}**

nd- not detected. TPA- Total phenolic acids; TF- Total flavonoids; TP- Total phenolic compounds. In each row different letters mean significant differences (p<0.05).