ANTIOXIDANT ACTIVITY, PHENOLS, FLAVONOIDS, FLAVONOLS AND ANTHOCYANINS CONTENT OF *TUSSILAGO FARFARA* (L.) GROWING WILD IN KOSOVO

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Abstract. Total phenols, flavonoids, flavonols and anthocyanis content and antioxidant activity were quantitatively determined to the *Tussilago fatfara* (L.) growing wild in Sharr (South part of Kosova). In the tested extracts of plant species *Tussilago fatfara* (L.) quantitative composition of total phenols, flavonoids, flavonols and anthocyanins were determined by spectrophotometric method. Total phenols determined by Folin-Ciocalteu reagent and their amounts in the range of 56.50 mg/g to 84.70 mg/g (expressed as gallic acid equivalent, mg GAE/g). The amounts of flavonoids in plant extracts are in the range of 2.75 mg/g to 8.76 mg/g (expressed as rutin equivalent, mg RE/g).The amount of total flavonols in *Tussilago farfara* (L.) extracts ranged from 0.21 mg RE/g to 0.51 mg RE/g. The contents of total anthocyanosides in the *Tussilago farfara* (L) ranged from 1.44 (ethanol extract) to 1.86 (acetone extract) anthocyanins determined as mg yanidin 3-glucoside/g dry weight. Antioxidative activity was determined in vitro, using the DPPH reagent by spectrophotometric method.The results demonstrated that extract RSC was dependent on the solvent polarity, and the type of free radical used in the reaction. The IC_{50} values extracts in DPPH• assay was from 0.10 mg/ml (ethanol extract) to 0.20 mg/ml (acetone extract).

*Keywords:*Tussilago farfara (L); phenols; flavonoids; flavonols; anthocyanins; antioxidant activity

Introduction

Oxidative compounds lead to the damage of bio molecules like DNA, RNA, lipids, proteins etc. (Dastmalchi et al., 2007) which can further initiate the development of diseases such as ageing, atherosclerosis, lipofuscinosis, oxygen toxicity, cancer and liver injury (Iyer & Devi, 2009; Smerq & Sharma, 2011). Antioxidants are compounds which inhibit oxidation, and therefore are potential quenchers of free radicals or reactive oxygen species. Recent investigations show that plant products are as potential antioxidants against various diseases, induced

by free radicals due to presence of these phytochemicals (Hou et al., 2003). These antioxidants in plants also react with other organisms in the environment, inhibiting bacterial or fungal growth hence responsible for the antimicrobial activity of plants (Bruneton, 1995). These substances are considered as basis for developing new antimicrobial drugs as they inhibit pathogens and have little toxicity to host cells.

In the last years, interest in medicinal plants is more and more increasing, particularly using them as antibacterial agents. The reason for that is the growth of antibiotic resistance of bacteria against synthetic drugs (Gruenwald, 2000).

Tussilago farfara (Asteraceae), commonly called coltsfoot has been traditionally used as a medicinal herb to treat lungailments, such as asthma as well as various coughs by way of smoking. *Tussilago farfara* leaves and flowers possess expectorant activity and are used for chronic dry cough and various pulmonary diseases (Uzun et al., 2004).Volatile constituents of T*ussilago farfara* were studied previously, while to the best of our knowledge the residues remaining after their distillation from the plant were not evaluated until now (Liu et al., 2006; Judzentiene & Budiene, 2011). The extracts of *Tussilago farfara* were shown to exhibit various activities, such as antioxidant and antimicrobial as well as inhibitory effects on NO synthesis in LPS-activated macrophage and diacylglycerol acyltransferase activity (Gaoet al., 2008; Park et al., 2008). Some phenolics, mucopolysaccharides and water-soluble polysaccharides were isolated from the flower buds of *Tussilago farfara* (Didry et al., 1980), however the presence of flavonoids has not been reported until now.

It should be noted that besides beneficial bioactive compounds present in the herb, it was found to contain toxic pyrrolizidine alkaloids (PAs), mainly senkirkine, which was recognized by The International Agency for Research on Cancer (IARC) as possessing "sufficient or limited evidence" for the carcinogenicity (Lebada et al., 2000). As a result, in order to minimize the amount of toxic PAs ingested, the German health authorities limited the daily intake of toxic PAs to 1 μg.[1)] Most recently Committee on Herbal Medicinal Products recommended to keep exposure to PAs as low as practically achievable, as recommended by IPCS 1988, EFSA 2007, BfR 589 2007.[2)] To achieve this requirement some free of the toxic compounds *Tussilago farfara* clones were selected and introduced (Wawrosch et al., 2000).

Our research group was interested to analyze the chemical profile of different medicinal plants, which are growing in the region of Kosovo and Albania (Haziri et al., 2009; 2010; 2017a; 2017b; Faiku & Haziri, 2013; 2015; Faiku et al., 2012; 2015; 2016; 2017a; 2017b; Rudhani et al., 2017).

The aim of this research was to determine the quantity of phenols, flavonoids, flavonols, anthocyanins and antioxidant activity of the different extracts from *Tussilago farfara* (L.) growing wild in Sharr (South part of Kosovo).

Material and methods

Plant material

The aerial part of *Tussilago farfara* (L.), growing wild in South part of Kosovo, was collected in 10.05. 2016. Voucher specimens were deposited in the herbarium of the Department of Biology, University of Prishtina. The plants were dried at room temperature.

Preparation of plant organic extracts

The volatile compounds were removed by hydrodistillation and the remaining solid residue was dried at 30 °C and extracted with acetone, methanol, ethanol and ethanol-water(50/50, v/v (%). The extractions were performed in triplicate.

Amount of total phenolic compounds

The amount of total phenolic contents in the extracts was determined spectrophotometrically with the FolinCiocalteu (FC) reagent using the method of Fukumoto & Mazza (2000), with small modifications (Bozin et al., 2008). The reaction mixture contained 1.0% dilution of examined extracts (100 μL), freshly prepared 0.2 mol/L FC reagent (2.5 mL) and 10% sodium carbonate solution (2 mL). The mixture was incubated in the dark at room temperature for 1 hour to complete the reaction. The absorbance of the resulting solution was measured at 760 nm on a UV/VIS spectrophotometer using distilled water as the blank. The concentration of total phenolic contents was expressed in mg gallic acid equivalents (GAE) per g dried extract (d.e.), using a standard curve of gallic acid (0.1-2.0 μg/mL). All measurements were replicated three times.

Amount of flavonoids

Total flavonoid content in the extracts was determined spectrophotometrically according to Zhishen et al. (1999), using a method based on the formation of a flavonoid-aluminium complex with an absorbance maximum at 430 nm. The examined extracts (1mL) were mixed with 2% $AlCl_3 \times 6H_2O$ (0.5 mL). After incubation at room temperature for 30 min, the absorbance of the reaction mixtures was measured. The blank sample was a 1:1 mixture of the examined extracts and distilled water. Flavonoid content was expressed in μg rutin equivalent (RE) per g dried extract by using a standard curve of rutin (concentration range 0.5–6.0 μg/mL). All measurements were replicated three times.

Amount of flavonols

The content of flavonols was determined by the previously reported method (Ermakov et al., 1987). Series of reference rutin solutions containing 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 mg/mL of rutin were prepared. Two mL of reference were mixed with 2 mL of aluminum trichloride solution (20 g/L) in95% ethanol and 6

mL of sodium acetate solution in ethanol (50 g/L) were added. The absorbance was read at 440 nm after 2.5 h at 20 °C and the calibration curve on the dependence of the absorbency on the concentration of rutin was drawn. Plant extract samples were prepared under the same conditions by using 2 mL of extract (10 g/L) in 95% ethanol instead of rutin. All determinations were performed in triplicate. The percentage of flavonols was calculated by the formula: $X = \frac{C \times V \times 100}{m \times 100}$, where C is the concentration of rutin, determined form the calibration curve (mg/mL); V is the volume of plant extract (mL); m is the weight of pure plant extract (g).

Amount of anthocyanins

Total anthocyanins were measured according to the methods described earlier (Lee at al., 2005) with slight modifications. The extracts were mixed with acidified methanol (1% HCl/methanol) for 2 h at room temperature in the dark and then centrifuged at 2000 × g for 10 min. The anthocyanin concentration in the supernatant was measured spectrophotometerically at 530 and 657 nm, respectively. The absorbance values for and 657 nm were indicated as A_{530} and A_{657}, respectively. Three different dilutions of the plant samples were prepared, one for pH 1.0 using 0.03 mol/L KCl buffer and the other for pH 4.5 using 0.4 mol/LCH_3COONa buffer. The absorbance of each sample was measured at 520 nm against distilled water as a blank. The samples had no haze or sediment and thus correction at 700 nm was omitted. The concentration (mg/l) of each anthocyanin was calculated according to the following formula and expressed as Cy-3-glc equivalents:

$$\text{Total anthocyanins (mg/mL, Cy-3-glc)} = \frac{\left(AxMWxDFx10^3\right)}{\varepsilon xL}$$

where A is the absorbance $A = (A_{520}\text{ nm} - A_{700}\text{ nm})_{\text{pH 1.0}} - (A_{520}\text{ nm} - A_{700}\text{ nm})_{\text{pH 4.5}}$, MW is the Cy-3-glcmolecular weight: 449.2 g/mol, DF is the dilution factor (0.2 ml sample is diluted to 2 ml, DF=10), ε is the extinction coefficient (L x cm^{-1}x mol^{-1}) =26.900for Cy-3-glc and L (pathlength in cm) =1.

Antioxidant activity- DPPH• *assay*

The DPPH assay was performed as described previously (Blois, 1958; Sanchez-Moreno et al., 1998), following the transformation of the DPPH radical to its reduced, neutral form (DPPH-H). The samples of all extracts of *Tussilago farfara* (L.) (from 2.50 to 50.00 μg/mL) were mixed with 90 μM DPPH• solution (1 mL) and made up with 95% methanol to a final volume of 4 mL. The absorbance of the resulting solutions was recorded spectrophotometrically at 515 nm after 1 h at room temperature, against the blank (with the same chemicals, except for the sample). The measurements were carried out in triplicate for each solvent.

ABTS•+ radical cation decolourisation assay

The RSC of extracts was also measured by ABTS•+ radical cation assay (Re et al., 1999). Stock solution of ABTS (2 mM) was prepared by dissolving in 50 mL of phosphate buffered saline (PBS) obtained by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of ultra-pure water. If the pH was lower than 7.4, it was adjusted with NaOH. Ultra-pure water was used to prepare 70 mM solution of $K_2S_2O_8$. ABTS•+ radical cation was produced by reacting 50 mL of ABTS stock solution with 200 µL of $K_2S_2O_8$ solution and allowing the mixture to stand in the dark at room temperature for15–16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. For the assessment of extracts, ABTS•+ solution was diluted with PBS to obtain the absorbance of 0.800±0.030 at 734 nm. Three mL of ABTS•+ solution were mixed with 30 µL ethanol solution of *Tussilago farfara* extract in 1 cm path length microcuvette. The absorbance was read at ambient temperature after 1, 4, 6 and 10 min. PBS solution was used as a blank sample. All determinations were performed in triplicate. The percentage decrease of the absorbance at 734 nm was calculated by the formulae: $I = \frac{AB - AA}{AB} \times 100$, where I is ABTS•+ inhibition, %; AB is the absorption of blank sample (t=0 min); AA is the absorption of extract solution (t=10 min). The RSC was expressed as a percentage of inhibition of Trolox (6-hydroxy-2.5.7.8-tetramethylchroman-2-carboxylic acid) mM equivalent per 1000 g using prepared calibration curve. The extent of quenching of ABTS•+ radical by extracts was compared with standard amounts of Trolox. The concentrations of Trolox standard used for calibration curve were 0.001, 0.01, 0.1, 0.5, 1, 1.5 mmol/L.

Ferric reducing antioxidant power (FRAP)

The ability of plant extracts to reduce ferric ion (FRAP assay) is another indicator frequently used for assessing antioxidant power (Benzie & Strain, 1996). Ferrous ion (Fe^{2+}) produced in this assay forms a blue complex (Fe^{2+}/TPTZ) absorbing at 593 nm. Briefly, the reagent was prepared by mixing acetate buffer (300mM, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$x $6H_2O$ at 10:1:1 (v/v/v).Firstly, 300 µL of freshly prepared FRAP reagent was heated to 37 °C and an absorbance (A_0) of a blank reagent was read at 593 nm in a Biotek EL808 microplate reader (Vermont, USA). Then 10 µL of 0.1% extract solution in water and 30 µL H_2O were added (final dilution of samples in the reaction mixture was 1:34) and the absorbance (A) was recorded every 1 min during the whole monitoring period which lasted up to 30 min. The change in the absorbance ($\Delta A_{593\ nm}$) between the final reading and A_0 was calculated for each sample and related to the ΔA_{593} nm of a Fe^{2+} reference solution which was measured simultaneously.

Results and discussion

Phenolic compounds are very important plant antioxidants due to the presence of hydroxyl groups in their structure. It was established that phenolic compounds are the major constituents with antioxidant activity in many plants which are able to adsorb and neutralize the free radicals (Jimoh et al., 2011). Among them flavonoids is the most abundant group of natural constituents found in various plants, which exhibit antioxidant activity through radical scavenging or chelating mechanisms (Pourmorad et al., 2006).

The total phenolic compounds, total flavonoids, total flavonols and the total anthocyanins were measured for all the samples. The results are given in Table 1.

Table 1. The content of total phenolic compounds, flavonoids, flavonols and anthocyanins in the extracts isolated from *Tussilago farfara* (L.)

Phytochemical Constituent	Ethanol	Methanol	Acetone	ethanol-water
Total phenolic compounds, mg GAE/g	56.50	84.50	84.70	58.72
Total flavonoids, mg RE /g	2.75	4.50	8.76	2.94
Total flavonols, mg RE /g	0.21	0.39	0.51	0.30
Anthocyanins, mg/ml (mg cyanidin 3-glucoside/g	1.44	1.72	1.86	1.55

The amount of total phenolics in *Tussilago farfara* (L.) extracts ranged from 56.50 mg GAE/g (ethanol extract) to 84.70 mg GAE/g (acetone extract). A significant amount of these compounds has also been observed in the methanol extracts 84.50 mg GAE/g. Polyphenolic compounds have been found to be one of the most effective antioxidant constituents in plant foods, including fruits, vegetables and grains (Velioglu et al., 1998).

The amount of total flavonoids in *Tussilago farfara* (L.) extracts ranged from 2.75 mg RE/g (ethanol extract) to 8.76 mg RE/g (acetone extract). A significant amount of these compounds has also been observed in the methanol extracts 4.50 mg RE/g.

The amount of total flavonols in *Tussilago farfara* (L.) extracts ranged from 0.21 mg RE/g (ethanol extract) to 0.51 mg RE/g (acetone extract). A significant amount of these compounds has also been observed in the methanol extracts 0.39 mg RE/g.

The contents of total anthocyanosides in the *Tussilago farfara* (L) ranged from 1.44 (ethanol extract) to 1.86 (acetone extract) anthocyanins determined as mg yanidin 3-glucoside/g dry weight (Table 1). It is to be expected that several activities might be related to a possible antioxidant action from anthocyanosides like polyphenol compounds (Einbond et al., 2004).

The highest concentration of all four compound groups was in acetone followed by methanol, ethanol-water and ethanol. It is obvious that acetone does not provide exhaustive extraction of phenolic compounds, flavonoids, flavonols and anthocyanins from the solid plant residue obtained after hydrodistillation; polar solvent should be applied as a second solvent. The amount of total phenolic, flavonoids, flavonols and anthocyanins in *Tussialago farfara* (L.) extracts were given in Fig. 1.

DPPH• and ABTS•+ radical scavenging and FRAP assays were used for assessing antioxidant activity of *Tussilago farfara* extracts (Table 2).

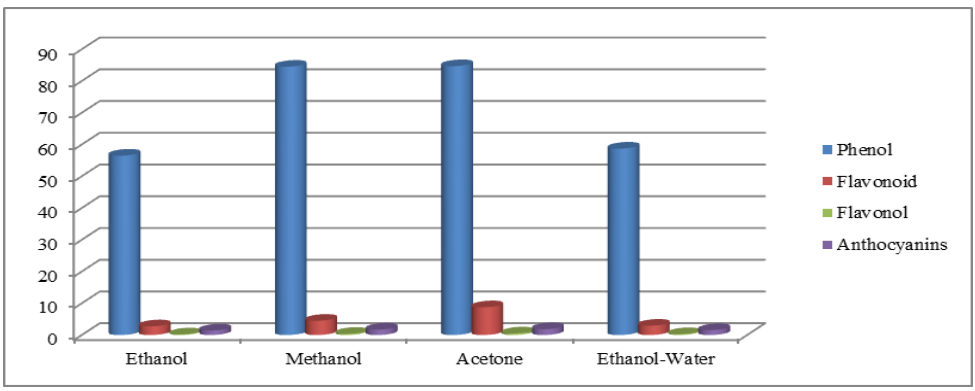

Figure 1. The amount of total phenolic, flavonoids, flavonols and anthocyanins in *Tussialago farfara* (L.) extracts

Table 2. Antioxidant activity of the extracts of *Tussilago farfara* (L.)

Characteristics	Ethanol	Methanol	Acetone	Ethanol-water
DPPH• scavenging (IC50), mg/mL	0.10	0.15	0.20	0.13
ABTS•+ scavenging, %	16.5	25.7	28.5	17.4
FRAP, mg/mL	0.34	0.40	0.48	0.35
TEAC, %, equivalent 1mmol Trolox	0.47	0.72	0.80	0.50

These methods are simple and widely used for the fast screening of plant antioxidant properties and they provide quite reliable preliminary information on the presence of antioxidatively active constituents in the extracts. In general, the reaction is based on the ability of radicals to accept either an electron or hydrogen atom. The extracts were isolated consecutively by using increasing polarity solvents, acetone, methanol or ethanol. Ethanol and methanol are quite similar protonic solvents; however both of them were tested because ethanol is more acceptable for the isolation of food and pharmaceutical grade ingredients.

The results demonstrated that extract RSC was dependent on the solvent polarity, and the type of free radical used in the reaction. The IC_{50} values of *Tussilago*

farfara (L) extracts in DPPH• assay were from 0.10 mg/ml (ethanol extract) to 0.20 mg/ml (acetone extract). In this case the extracts isolated with polar solvents were stronger DPPH• radical scavengers, which is in agreement with previously published results (Bandonienė et al., 2002; Dapkevicius et al., 2002). However, ethanol extracts were less effective in scavenging ABTS•+ than acetone, methanol and ethanol-water. Although the principle of DPPH• and ABTS•+ assays is similar, the latter is applicable both for lipophilic and hydrophilic antioxidants; in addition DPPH• can be dissolved only in organic solvents (e.g., methanol), while ABTS•+ is soluble in both aqueous and organic media. Therefore, ABTS•+ assay can be performed in hydrophilic and lipophilic systems (Re et al., 1999).

FRAP assay is a versatile method and can be readily applied to aqueous, alcohol and acetone extracts. In this assay, the antioxidant activity is determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron and the results are expressed in mg ferrous iron equivalents per mL of sample. In FRAP assay, similarly to ABTS•+, acetone extracts were significantly stronger antioxidants than methanol, ethanol-water and ethanol. The ferric reducing antioxidant power of different extracts was in the range of 0.34–0.48 mg Fe (II)/mL. In FRAP assay a ferric salt (Fe III) is used as an antioxidant and its redox potential (0.70 V) is comparable to that of ABTS•+ (0.68 V), therefore the results of TEAC and FRAP assays showed similar trends (Huang et al., 2005).The amount of DPPH•, FRAP and TEAC in *Tussialago farfara* (L.) extracts in *Tussialago farfara* (L.) extracts were given in Fig. 2.

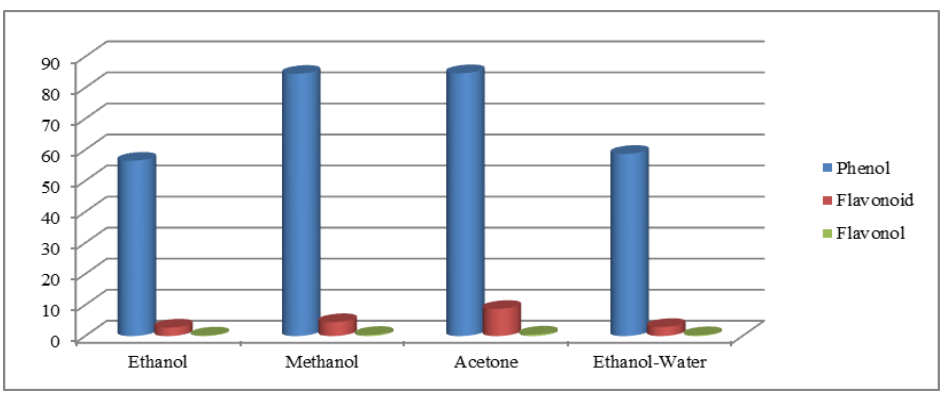

Figure 2. The amount of DPPH•, FRAP and TEAC in *Tussialago farfara* (L.) extracts

Conclusion

Total phenols, flavonoids, flavonols and anthocyanis content and antioxidant activity were quantitatively determined to the *Tussilago fatfara* (L.) growing wild in Sharr (South part of Kosova).The amount of total phenols in organic and aqueous-or-

ganic extracts of *Tussilago farfara* (L.) was in the region of 56.50 mg GAE/ g (ethanol extract) to 84.70 mg GAE/ g (extract of acetone). Also, great amount of flavonoids was found in acetone and methanol. Small amounts of flavonoids were found in the ethanol-water extract until very small amount we found in the ethanol. The amount of total flavonols in *Tussilago farfara* (L.) extracts ranged from 0.21 mg RE/g (ethanol extract) to 0.51 mg RE/g (extract of acetone).The contents of total anthocyanosides ranged from 1.44 (ethanol extract) to 1.86 (acetone extract) anthocyanins determined as mg yanidin 3-glucoside/g dry weight. The highest concentration of all four compound groups was in acetone followed by methanol, ethanol-water and ethanol.

The IC_{50} values of *Tussilago farfara* (L) extracts in DPPH• assay were from 0.10 mg/ml (ethanol extract) to 0.20 mg/ml (acetone extract). Results obtained from acetone extracts of *Tussilago farfara* (L.)is very logical, since we found that this extract contains the largest amount of total phenolic and flavonoid content. In this case the extracts isolated with polar solvents were stronger DPPH• radical scavengers, which is in agreement with previously published results. However, ethanol extracts were less effective in scavenging ABTS•+ than acetone, methanol and ethanol-water. In FRAP assay, similarly to ABTS•+, acetone extracts were significantly stronger antioxidants than methanol, ethanol-water and ethanol.

NOTES

1. https://www.aerzteblatt.de/pdf/89/23/a2145.pdf?ts=11%2E07%2E2011+15%3A50%3A03
2. http://www.ema.europa.eu/docs/en_GB/document_library/Public_statement/2012/10/WC500134311.pdf

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