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HPLC determination of phenolic acids and antioxidant activity in concentrated peat extract—a natural immunomodulator

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Abstract

The aim of this study was to devise a method for identification and quantification of phenolic acids in concentrated peat extract samples. The simple reversed-phase HPLC method for simultaneous determination of several phenolic acids was developed. The method was validated and it was suitable for the analysis of phenolic acids in peat extracts. This method allowed identifying eight phenolic acids in peat extracts. Phenolic profiles of two samples of peat extract obtained from different medicinal peats were similar, although variations in amounts of individual phenolic acids were observed. Also, slight variations in total phenolic content were detected. The antioxidant activity of peat extracts was evaluated with spectrophotometric ABTS assay. Differences in antioxidant activity were observed for two samples of peat extract produced from different peat varieties. This differences probably reflected phenolic composition of peat extracts.

Keywords: Phenolic acids; HPLC; Antioxidant activity; ABTS; Peat extract

1. Introduction

Antioxidant can be defined as a molecule, which significantly delays or inhibits the oxidation of other molecules. In biological systems as a natural consequence of oxidation reactions during cellular respiration reactive oxygen species (ROS) are formed, e.g. hydroxyl radical ($^{\bullet}OH$), superoxide anion ($^{\bullet}O_2^-$), and hydrogen peroxide (H_2O_2) [1]. Some of the reactive oxygen species are free radicals. Free radicals are any chemical species containing one or more unpaired electrons and capable of independent existence. Radicals are more reactive than nonradicals, and they can react with other molecules in several ways. Free radicals damage living cells causing lipid, protein, and DNA oxidation. They are involved in the development of various diseases such as cancer, cardiovascular or neurodegenerative disorders, and also accelerate the ageing process. Human organism has developed a number of defence mechanisms against free radicals and other oxidising agents. The first group includes enzymes such as superoxide dismutase, catalase, and glutathione peroxidase; the second group consists of nonenzymatic factors such as albumin, glutathione, ascorbic acid, α-tocopherol, uric acid, bilirubin, iron, and cooper ions. The oxidative stress resulting from imbalance of oxidising species and antioxidants may lead to pathologies, and dietary intake of antioxidants helps to scavenge free radicals and oxidants, and protects against disease. Epidemiological data suggest that consumption of plant-derived antioxidants such as flavonoids and phenolic acids prevents various diseases [2], and, accordingly, they have attracted special attention recently [3].

Phenolic acids constitute a large group of organic compounds, which are widely distributed in nature and show a broad spectrum of pharmacological activity [4]; they reportedly have antioxidant, antimutagenic, antitumor, and anticarcinogenic properties [5]. Phenolic acids are simple phenolic compounds of the nonflavonoid family (Fig. 1). They are synthesised through the shikimic acid pathway and may occur in the bound or free form. We can distinguish two main groups of phenolic acids, both of which are hydroxy derivatives of aromatic carboxylic acids: (1) benzoic acid and (2) cinnamic acid. They differ according to the number and position of hydroxylations

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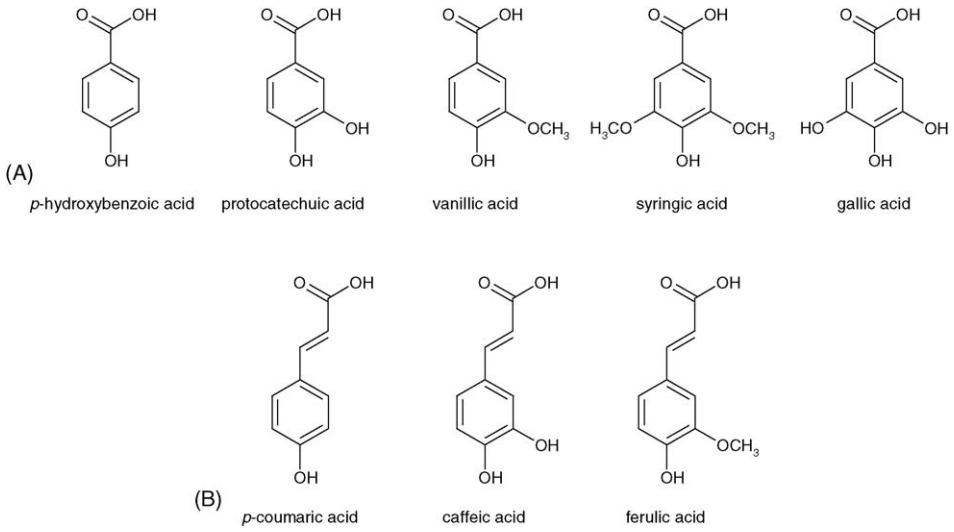

Fig. 1. Chemical structures of phenolic acids analysed in this study. (A) Derivatives of benzoic acid; (B) derivatives of cinnamic acid.

and methoxylations of the aromatic ring. Phenolic compounds are naturally present in virtually all plant material [6] (e.g. vegetables, fruit, herbs) [7] and plant related products (e.g. beer, wine, tea) [8]. Phenolic acids were also detected in peat [9], as peat is formed by partial decomposition of plant tissue.

Concentrated peat extract is a natural immunomodulator. It is produced by Torf Corporation from the therapeutic peat on the basis of patented technology developed by Prof. Stanislaw Tolpa and his co-workers. Briefly, production technology involves alkaline extraction of peat, acidification to remove humic acid fraction, and concentration connected with thermal processing. Scientific studies conducted by different research groups over many years confirmed biological activity of peat extract, but the exact mechanism of its action is still unclear. In particular, peat extract activates neutrophils, macrophages, synthesis of INF-β, INF-γ and TNF-α, as well as NK cells [10–12]. Many researches also showed a remarkably low toxicity of the peat extract, the extract having no mutagenic, teratogenic or carcinogenic effects [13,14]. It was used with good results in therapy of chronic disorders with inflammatory basis, for example respiratory tract infections [15]. Torf Corporation is the only manufacturer of concentrated peat extract, which serves as a raw material for production of Immuno-Complex Tablets and other preparations. Immuno-Complex Tablets are used as a regenerating and strengthening dietary supplement, and other wares containing peat extract include various cosmetics and oral cavity hygiene products.

Concentrated peat extract is a very complex mixture: it contains amino acids, carbohydrates, fulvic acids, and mineral salts (based on Torf Corporation Ltd. unpublished data). It is thought that the fraction of peat extract responsible for immunotropic activity contains Maillard reaction products, mainly Amadori compounds and advanced glycation end products formed during technological processing.

The aim of this study was to elucidate phenolic acids composition and contents, and show that the concentrated peat extract is characterized by antioxidant activity.

2. Materials and methods

2.1. Chemicals

The ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt], ethylenediaminetetraacetic acid (EDTA), and syringic acid were purchased from Sigma Chemical Co. (USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich (Germany). Potassium peroxodisulfate, gallic acid, ferulic acid, *p*-hydroxybenzoic acid, and *p*-coumaric acid were purchased from Fluka (Switzerland). Caffeic acid was purchased from Extrasynthese (France). Ethyl acetate (p.a.), diethyl ether (p.a.), ethanol (99.8%), Folin-Ciocalteu reagent, sodium carbonate, sodium hydroxide, and sodium sulfate were purchased from Polish Chemical Reagents (Poland). Methanol (HPLC gradient grade) and ascorbic acid were purchased from Merck (Germany). Acetic acid (HPLC grade) was purchased from J.T. Baker (Netherlands). Vanillic acid (≥97%) and protocatechuic acid (≥97%) were synthesised as described earlier by Pearl [16,17]; the purity was confirmed by determining the melting point and by HPLC analysis. Milli-Q water (18.2 MΩcm) was used in all experiments.

2.2. Samples

Two different samples of concentrated peat extract were analysed, both of which were produced in 2004, according to the patented technology [18]. Samples were taken from succeeding batches, and each batch of concentrated peat extract was produced from peat taken from different bog. Differences in the dry weight of the extracts were taken into account in the calculations.

2.3. Phenolic acids extraction

Sample of 5 ml of concentrated peat extract (see Section 2.2) was mixed with 5 ml of water and subsequently extracted four

times using 20 ml of ethyl acetate each time. The clear organic extracts were combined and dried over anhydrous sodium sulfate. Then the extract was filtered and concentrated to dryness in a rotary vacuum evaporator at a temperature not higher than 50 °C; the residue was dissolved in 0.5 ml of methanol, then 1.5 ml of mobile phase consisting of 2% (v/v) acetic acid in water–methanol 82:18 (v/v) was added. Finally, the resulting solution was filtered through the syringe filter 0.45 μm pore size (Titan) and left for chromatographic analysis.

2.4. Chromatographic analysis of phenolic acids

A simple and quick reversed phase HPLC method for determination of phenolic acids was developed. Chromatographic analysis was performed with the use of liquid chromatographic system, which consisted of Knauer HPLC Pump, Knauer UV–vis Variable Wavelength Monitor, Knauer Injection Valve, Knauer Degasser and Shimadzu CTO-6A Column Oven. Chromatographic system was connected through the Dionex Universal Chromatography Interface to the PC computer. Software used for data acquisition and evaluation was Dionex Chromeleon Version 6.40 SP1. The separation was carried out on 250 × 4.6 mm, 4 μm Hypersil BDS C18 (Thermo Hypersil-Keystone) reversed phase column. Column temperature was maintained at 50 °C. Elution was performed isocratically with the mobile phase consisting of 2% (v/v) acetic acid in water–methanol 82:18 (v/v), and the flow rate was 1 ml/min. The injection volume for all samples was 20 μl. For detection chromatograms were monitored at 280 nm. Identification of phenolic acids was based on retention times in comparison with standards. The quantification was carried out using the external standard method. Stock solution of standard compounds at concentration 1 mg/ml each was prepared in methanol, and several dilutions with mobile phase were made. The solution of standards at various concentrations (0.005, 0.010, 0.020, 0.050, 0.100 mg/ml) was injected into the HPLC system and the calibration curves were established for each standard compound. The concentration of the compound was calculated from peak area according to calibration curves. The amount of each phenolic acid was expressed as milligram per gram of dry weight (DW).

2.5. Antioxidant activity assay

The assay was performed essentially as described previously [19–21]. All measurements were made at room temperature on the Shimadzu UV–vis spectrophotometer model UV-2100. ABTS and potassium persulfate were dissolved in water to a 14 and 5 mM concentration, respectively. ABTS radical cation was produced by mixing 1 ml of both stock solutions, and the mixture was allowed to stand in the dark at room temperature for 15–20 h before use. The ABTS radical cation solution was than diluted with water, approximately 100×, to an absorbance of 0.750 (±0.050) at 734 nm. For measuring antioxidant activity 50 μl sample was mixed with 4000 μl of the diluted ABTS radical cation solution. Absorbance was recorded before addition of the antioxidant sample and exactly 10 min after mixing. For analysis of peat extracts, 5 min after addition of 50 μl of concentrated peat extract (see Section 2.2) to 4000 μl of the diluted ABTS radical cation solution, the reaction mixture was centrifuged 2 min, at 12,000 rpm, and exactly 10 min after mixing absorbance reading for the supernatant was taken. Trolox stock solution (8.1 mM) was prepared in ethanol, 5 ml of this solution was diluted with ethanol to 50 ml. A series of Trolox dilutions was prepared to give final concentrations 2, 4, 6, 8 and 10 μM in the reaction mixture. Appropriate solvent blanks were run in each assay. Absorbance values were corrected for the solvent blanks. The calibration curve describing inhibition of the absorbance as a function of Trolox concentration was prepared. When the absorbance of the reaction mixture decreased below 0.150, peat extract sample was diluted properly. Measurements for calibration curve were performed in at least three replicates, and other samples in triplicate. The antioxidant activity was expressed as TEAC (in micromoles of Trolox) per gram of dry weight (DW). Additionally antioxidant activity of standard substance as ascorbic acid was measured, a stock solution of ascorbic acid (8.1 mM) in water was prepared, it was diluted 10× to give final concentration of 10 μM in reaction mixture, the whole experiment was performed as described above.

2.6. Determination of total phenolics

Total phenolics were determined using Folin-Ciocalteu reagent as described earlier by Singleton [22]. Samples of concentrated peat extract (1 ml) (see Section 2.2) were diluted with water to 100 ml. Stock solution of gallic acid (5 mg/ml) in methanol was prepared as a standard. Gallic acid stock solution was used to prepare serial dilutions containing 0.10, 0.08, 0.06, 0.04 and 0.02 mg/ml gallic acid. Than 1 ml of a sample (standard, blank, extract) was introduced into the test tube, and next 5 ml of diluted Folin-Ciocalteu reagent (1:10 with water) was added. After 2 min 4 ml of 7.5% (m/v) sodium carbonate solution was added and mixed well. The absorbance of all samples was measured at 765 nm after incubating at room temperature for 2 h. All measurements were made on the Shimadzu UV–vis spectrophotometer model UV-2100. Results were expressed as milligram of gallic acid equivalent (GAE) per gram of dry weight (DW).

3. Results and discussion

Analysed concentrated peat extract samples were produced from two types of medicinal peat from different peat bogs, using the same technology. Peats differed in pH, water content and contents of organic matter. Both concentrated peat extracts were biologically active in vitro and in vivo (data not shown). We have decided to investigate contents of phenolic acids and antioxidant activity of peat extracts.

Literature specifies many methods, used with good results, for chromatographic analysis of phenolic compounds [23–26], but most of them involve gradient separations. We wanted to introduce isocratic method. The intention was to develop a simple and fast chromatographic method for detection of several phenolic acids. Various mobile phases including methanol–water and acetonitrile–water with acetic acid were tested. Eventually, it was established that the methanol–water system containing

Table 1
HPLC method characteristics

Compound	Retention time (min)	Number of theoretical plates	Capacity factor (k')	Resolution	Asymmetry
Gallic acid	3.00	10061	0.17	8.1	1.3
Protocatechuic acid	4.09	12033	0.59	10.8	1.2
p-Hydroxybenzoic acid	5.93	15274	1.31	4.5	1.1
Caffeic acid	6.86	15154	1.67	2.4	1.1
Vanilic acid	7.41	16875	1.88	4.3	1.1
Syringic acid	8.44	16816	2.29	10.1	1.1
p-Coumaric acid	11.48	17991	3.47	9.2	1.1
Ferulic acid	15.10	18368	4.88	nd	1.0

All calculations based on EU standards; nd: not determined.

acetic acid (see Section 2.4) resulted in good separation in isocratic elution (Table 1). It can be noticed that satisfactory separation with good resolution can be achieved within less than 20 min (Fig. 2A).

Different solvents used for extraction of concentrated peat extract, such as ethyl acetate and diethyl ether were tested. Standard mixture of phenolic acids was extracted with diethyl ether and ethyl acetate to analyze the effect of the solvent on extraction. Both organic extracts were treated as described under Section 2, after HPLC analysis quantities of each phenolic acid were compared by *t*-test ($p < 0.05$), it was found that there was no significant difference, and, accordingly, ethyl acetate was used in further experiments. Extraction conditions were checked, and it was discovered that four-time extraction with 20 ml of ethyl acetate is sufficient to obtain nearly complete recoveries (Table 2). The results show that the extraction efficiency is very high and highly reproducible.

There were no endogenous interfering peaks in the peat extract that co-eluted with analyzed phenolic acids indicating that the method is selecitve and specific; fractions from HPLC separation of phenolic acids in concentrated peat extract, corresponding to each analysed compound were collected, UV spectra were recorded, compared with spectra of standard substances and on this basis the identity and purity was confirmed. The HPLC assay for phenolic acids were found to be linear in the range of 0.005–0.100 mg/ml, with very high (>99.99) correlation coefficients (Table 2), the slope and correlation coefficient were determined using the least-squares linear regression analysis method. Table 2 summarises the precision and accuracy of the simultaneous assay for eight phenolic acids. The results suggests satisfactory intra-day and inter-day precision and accuracy for all analytes. The sensitivity of the method was evaluated, LOD and LOQ were estimated and are shown in Table 2. Samples stored in mobile phase in 4 °C were stable at least for 48 h.

The HPLC analysis of phenolic acids in peat extracts showed that protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, and *p*-coumaric acids were present in both samples (Fig. 2B). Also, traces of gallic, caffeic, and ferulic acids were detected (Fig. 2B). Amounts of all phenolic acids in samples No. 1 and 2 are shown in Table 3; the reported values are means of three replicates ± standard deviation (S.D.). The sum of phenolic acids determined by HPLC analysis was 0.865 ± 0.010 and

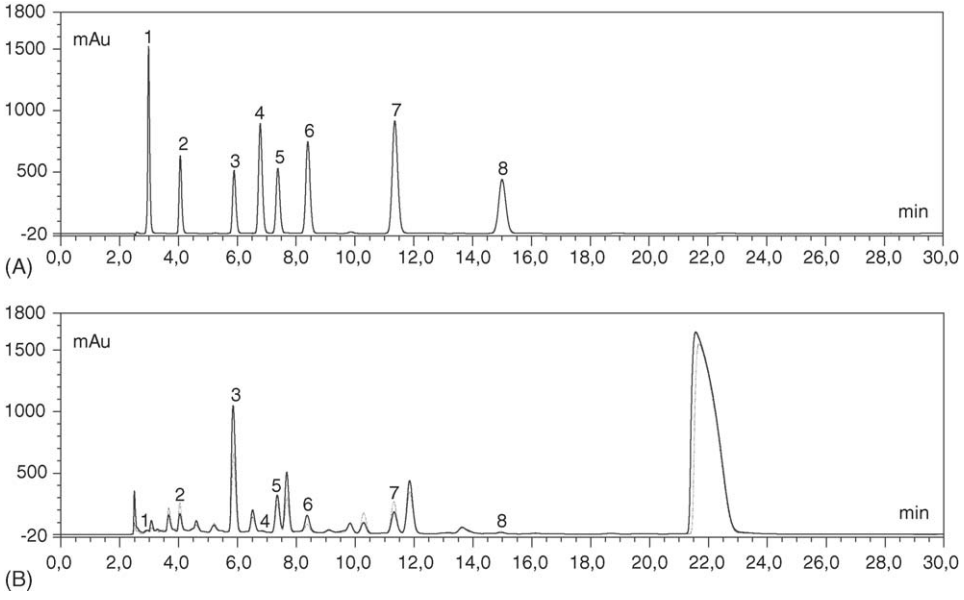

Fig. 2. Representative HPLC separations of a mixture of phenolic acids standards (A) and phenolic acids from peat extract (solid line-sample No. 1; dotted line-sample No. 2) (B). Peak identification: (1) gallic acid; (2) protocatechuic acid; (3) *p*-hydroxybenzoic acid; (4) caffeic acid; (5) vanillic acid; (6) syringic acid; (7) *p*-coumaric acid; (8) ferulic acid. Conditions as described in the text.

Table 2
HPLC method validation data

Compound	Precision[a] (%)		Accuracy[b] (%)	Slope	Correlation coefficient (%)	Linearity range (mg/ml)	LOD[c] (mg/ml)	LOQ[d] (mg/ml)	Recovery[e] (%)
	Intra	Inter							
Gallic acid	1.06	1.11	101.89	1119.64	99.9962	0.005–0.1	0.002	0.005	94.6 ± 1.1
Protocatechuic acid	1.17	1.04	101.08	619.39	99.9993	0.005–0.1	0.001	0.002	96.6 ± 1.9
p-Hydroxybenzoic acid	1.92	0.86	101.40	656.19	99.9977	0.005–0.1	0.002	0.006	97.2 ± 2.2
Caffeic acid	1.03	2.05	98.99	1285.26	99.9990	0.005–0.1	0.001	0.002	94.9 ± 1.8
Vanilic acid	1.50	2.29	100.35	765.33	99.9921	0.005–0.1	0.002	0.005	97.7 ± 2.1
Syringic acid	0.89	1.00	100.22	1221.01	99.9985	0.005–0.1	0.001	0.002	103.1 ± 0.9
p-Coumaric acid	1.40	2.30	98.57	1977.92	99.9990	0.005–0.1	0.001	0.002	94.5 ± 1.7
Ferulic acid	1.65	2.94	98.71	1190.63	99.9951	0.005–0.1	0.002	0.005	95.6 ± 2.1

[a] Precision (R.S.D.) = (S.D./mean) × 100% (n = 6), at medium analyte concentration (0.020 mg/ml each).
[b] Accuracy = (found/nominal) × 100% (n = 6), at medium analyte concentration (0.020 mg/ml each).
[c] Detection limit was expressed as: LOD = (3.3s)/a, where a is the slope, and s is residual standard deviation of a regression line.
[d] Quantitation limit was expressed as: LOQ = (10s)/a, where a is the slope, and s is residual standard deviation of a regression line.
[e] Mean (n = 3), ±S.D., at medium analyte concentration (0.020 mg/ml each).

Table 3
Content of phenolic acids in two samples of concentrated peat extract determined by HPLC analysis

Compound	Amount of phenolic acids[a]	
	Sample 1	Sample 2
Gallic acid	0.005 (0.6%) ± 0.000	0.004 (1.1%) ± 0.000
Protocatechuic acid	0.074 (8.5%) ± 0.002	0.074 (17.7%) ± 0.001
p-Hydroxybenzoic acid	**0.536** (61.9%) ± 0.005	**0.204** (48.7%) ± 0.006
Caffeic acid	**0.005** (0.6%) ± 0.001	**0.001** (0.3%) ± 0.000
Vanilic acid	**0.143** (16.5%) ± 0.002	**0.070** (16.8%) ± 0.003
Syringic acid	**0.053** (6.1%) ± 0.002	**0.017** (4.1%) ± 0.001
p-Coumaric acid	0.045 (5.2%) ± 0.003	0.040 (9.6%) ± 0.002
Ferulic acid	**0.006** (0.7%) ± 0.000	**0.008** (1.8%) ± 0.000
Sum of phenolic acids	**0.865** (100%) ± 0.010	**0.419** (100%) ± 0.014

[a] Results are expressed as mg per gram of dry weight and are means of three replicates ± S.D., percent values are given in the brackets; values in bold are significantly different (t-test, $p < 0.05$).

0.419 ± 0.014 mg/g of dry weight (DW), for sample No. 1 and 2, respectively (Fig. 3B). Contents of phenolic acids in sample No. 2 are more than 50% less than in sample No. 1. Interestingly, differences in contents of individual phenolic acids were not equal. Amounts of protocatechuic and *p*-coumaric acids were almost the same in both samples (Table 3), but amounts of vanillic, *p*-hydroxybenzoic and syringic acids were 2–3 times higher in sample No. 1 (Table 3). Wide peak that appears at about 22 min (Fig. 2B) corresponds to methyl and propyl esters of *p*-hydroxybenzoic acid, which serve as preservatives for peat extract. Chromatograms revealed some unidentified peaks that may correspond to unknown phenolic compounds (Fig. 2B).

Exact source of phenolic compounds in peat extract is unknown, it was supposed that the concentrated peat extract, as plant material, may also contain esters of phenolic acids, so in the next step it was tried to liberate bound phenolic acids from the concentrated peat extract by means of alkaline hydrolysis with 2 M sodium hydroxide solution containing 0.01 M ethylenediaminetetraacetic acid (EDTA) and 1% (m/v) ascorbic acid to prevent degradation of phenolic acids during the hydrolysis [27]. Chromatographic analysis of hydrolysed and non-hydrolysed samples of concentrated peat extract showed no significant differences in phenolic acids composition. In hydrolysed samples the peak corresponding to *p*-hydroxybenzoic acid was higher, due to the hydrolysis of its esters; additionally, a few unidentified peaks appeared at the front of the chromatogram and the baseline was elevated (data not shown).

Folin-Ciocalteu method is widely used to determine total phenolic compounds. The content of phenolic compounds was expressed as milligram of gallic acid per gram of peat extract dry weight. Determinations were made in three replicates; the reported values are means ± standard deviation. The amounts of total phenolics were quite similar for both samples of peat extract: 12.112 ± 0.282 and 11.157 ± 0.125 mg GA/g of dry weight (DW), for sample No. 1 and 2, respectively (Fig. 3A). Contents of phenolic compounds in sample No. 2 were near 8% less then in sample No. 1. Higher amounts of phenolic compounds determined by this method were due to less selectivity of this assay, not only phenolic acids, but all phenolic constituents

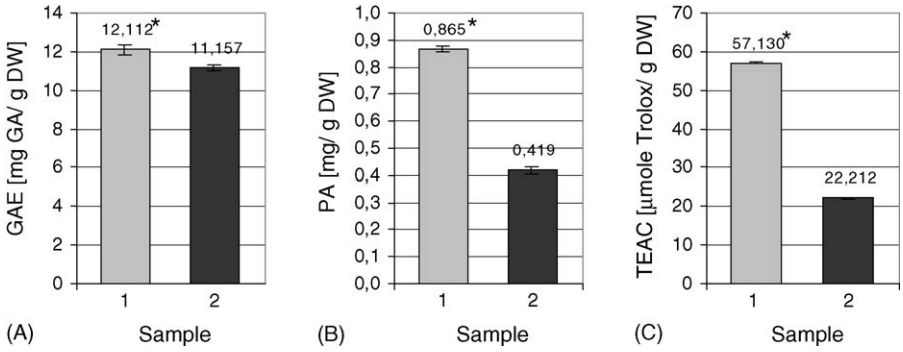

Fig. 3. Comparison of two samples of concentrated peat extract produced from different types of medicinal peat: (A) total phenolic compounds as gallic acid eqiuvalents (GAE) determined by means of Folin-Ciocalteau reagent; (B) sum of phenolic acids (PA) determined by HPLC analysis; (C) total antioxidant activity as Trolox equivalents (TEAC) determined by ABTS assay; asterisk denotes statistically significant difference (t-test, $p < 0.05$).

give positive reaction. Contents of phenolic acids measured by HPLC method is only a part of total phenolic compounds.

Various methods have been introduced for the measurement of the total antioxidant capacity [28]. In this study antioxidant activity was estimated by the improved Trolox equivalent antioxidant capacity (TEAC) assay. We measured the relative capacity of antioxidants to scavenge the long-lived ABTS radical cation in comparison to the antioxidant activity of Trolox, a water-soluble Vitamin E (α-tocopherol) analogue. This technique involves direct production, through the reaction between ABTS and potassium persulfate, of blue-green ABTS radical cation, which can easily be detected spectrophotometrically at 734 nm. Addition of antioxidants to the preformed ABTS radical cation reduces it, and simultaneously decolorization of reaction mixture is observed. The decrease in absorbance is related to the concentration of antioxidants, and it also depends on duration time of the reaction. In predetermined reaction time conditions the extent of decolorization can be expressed as a function of concentration of standard substance (Trolox) and represented as TEAC.

Peat extracts are suspensions; it was necessary to check if the precipitate has influence on antioxidant activity because it also interferes with photometric measurement. We have tested whole peat extract and the same extract in which suspended particles were removed by centrifugation, we have observed higher antioxidant acivities for the whole extract (data not shown), which meant that the suspended particles also contribute to antioxidant activity. The solubility of the centrifuged precipitate was checked, it was found to be insoluble in tested solvents, it was slightly dissolving only in 1 M HCl and 1 M NaOH, which are incompatible with ABTS assay, so we introduced a centrifugation step to remove the precipitate before spectrophotometric measurement. Antioxidant activities of concentrated peat extracts were 57.130 ± 0.091 and 22.212 ± 0.059 (μmoles of Trolox per gram of dry weight ± standard deviation) for sample No. 1 and 2, respectively (Fig. 3C), for comparison antioxidant activity of standard substance as ascorbic acid was measured and acounted to 5.671 ± 0.032 mmoles of Trolox per gram, which was in agreement with calculated theoretical activity. Antioxidant activity of sample No. 2 was more than 60% less than in sample No. 1; this observation may indicate positive correlation between contents of phenolic compounds, especially phenolic acids, and antioxidant activity of concentrated peat extract. However, this dependence is uncertain because unknown compounds may also contribute to the antioxidant activity of peat extracts.

4. Conclusions

In this study rapid and reliable reversed-phase HPLC method with UV detection, involving single step liquid extraction was described. The method is convenient for quality control of complex matrices such as peat extracts. This method is simpler than previously described because it uses isocratic elution versus gradient. This is also a first report showing such properties of peat-derived preparation. It was showed that both extracts produced from different types of peat had similar phenolic profiles and antioxidant activities. To answer the question, what is the source of differences in studied parameters, it is necessary to pose a question about the source of phenolic compounds in concentrated peat extract. There are three possibilities: phenolic acids may originate from disintegrated biomass, or from decomposition of humic substances, or be a product of Maillard reactions. It is thought that minor differences in peat extracts properties might result from numerous factors: mainly from the type, chemical composition and extraction efficiency of peat. To answer the above questions further studies are required. In future we are going to investigate other biologically active components of concentrated peat extract.

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