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DETERMINATION OF ANTIOXIDANTS IN MEDICINAL HERBS

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Abstract: *The evaluation of antioxidant capacity, total phenolics, vitamin C content and identification of several phenolic acids and flavonoids was performed in the following fresh and dried herbs widely used in folk medicine throughout Slovenia and elsewhere: elder berries (Sambucus nigra L.), barberries (Berberis vulgaris L.), rowan tree berries (Sorbus aucuparia L.), coltsfoot flowers (Tussilago farfara L.), linden flowers (Tilia platyphyllos Scop.), thyme flowers (Thymus vulgaris), milfoil flowers (Achillea millefolium) and plantain leafs (Plantago lanceolata L.). It was found out that selected medicinal herbs contain different antioxidants with different polarity and exhibit different antioxidant capacity. Their levels decrease with the drying process and during preparation of their infusions.*

Key words: *antioxidant capacity, total phenolics, vitamin C, medicinal herbs.*

1. Introduction

Herbs are usually considered as plants with aromatic properties and are mainly used in spicy foods and for preparation of herbal teas in folk medicine. Medicinal plants have always been considered as a source of health. They are among our oldest medicines and their increasing use in recent years is evidence of public interest in alternatives to conventional medicine. Since prehistoric times, herbs have also been the basis for nearly all medicinal therapy until synthetic drugs were developed in the 19th century. Today, herbs are still found in 40% of prescription drugs. In addition, herbs are used for many other purposes including beverages such as tea, dyeing, repellents, fragrances, cosmetics, charms, smoking and industrial uses.

Many recent studies found out that herbs contain various phytochemicals including antioxidants [3], [8], [9]. At the same time it has been shown that the free radicals are the major contributors to aging, and to degenerative diseases of aging, such as cancer, cardiovascular diseases, cataracts, immune system decline and brain dysfunction. It was also found out that free radical formation is controlled naturally by various beneficial compounds, namely antioxidants. There has been lots of evidence that consuming foods of plant origin (fruits, vegetables, tea, coffee and others) is associated with reduced incidence of these diseases. Moreover, knowledge and application of such potential antioxidant activities in reducing oxidative stresses *in vivo* has prompted many investigators to search for potent and cost-effective antioxidants from various

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plant sources [1], [5], [6]. These research activities have contributed to new or renewed public interests worldwide in herbal medicines, health foods, and nutritional supplements. It is therefore of big interest to systematically check for the presence of antioxidants and their antioxidative capacity in medicinal herbs that are widely used in local folk medicine.

2. Objectives

- to evaluate the total phenolics (TP), antioxidative capacity (AOC) and vitamin C content of eight different medicinal herbs that are used in folk medicine in Slovenia and elsewhere
- to compare AOC in extracts prepared by different extraction methods (cold, hot water and methanol)
- to evaluate the differences in AOC of fresh and dried plants as well as their infusions
- to evaluate the ratio between oxidised and reduced form of vitamin C in all samples
- to identify some of the most common antioxidants using HPLC-DAD-MS system

3. Material and Methods

3.1. Chemicals

Luminol (3-aminophthalhydrazide) and Folin-Ciocalteu reagent were purchased from Fluka, Germany. HRP (horseradish peroxidase) E.C. 1.11.1.7 (269 U mg^{-1}), chlorogenic acid, AA (ascorbic acid), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), MFK (metaphosphoric acid), $DPPH^{\bullet}$ (2,2-diphenyl-1-picryhydrazy radicals) and Tris [2-carboxyethyl] phosphine hydrochloride (TCEP) were from Sigma-Aldrich, Germany. Dimethyl-sulfoxide (DMSO), methanol, HCOOH, H_2SO_4, acetonitrile, Na_2CO_3, H_2O_2 and Na_2HPO_4 were from Merck, Germany.

3.2. Total phenolics assay by using Folin-Ciocalteu reagent

Total phenolics (TP) were determined spectrophotometrically using Folin-Ciocalteu (F-C) reagent. To obtain the calibration curve the reaction mixture contained 725 µL of standard solution of chlorogenic acid (3.4 – 58 µmol L^{-1}) and 125 µL of F-C reagent (freshly diluted with water; 1:2). After exactly 5 minutes 125 µL of 20% water solution of Na_2CO_3 was added. Solutions were mixed well and kept in the dark at ambient temperature for 1 hour to complete the reaction. Absorbance at 746 nm was measured by a spectrophotometer (Hewlett- Packard, model HP-8453, USA) against blank sample. To determine TP in herbal extracts, 5 µL of each extract was diluted with 2% MFK to 725 µL and the assay was performed as described above. With all samples assay was conducted in triplicate and the results were averaged. TP of the herbal extracts was expressed as equivalent of chlorogenic acid that gives same signal as the sample. In all figures TP is expressed as AOC since F-C assay is not selective solely to phenolics, but also all the rest of reducents (namely antioxidants) present in the sample react with F-C reagent.

3.3. $DPPH^{\bullet}$ scavenging capacity assay

One way to evaluate the effect of antioxidants is through their antiradical activity. The decrease of $DPPH^{\bullet}$ concentration is in linear correlation with antioxidant capacity (AOC) of the sample.

The reaction mixture was prepared as follows: 0.081 mM methanol solution of $DPPH^{\bullet}$ was prepared fresh each day and kept in dark to prevent decomposition. To

make the calibration curve the reaction mixture was prepared by mixing 70 μL of standard solution of chlorogenic acid (0.13 – 0.90 mmol L^{-1}) and 1 mL of methanol solution of $DPPH^{\bullet}$. The absorbance of reaction mixture was measured using UV–Vis spectrometer at 517 nm after 1 hour. For each analysis 5 μL of extract of fresh herbs and 10 μL of extract of dried herbs was used. With all samples assay was conducted in triplicate and the results were averaged. AOC of the herbal extracts was expressed as equivalent of chlorogenic acid.

3.4. Chemiluminescence assay

Chemiluminescence was followed by a fluorimeter (Cary Eclipse, Varian, Australia), having the excitation lamp off and using only the photomultiplier at 420 nm.

According to previously optimized chemiluminescent assay [4] the reaction mixture was prepared as follows: 0.188 M luminol stock solution was prepared in DMSO and kept on dark and cold until diluted 1:100 (making 1.88 mM solution) with 0.1 M phosphate buffer (pH 7.4) prior to experiments. Afterwards, 4.5 mL of previously prepared 1.88 mM luminol solution and 0.5 mL of 80 mM H_2O_2 in phosphate buffer (pH 7.4) were mixed and 2 mL of the so prepared mixture was then put to cuvette, to which 70 μL of phosphate buffer (pH 7.4) and 100 μL of HRP solution (3 U/mL) was added. After addition of the sample (standard solutions of chologenic acid or herbal extracts), which contained antioxidants, the chemiluminescence of the mixture was inhibited for the time that corresponded to AOC of the sample.

For analysis of herbal extracts 7 μL of fresh herb extracts and 15 μL of extracts of dried herbs were used. With all samples the assay was conducted in triplicate and the results were averaged. AOC of the herbal extracts was expressed as equivalent of chlorogenic acid that gives same signal as the sample.

3.5. Chromatographic analysis of ascorbic acid

Samples were analysed by HPLC (Marathon-XTautosampler, Knauer isocratic Pump (K-1001), X-Act degassing unit from Jour research, Knauer UV–VIS detector, Wellchrominterface box and PC running EuroChrom 2002 software).

Ascorbic acid (AA) was analysed on a Synergy Hydro-RP 80 (4 μm, 250×4.60 mm, Phenomenex) chromatographic column, equilibrated with 2.5 mM H_2SO_4 in Milli-Q water at a flow rate of 1 mL/min. 20 μL samples were injected on the column. AA, eluted after 4.9 min, was detected at 250 nm.

To determine the total vitamin C in herbal extracts all dehydroascorbic acid was fully reduced with TCEP prior to HPLC analysis. For this purpose 600 μL of 11 mM TCEP in 2% metaphosphoric acid was added to 600 μL of water herbal extracts.

3.6. HPLC-DAD-MS

To identify some of the polyphenols present in herbal extracts Agilent 1100 HPLC with DAD and MS detector coupled to an Agilent NDS ChemStation (Agilent Technologies, Palo Alto, USA) was used. The HPLC separations was carried out using a Phenomenex (Torrance, CA, USA), Luna C18 (2) column (150 mm×2.0 mm I.D., 3 μm particle size), protected by a Gemini C18 guard cartridge (4.0 mm× 2.0 mm I.D.). A Micromass Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source was operated in negative ion mode (Waters, Milford, MA). The mobile phase of formic acid in water (A) and

acetonitrile:methanol (B) was used in a gradient mode to achieve a satisfactory separation of polyphenolic compounds.

UV-Vis absorption spectra were recorded on-line during each HPLC analysis. The mass spectra were obtained by electrospray ionization.

The polyphenols were identified by comparisons of their retention times and spectral characteristics with the reference compounds, using the MassLynx 4.0 software (Micromass).

3.7. Extraction of herbs

For the purpose of this work extracts of the following medicinal herbs were analysed: elder berries (*Sambucus nigra* L.), barberry (*Berberis vulgaris* L.), rowan tree berries (*Sorbus aucuparia* L.), coltsfoot flowers (*Tussilago farfara* L.), linden flowers (*Tilia platyphyllos Scop*.), thyme flowers (*Thymus vulgaris* L.), milfoil flower (*Achillea millefolium*) and plantain leafs (*Plantago lanceolata* L.).

Water extracts

6 g of each herb was homogenised with 18 g of 2% metaphosphoric acid (in water), filtered through cellulose filters, and centrifuged for 5 min at 14,000× g at room temperature. The supernatants were filtered through CA filters (0.20 μm). Extracts were stored frozen (-20 °C) until analysed.

Methanol extracts

Same procedure as for the preparation of water extract was applied; water was replaced by methanol.

Preparation of infusions

Six g of dried herb was poured over by 18 g of boiling water and let 5 minutes to extract. The infusion was filtered through CA filters cooled down and analysed immediately.

4. Results and Discussions

4.1. AOC of fresh herbs

Water extracts

AOC and TP were determined and compared for all water extracts of selected fresh herbs. It can be seen on Figure 1 that the highest AOC was determined in thyme and plantain extracts, whereas the least antioxidants were determined in rowan tree and coltsfoot extracts. It can be noted that the results differ not only on the origin of extract but also on the method chosen for the evaluation of antioxidants. The highest AOC were in almost all extracts determined by F-C assay which besides polyphenols evaluate also all the rest of antioxidants present in the sample (see 3.2.). In general, lower AOCs were determined with the other two methods ($DPPH^{\bullet}$ and chemoluminescence) used in this study. The values depend on the antioxidative profile of each extract. It is therefore important to make evaluation of AOC with different methods that are differently sensitive to antioxidants with different antioxidative properties.

Methanol extracts

Similar results were obtained when methanol extracts of fresh herbs were analysed by the three methods for evaluation of AOC. The highest levels of antioxidants were determined in methanol extracts of linden and thyme and the lowest in coltsfoot and elder. It can be noted on Figure 2 that AOC of methanol extracts is in all cases considerably higher (up to 7-fold) than AOC of the same herb extracted by 2% MFK, hence it can be concluded that selected medicinal herbs contain more less-polar antioxidants that extract better with less-polar methanol and less highly-polar antioxidants that extract better with the highly-polar water solvent.

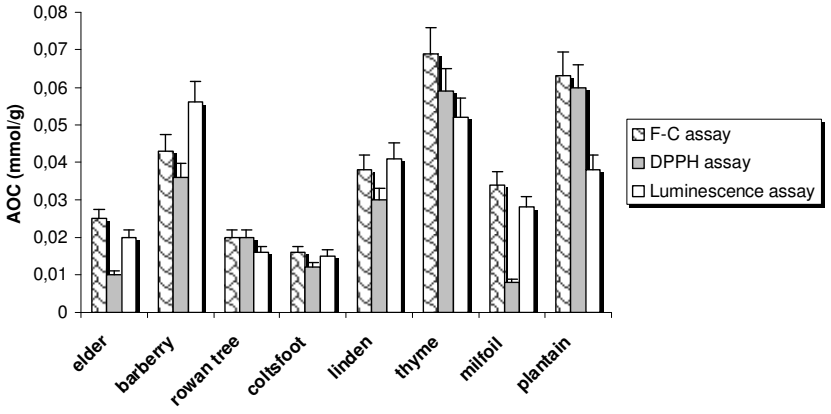

Fig. 1. *AOC of water extracts of fresh herbs determined by different methods. AOC is expressed as equivalent of chlorogenic acid.*

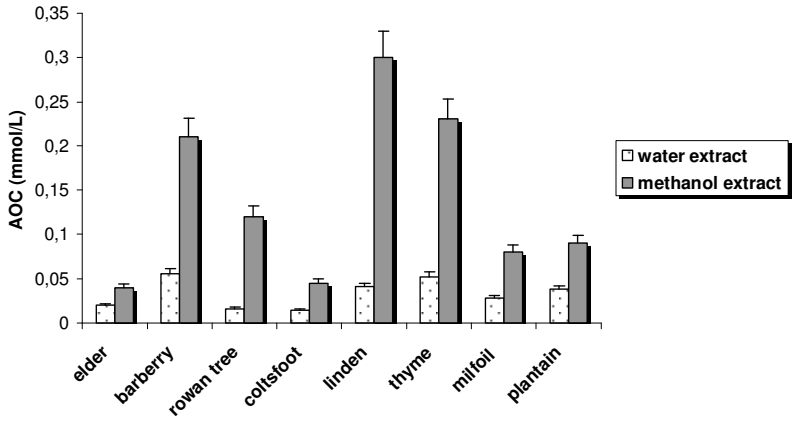

Fig. 2. *AOC, determined by chemiluminescence assay, of water and methanol extracts of fresh herbs. AOC is expressed as equivalent of chlorogenic acid.*

4.2. AOC of fresh and dried herbs and their infusions

For the purpose of this study all samples of medicinal plants were dried at ambient temperature until all the water was evaporated. It was found out that all herbs contained big proportion of water; from 55.3 % (barberry) to 86.8% (coltsfoot flowers). From the dried herbs infusions were prepared in the way they are usually prepared in folk medicine (see 3.7.). The analyses showed that drying process and preparation of herbal infusions influence the levels of AOC considerably (Figure 3). The highest AOC was determined in fresh herbs in all cases except of linden extracts (dried herbs showed higher AOC than fresh plants) and the least antioxidants were determined in infusions. The drying procedure lowered AOC down to 40% (thyme) of initial AOC and preparation of infusion resulted in decrease of AOC to as low as 5% (plantain) of initial AOC.

Similar results were obtained when extracts of fresh and dried herbs as well as their infusions were analysed by other two methods for evaluation of AOC (results not shown).

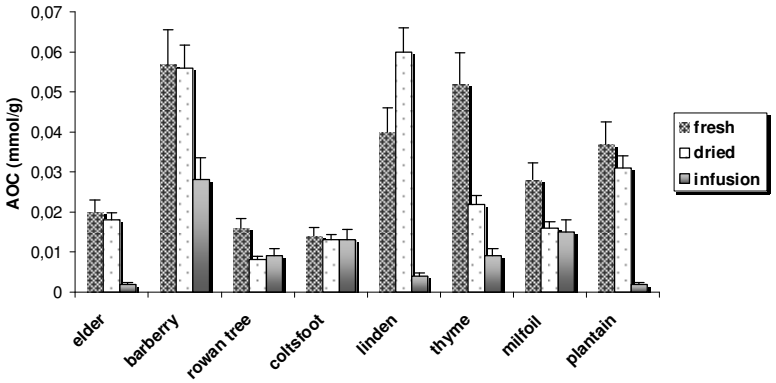

Fig. 3. *AOC of water extracts analysed by chemoluminescence assay. AOC is expressed as equivalent of chlorogenic acid per gram of fresh herb.*

4.3. Vitamin C content in extracts of fresh herbs

Vitamin C is considered one of the major antioxidants in medicinal herbs, therefore we decided to determine the levels of this important vitamin in water extracts of fresh and dried herbs and their infusions. As expected, the selected medicinal herbs differ not only on AOC, but also on the vitamin C levels (Figure 4). The highest concentration of vitamin C was determined in rowan tree fruits (0.137%) and the lowest in thyme flowers (0.014%), which do not correspond to the results of AOC. The results show that there is no correlation between determined vitamin C and AOC, which means that there are many other antioxidants contributing to overall AOC of the extracts.

It can additionally be noted from Figure 4 that in some cases the vast majority of vitamin C is in its reduced form (up to 97%), namely ascorbic acid (elder, barberry and thyme), whereas in the other herbs big portion (up to 86%) of vitamin C is in its oxidised and thus less stable dehydro-form (rowan tree, coltsfoot, linden, plantain).

The results on Figure 5 show that most of the vitamin C is lost already during the drying process and only infusions of linden flowers and rowan tree berries, the richest sources of vitamin C among selected medicinal herbs, contain detectable levels of this antioxidant. The reduction in vitamin C is much higher than reduction in overall AOC levels (Figure 3), which again leads to same conclusion that vitamin C is not the major antioxidant in dried herbs and their infusions and not even in fresh herbs.

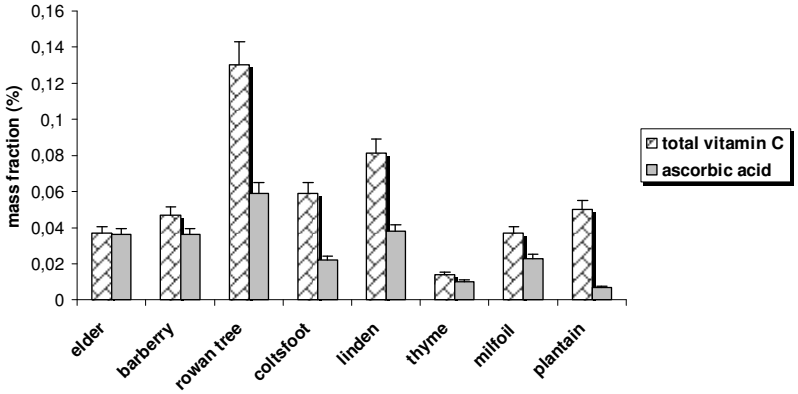

Fig. 4. *Vitamin C content in extracts of fresh herbs.*

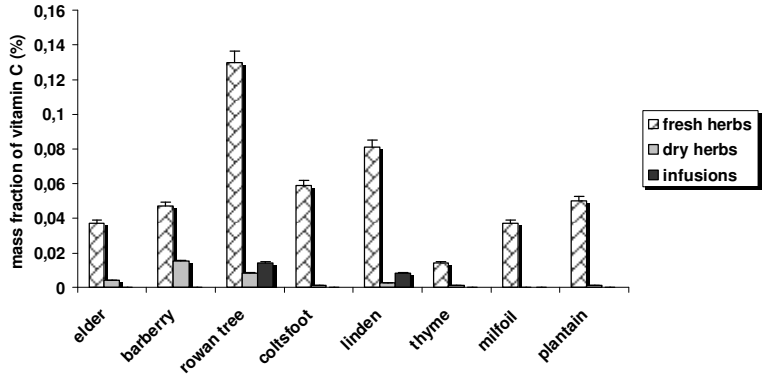

Fig. 5: *Total vitamin C content in medicinal herbs*

4.4. Identification of antioxidants in herbal extracts

The methanol extracts of fresh and dried herbs were analysed by HPLC-DAD-MS spectrometer, which resulted in identification of four phenolic acids and seven flavonoids. It was found out that chlorogenic acid, ellagic acid, luteolin, quercetin, galangin are present in all herbs, whereas chrysin is present only in elder, barberry, rowan tree, thyme, milfoil and plantain. Pinocembrin is not present in dried elder, barberry and milfoil but in all the rest of the herbs. It was additionally found out that the chrysin, galangin and luteolin levels rise during the drying process, whereas the pinocembrin, apigenin, p-coumaric acid, chlorogenic acid and caffeic acid levels are reduced. It was additionally found out that the extracts with the highest AOC (fresh and dried barberry, fresh milfoil flower and fresh linden flower) contain the highest concentrations of phenolic compounds identified in this study.

5. Conclusions

In the course of this study some of the most commonly used medicinal herbs in Slovenia and elsewhere were analysed for

the presence of antioxidants and their antioxidative capacity was evaluated. The results show that the drying process and preparation of herbal infusions resulted in a significant reduction of the AOC and vitamin C concentration. The content of TP in the fresh herbs varies from 0.016 mmol/g (coltsfoot) to 0.069 mmol/g (thyme) and in dried herbs from 0.004 mmol/g (elder) to 0.077 mmol/g (barberry), expressed as chlorogenic acid equivalent. In the herbal infusions on the other hand only between 0.004 mmol/g (elder) and 0.032 mmol/g (coltsfoot) of TP was determined. Four phenolic acids and seven flavonoids we identified in the herbal extracts. It was found out that with the drying process, the amount of the most of the phenolic compounds was reduced, so they have to be treated carefully.

It can be concluded from the obtained results that all investigated medicinal herbs have high levels of various antioxidants, which was already found out by several other research groups [2], [6]. Nevertheless, all the other studies are mainly focused on one particular plant rather than to the comparison of different herbs. In this study some antioxidants were identified for the first time in these herbs, but more research has to be performed in order to quantify those components.

Acknowledgements

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