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Achillea millefolium L. – phytochemical profile and *in vitro* antioxidant activity

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Abstract

Nowadays, medicinal plants used in folk medicine are being increasingly studied and used on pharmaceutical, food and nutraceutical fields. Yarrow (*Achillea millefolium* L.) is widely used in both folk and official medicine. Therefore, in the present paper different extracts of yarrow - inflorescences and upper leaves were investigated for their total polyphenolic content and antioxidant activity using several reliable assays, namely DPPH-, ABTS-, FRAP- and CUPRAC assays. The phytochemical profile of the extracts was assessed by RP-HPLC methods as well in order to evaluate the influence of the single constituents. The total polyphenolic content of the extracts was established to be in range 2.74 ± 0.01 and 7.92 ± 0.09 mg GAE/g dw. Among the four applied techniques for extraction the decoction seems to be still the most effective ones. However, the results revealed that the extracts of the *Achillea millefolium* could be used as easily accessible source of natural antioxidants, but also as a possible food supplement or in pharmaceutical industry.

Introduction

Herbs are used in many domains, including medicine, nutrition, flavouring, beverages, dyeing, repellents, fragrances, cosmetics (Djeridane *et al*., 2006; Najafi and Deokule, 2010). Many species were used in folk and human medicine, because they have been recognized to have medicinal properties and beneficial impact on health, e.g. antioxidant activity, digestive stimulation action, antiinflammatory, antimicrobial, hypolipidemic, antimutagenic effects and anticarcinogenic potential (Aaby *et al.*, 2004; Luo *et al*., 2004).

Achillea millefolium L. (yarrow), belongs to Asteraceae family and it is represented by about 85 species mostly found in Europe and Asia and a handful in North America (Turner and Wasson, 1999). In the Bulgarian flora the genus *Achillea* is presented by 22 species, as *Achillea millefolium* most distributed (Assyov *et al*., 2012). In the folk medicine yarrow is used for treatment of fever, asthma, bronchitis, cough, skin inflammation, jaundice, diabets, hepatobiliary diseases, healing of wounds, menstrual regulation, flatulence, dyspepsia, hemorrhoids, dysmenorrhoea and gastritis and also consumed for its antitumour, antimicrobial, anti-inflammatory and antioxidant properties (Cavalcanti *et al*., 2006; Yassa *et al*., 2007; Candan *et al*., 2010; Potrich *et al*., 2010; Dall'Acquaa *et al.*, 2011; Jonsdottir *et al*., 2011; Trumbeckaite *et al*., 2011; Baretta *et al.*, 2012). Antioxidant properties of *A. millefolium* have previously been reported in hydroalcoholic, methanolic and aqueous extracts, as also in the essential oils (Candan *et al*., 2010; Kintzios *et al.*, 2010; Trumbeckaite *et al*., 2011; Vitalini *et al*., 2011).

Some articles have described potential of water extracts- decoction and infusion from *Achillea millefolium* as well activity and total phenols (Keser *et al*., 2013), chemical composition, bioactivity and phenolic profile (Dias *et al.*, 2013) as skin and mucosa inflammations (Rauchensteiner *et al.*, 2004). The aim of the present study was to investigate the antioxidant potential and the phytochemical profile in terms of phenolic acids and flavonoids content of different water extracts obtained from Bulgarian *Achillea millefolium* L.

Materials and Methods

Plant material

Achillea millefolium L. as dry herb was obtained

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from a local pharmacy (Plovdiv, Bulgaria). After additional drying the plant parts – leaves and stems were roughly grounded and stored in air-tight dark containers until extraction.

Preparation of plant extracts

In order to explore the most suitable form of extract obtaining for everyday consumption by human, four different extraction procedures with water were investigated in the present study. Grounded plant mass of *A. millefolium* was extracted with water (ratio of solvent to raw material was 1:20). In order to investigate more properly the biological activity of the plant, different extraction procedures were carried out as follow:

- Infusion - For the preparation of aqueous *A. millefolium* infusion plant material was infused into boiled solvent for 30 min.
- Decoction was conducted by boiling of the plant material with the solvent for 30 min;
- Ultrasound-assisted extraction (UAE) was carried out in an ultrasound cleaning bath (UST 5.7-150, rated power 240 W, temperature 60°C, Siel, Gabrovo, Bulgaria) for 30 min.
- Microwave-assisted extract (MAE) experiments were performed with a domestic microwave oven (LG MB4047C). The extraction was carried out at output power 800 W for 30 sec (with frequency of the waves 2450 MHz).

All obtained extracts were filtered after incubation and stored at 4°C without adding any preservatives until analyses.

Antioxidant activity (AOA)

Total polyphenol content analysis (TPC)

The total polyphenol content was analyzed using the Folin-Ciocalteu method of Kujala *et al.* (2000) with some modifications. Each sample extract (1 ml) was mixed with 5 ml of Folin-Ciocalteu`s phenol reagent and 4 ml of 7.5% Na_2CO_3. The mixture was vortexed well and left for 5 min at 50°C. After incubation, the absorbance was measured at 765 nm. The TPC in the extracts was expressed as mg gallic acid equivalent (GAE) per g dry weight.

DPPH radical scavenging activity

The ability of the extracts to donate an electron and scavenge DPPH radical was determined by the slightly modified method of Brand-Williams *et al.* (1995). Freshly prepared $4x10^{-4}$ M methanolic solution of DPPH was mixed with the samples in a ratio of 2:0.5 (v/v). The light absorption was measured at 517 nm at room temperature after 30 min incubation. The DPPH radical scavenging activity was presented as a function of the concentration of Trolox. Trolox equivalent antioxidant capacity (TEAC) and was defined as the concentration of Trolox having equivalent AOA expressed as the μM Trolox per g dw.

ABTS radical cation decolorization assay

The radicals scavenging activity of the extracts against radical cation ($ABTS^{\bullet+}$) was estimated according to a previously reported procedure with some modifications (Re *et al*., 1999). The results were expressed as TEAC value (μM TE/g dw).

Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was carried out according to the procedure of Benzie and Strain (1999). The FRAP reagent was prepared fresh daily and was warmed to 37°C prior to use. The absorbance of the reaction mixture was recorded at 593 nm after incubation at 37°C for 4 min. The results were expressed as μM TE/g dw.

Copper reduction assay (CUPRAC)

CUPRAC assay was performed according to the method of Ak and Gülçin 2008. To a test tube were added 1 ml of $CuCl_2$ solution (1.0×10^{-2}M), 1 ml of neocuproine methanolic solution (7.5×10^{-3}M), and 1 ml NH_4Ac buffer solution (pH 7.0), and mixed; 0.1 ml of herbal extract (sample) followed by 1 ml of water were added (total volume of 4.1 ml), and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. Trolox was used as standard and total antioxidant capacity of herbal extracts was measured as μM TE/g dw.

HPLC analysis

The HPLC analysis of phenolic acids and flavonoids were performed by a Waters HPLC system, (Milford, MA, USA) equipped with binary pump (Waters 11525), a UV-VIS detector (Waters 2487) and Breeze 3.30 SPA software. Detailed conditions of HPLC analyses are reported previously (Marchev *et al*., 2011). Concentration of each individual compound was calculated based on external standard method and was converted to μg compound per g dry weight (dw).

Statistical analysis

The presented results are average from two independent experiments carried out in triplicates. The results were expressed as mean ± SD, analyzed using MS Excel 2003 software.

Results and Discussion

Antioxidant properties of the most frequently consumed forms of *A. millefolium,* infusion and decoction, in addition to two alternative extraction forms (microwave and ultrasound extracts) were established in the present study and the results are shown in Table 1.

Determination of total polyphenolic content (TPC)

The total polyphenolic concentration of the water extracts from A. millefolium were in range from 2.74 ± 0.01 to 7.92 ± 0.09 mg GAE/g dw, respectively (Table 1). Among all applied methods of extraction decoction technique revealed as most effective in terms of total polyphenolic substances. Other authors (Dias *et al*., 2013) reported different results regarding contents of polyphenolic compounds depending on the origin of the sample (wild or commercial) and the type of preparation of the extract. They established higher amount of total polyphenolic compounds in the methanolic extract of wild *A. millefolium* than the commercial sample. Wojdyło *et al.* (2007) reported total polyphenolic content in herbal parts of *A. millefolium* - 9.55 ± 0.11 mg GAE/100 g dw.

Determination of antioxidant activity (AOA) - DPPH, ABTS, FRAP and CUPRAC assays

In order to investigate the AOA, experiments with two stable radicals - DPPH• and $ABTS^{\bullet+}$ were conducted, in addition to the FRAP and CUPRAC assays. $TEAC_{DPPH}$ values were in range from 24.15 ± 0.15 to 116.74 ± 0.21 µM TE/g dw and the $TEAC_{ABTS}$ values were from 18.59 ± 0.22 to 125.75 ± 2.24 µM TE/g dw, which is in agreement with the established total polyphenolic content (Table 1). Higher TEAC value indicates that a sample has stronger AOA.

However, the established results were in prevalence of decoction extracts among all and the infusions posses much lower ability to scavenge the studied free radicals. Dias *et al*. (2013) by comparing decoctions and infusions of wild and commercial *A. millefolium* outlined that decoctions showed the higher DPPH scavenging activity, β-carotene bleaching inhibition and TBARS inhibition, while infusions presented the highest reducing power.

In another antioxidant study of *A. millefolium* herbal parts (Wojdylo *et al*., 2007) was demonstrated that the $TEAC_{ABTS}$ was 11.2 ± 0.77 µM TE/100 g dw, $TEAC_{DPPH}$ - 200 ± 3.33 µM TE/100 g dw and $TEAC_{FRAP}$ - 191 ± 4.51 µM TE/100 g dw, respectively.

The FRAP values for the investigated extracts of *A. millefolium* were in accordance with the above mentioned results as follows: from 29.57 ± 0.40 to 132.71 ± 1.86 µM TE/g dw. Using the CUPRAC assay the established results were from 28.23 ± 0.34 to 148.99 ± 1.94 µM TE/g dw for the four different types of water extracts.

Table 1. Total polyphenolic content (mg GAE/g dw) and antioxidant activity (µM TE/g dw) of different water extracts from *A. millefolium*

Extract/assay	infusion	decoction	UAE	MAE
TPC	2.77 ± 0.03	7.92 ± 0.09	2.74 ± 0.01	3.47 ± 0.03
$TEAC_{DPPH}$	24.15 ± 0.15	116.74 ± 0.21	32.00 ± 0.63	90.07 ± 0.75
$TEAC_{ABTS}$	30.54 ± 0.01	125.75 ± 2.24	18.59 ± 0.22	63.01 ± 0.53
$TEAC_{FRAP}$	38.16 ± 0.47	132.71 ± 1.86	29.57 ± 0.40	76.41 ± 0.53
$TEAC_{CUPRAC}$	55.38 ± 0.85	148.99 ± 1.94	28.23 ± 0.34	103.64 ± 1.27

The results of the total antioxidant capacity assays (Table 1) showed that the investigated extracts possessed AOA, which for the decoction extract was approximately two/three times higher than the other investigated type extracts. This confirmed the results obtained from the TPC assay. Interestingly, the highest AOA values were measured by the CUPRAC assay. A slight difference among the results obtained by the DPPH, ABTS, FRAP and CUPRAC assays was observed. The probable reason for this could be the unique mechanism and the unequal sensitivity of each method applied. Therefore, the authors suggested that, when analyzing the antioxidant activity of samples, it is better to use at least two methods due to the differences between the test systems (Ou *et al.*, 2001).

HPLC analysis

The identification of phenolic acids and flavonoids present in the studied extracts of *A. millefolium* and their quantification was of interest, in order to establish their influence over each extract properties and thus to reveal the most suitable extraction procedure.

Thus the decoction and microwave extracts show the highest total polyphenolic content and antioxidant potential those two samples were chosen for further analyses. The conducted experiments showed presence of several flavonoids and phenolic acids in the extracts of *A. millefolium* (Tables 2 and 3).

The most abundant phenolic acids in all

Table 2. Phenolic acids content in extracts from *A. millefolium,* µg/g dw

Phenolic acids/extract	*Microwave extr.*	*Decoction extr.*
Gallic acid	-	27.22
3,4-OH-benzoic acid	-	-
2-OH-benzoic acid	89.10	144.99
Chlorogenic acid	403.86	784.11
Vanillic acid	8.29	46.94
Caffeic acid	171.87	15.28
Syringic acid	-	300.82
p-Coumaric acid	266.39	475.24
Sinapic acid	46.54	382.87
Ferulic acid	145.76	19.27
Cinnamic acid	-	6.43

Table 3. Flavonoid aglycones and glycosides content in extracts from *A. millefolium*, µg/g dw

Flavonoids aglycones and glycosides/extract	*Microwave extr.*	*Decoction extr.*
Myrcetin	52.00	45.44
Hesperidin	-	-
Quercetin	-	-
Luteolin	95.21	128.97
Kaempherol	15.91	14.64
Apigenin	-	-
Rutin	54.03	94.80
Hyperoside	453.25	604.95

investigated samples were chlorogenic acid (784.11 and 403.86 µg/g dw, resp.) and p-coumaric acid (475.24 and 266.39 µg/g dw, resp.) (Table 2). On the other hand Dias *et al.* (2013) reported that phenolic acids were the major phenolic compounds present in both wild and commercial samples of *A. millefolium*, being caffeoylquinic and dicaffeoylquinic acids derivatives the most abundant ones; cis and trans 3,5-O-dicaffeoylquinic acids 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. Hyperoside was the flavonoid compound present at high concentrations among the investigated standards (604.95 and 453.25 µg/g dw,

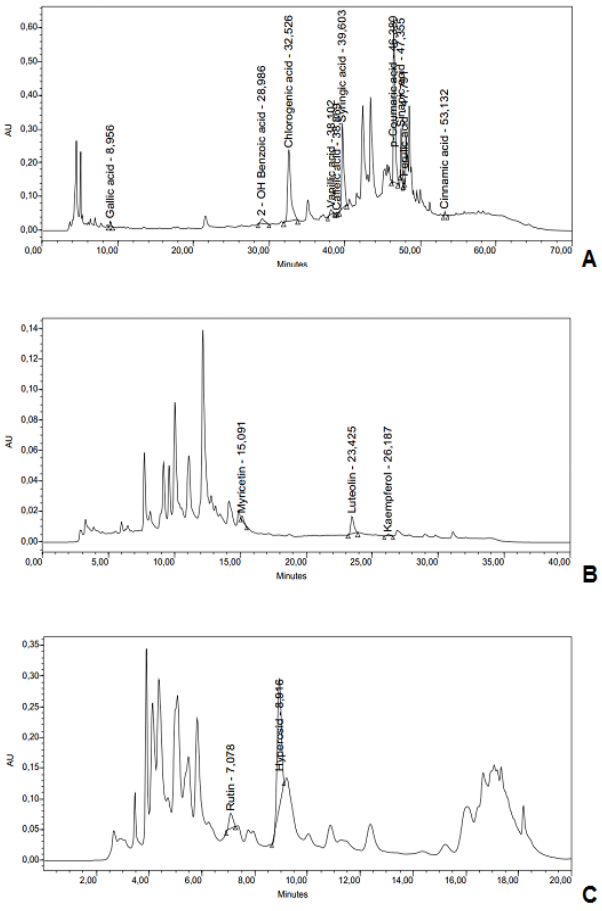

Figure 1. HPLC profile of microwave extract from *A. millefolium*: (A) phenolic acids; (B) flavonoid aglycones and (C) flavonoid glycosides

respectively).

The presence of the established constituents can contribute to the potential antioxidant properties of the examined plant extracts. The observed variations of the compounds present could be due to different extraction process. The extraction properties could be clearly demonstrated by HPLC chromatograms of phenolic acids and flvonoids profiles (Figures 1 and 2).

From the results it can be concluded that the decoction technique is more efficient compared to the microwave-assisted extraction, ultrasound extraction and the infusion obtaining in order to obtain extract with higher content of biologically active substances in terms of antioxidant potential. It should be noted that the results of microwave extracts were also relatively higher compared to infusions and ultrasound extracts. Thus nowadays the microwave extraction is increasingly used, it can be concluded that this type of herbal extract of *A. millefolium* is reasonable to be recommended in terms of total polyphenolic content in addition to the decoction technique.

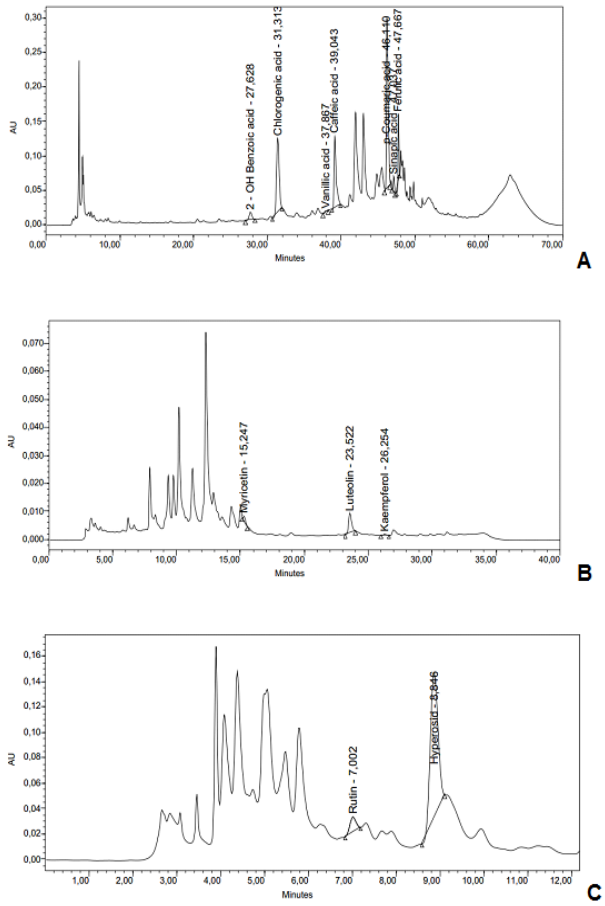

Figure 2. HPLC profile of decoction extract from *A. millefolium*: (A) phenolic acids; (B) flavonoid aglycones and (C) flavonoid glycosides

Conclusions

In summary, the present paper investigates the different possibilities of water extracts obtaining from *A. millefolium* in terms of biologically active substances intake in everyday life. Among the four investigated methods the decoction and microwave extraction techniques revealed as most effective with their total polyphenolic content and antioxidant capacities. Furthermore, the established phenolic constituents present in the both extracts could contribute to the potential properties of the *A. millefolium* extracts. However, the exact mechanism of action of the extracts *in vivo* definitely must be conducted as further research.

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