EFFECT OF EXTRACTION SOLVENTS ON PHENOLIC COMPOUNDS CONCENTRATION, ANTIOXIDANT ACTIVITITY AND COLOUR PARAMETERS OF SELECTED MEDICAL PLANTS

LENKA BURDEJOVA[1,2], MARTIN POLOVKA[1]
[1]National Agricultural and Food Centre
VUP Food Research Institute
Priemyselna 4, 821 08 Bratislava
SLOVAKIA
[2]Institute of Food Science and Biotechnology
Brno University of Technology, Brno
Purkynova 118, 612 00 Brno
CZECH REPUBLIC

xcbutorova@fch.vutbr.cz

Abstract: The impact of extraction solvents on concentration of selected polyphenols, antioxidant activity and colour parameters was evaluated for group of 10 the most popular medical plants conventionally produced in the Czech Republic. High Performance Liquid Chromatography, Electron Paramagnetic Resonance and Ultra Violet Visible Near Infrared Spectroscopy were employed. The entire experimental characteristics were processed by multivariate statistical methods in order to assess the influence of extraction conditions on monitored characteristics and for mutual differentiation of medical plant samples according to the extraction solvent used. Results obtained clearly proved the successful differentiation of medical plant samples by means of canonical discrimination analysis, reaching 96.7% correctness, as well as by k-th nearest neighbour method reaching 100% for k = 1 and 60% for k = 2. The results unambiguously confirmed also the importance of selection of the most proper extraction conditions to obtain the highest possible yields of target compounds/molecules with health-promoting and antioxidant properties.

Key Words: medical plants, solvent extraction, phenolic compounds, antioxidant activity

INTRODUCTION

Medical plants are natural valuable source of phytochemicals, many of which possess also antioxidant activity. The most abundant group of antioxidants in herbs are phenolic compounds, comprising flavonoids, phenolic acids, tannins, chalcones, coumarins or stilbens (Huanq et al. 2010). Polyphenols and flavonoids are of considerable interest to scientists, manufacturers and consumers due to their health promoting properties (Milevskaya et al. 2017, Sytar et al. 2016).

Extraction is the first important step to separate bioactive constituents from plant materials. Further selection of a suitable extraction technique is considerable for the standardization of plant products. However, extraction yield and antioxidant activity not only depend on the extraction method but also on the solvent used for extraction (Dhanani et al. 2017). The most suitable solvents for extraction phenolic compounds are aqueous mixtures containing ethanol, methanol, acetone, ethyl acetate, dimethyl sulfoxide (DMSO), methanol-DMSO mixtures, and dimethylformamide (Magwaza et al. 2016, Ngo et al. 2017). Ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption and water is certainly the safest and the most environmentally friendly and accessible solvent. It is also significantly less expensive and effective in extraction of phenolic compounds than organic solvents (Addai et al. 2013, Tan et al. 2014).

The main aim of this contribution was to determine the effect of extraction solvent on the concentration of phenolic compounds, their antioxidant properties and colour parameters by using modern analytical techniques (Ultra Violet Visible Near Infrared

Spectroscopy - UV-VIS-NIR, Electron Paramagnetic Resonance - EPR and High Performance Liquid Chromatography - HPLC) with combination of multivariate statistical analysis.

MATERIAL AND METHODS

Medical plant material and its pre-treatment

Ten selected medical plants, the most commonly used in the Czech Republic were analyzed (Table 1). The samples originated from the Medical Herbs Centre in Brno and were harvested in their full ripeness by experienced botanists preferably in the sunny morning time (8:00–10:00) during the summer and autumn 2016. The weather conditions at localities were more-less constant (temperature: 20–28 °C; rainfall: 5–15 mm; humidity: 40–80%). After harvest each herbal sample was air-dried on trays at 30 °C and stored separately in paper bags and dark room until analysis.

Table 1 List of medical plants used in the study

Abbreviation*	Botanical name	Family	Part used
LA	*Lavandula angustifolia*	Lamiaceae	flower
CO	*Calendula officinalis*	Asteraceae	flower
HP	*Hypericum perforatum*	Hypericaceae	flower
SS	*Salvia sclarea*	Lamiaceae	flower
MO	*Melissa officinalis*	Lamiaceae	leaf
GO	*Galega officinalis*	Fabaceae	flower
HO	*Hyssopus officinalis*	Lamiaceae	flower
MP	*Mentha piperita*	Lamiaceae	leaf
SO	*Salvia officinalis*	Lamiaceae	leaf
SM	*Silybum marianum*	Asteraceae	seed

*Legend: * For practical reasons, the abbreviations of medical plants based on their botanical name are further used throughout the text.*

Medical plant extraction

0.5 g of homogenized herbal sample was placed into centrifuge tubes for solvent extraction. 20 ml of extraction solvent (three different solvents were tested: water; 50% (v/v) ethanol water solution; dimethylsulfoxide) was poured over the respective herbal sample. Mixture was shaken on laboratory shaker for 1 hour at 150 rpm at laboratory temperature. After that, the mixture was centrifuged using the laboratory ultracentrifuge at 5 000 rpm and 20 °C for 10 minutes, filtered through a fluted filter into a dark vial. The repeated filtration of sample through 0.22 μm nylon filter was applied prior the HPLC experiments. Four extracts from each herb were prepared.

Determination of total phenolic content, total flavonoid content and colour by UV-VIS-NIR

The entire UV-VIS-NIR experiments were performed using UV-VIS-NIR spectrophotometer Shimadzu 3600 with accessories.

Total phenolic compounds content (TPC) was determined applying the Folin-Ciocalteu method (Tobolková et al. 2014). Standard solutions of gallic acid were used for calibration curve construction and the results were expressed as gallic acid equivalents (GAE, g/kg).

Total flavonoids content (TFC) was determined following the modified method using 2-aminoethyl-diphenylborate reagent (Tobolková et al. 2014). Standard solutions of rutin were used for calibration curve construction and the results were expressed as rutin equivalents (RE, g/kg).

Colour characteristics were determined directly from the measured spectra by means of Colour Lite Panorama Shimadzu software under the standardized conditions: D65 day light illuminant and 10° standard observer. Every spectrum was recorded at wavelength range 200–1000 nm, with 0.5 nm interval and slot width 1 nm. For these purposes, 1 mm quartz cuvette was used for measurements. Colour parameters *L*a*b** were used to assess the objectively the colour of medical plant extracts. Additionally, the chroma (C*) and hue angle (h°) were calculated according to the procedure suggested by Kortei et al. (2015).

Determination of antioxidant activity by EPR

The entire experiments were performed using a portable X-band EPR spectrometer e-scan with accessories. The experimental EPR spectra processing and evaluation was carried out using WINEPR.

Antioxidant activity of extracts was tested by 2,2´-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid cation radical ($ABTS^{\bullet+}$) assay. Radical scavenging activity of respective sample was expressed as TEAC (Trolox equivalent antioxidant capacity) value in the same manners as previously described by Polovka and Suhaj (2010).

The capability of extracts components to terminate the hydroxyl radicals ($^{\bullet}OH$) generated by chemical reaction directly in the experimental system via the thermal decomposition of potassium persulfate radical initiator in the presence of DMPO as spin trap was investigated according the method described by Butorová et al. (2017). The antioxidant/prooxidative activity of the samples tested by the spin trap technique in the presence of DMPO/$K_2S_2O_8$ was expressed as % RS (% of radicals scavenged). The % RS was calculated for samples in deionized water and DMSO according to the equation (1):

$$\%RS = \left(1 - \frac{I_{sp(15)OH\cdot} - I_{sp(1)OH\cdot}}{I_{ref\,(15)OH\cdot} - I_{ref\,(1)OH\cdot}}\right) \cdot 100 \quad (1)$$

Where: $I_{sp/ref\ (15)OH\cdot}$ represents the intensity of the 15th EPR spectra of the $^{\bullet}OH$ of the sample respectively reference

$I_{sp/ref\ (1)OH\cdot}$ represents the intensity of the 1st EPR spectra of $^{\bullet}OH$ of the sample respectively reference

For samples in 50% ethanol, the% RS was calculated according to the equation (2) due to the presence of both $^{\bullet}OH$ and carbon-centered radicals:

$$\%RS = \left(\frac{I_{sp(15)C\cdot}}{I_{sp(15)OH\cdot}} - \frac{I_{ref\,(15)C\cdot}}{I_{ref\,(15)OH\cdot}}\right) \cdot 100 \quad (2)$$

Where: $I_{sp/ref\ (15)C\cdot}$ represents the intensity of the 15th EPR spectra of the $C^{\bullet}$ of the sample respectively reference

$I_{sp/ref\ (15)OH\cdot}$ represents the intensity of the 15th EPR spectra of $^{\bullet}OH$ of the sample respectively reference

Determination of individual phenolic compounds by HPLC

HPLC Agilent 1260 Infinity apparatus equipped with diode array detector (DAD) with 10 mm absorption cell, autosampler, quaternary pump, column thermostat and degasser was used.

Individual phenolic compounds (gallic acid, chlorogenic acid, caffeic acid, ferrulic acid, sinapic acid, catechin, rutin, hesperidin, myricetin, quercetin, luteolin) were separated on Poroshell 120 Agilent C18 column (150 mm × 4.6 mm, particle size 2.7 nm) at 45 °C. The mobile phase consisted of acetonitrile (solvent A) and 2.5% solution of formic acid (v/v) (solvent B) was used. The following gradient was used for the determination of phenolic compounds: 92% B, 0–25 min; 85% B, 25–30 min; 88% B, 30–45 min; 85% B, 45–53 min; 80% B, 53–56 min; 92% B, 56–65 min. Additional parameters: flow rate, 0.75 ml/min; injection volume, 5 µl; wavelengths of detection: 280, 290, 330, 350 nm; scan range: from 200 to 450 nm. The data were collected by the Agilent 1260 Infinity chromatography data system. Identification of the individual phenolic compounds was based on the comparison of the retention times and the UV spectra obtained by DAD of unknown peaks to those of reference authentic standards. Quantification of individual phenolic compounds was performed via calibration curve of respective standard.

Statistical analysis

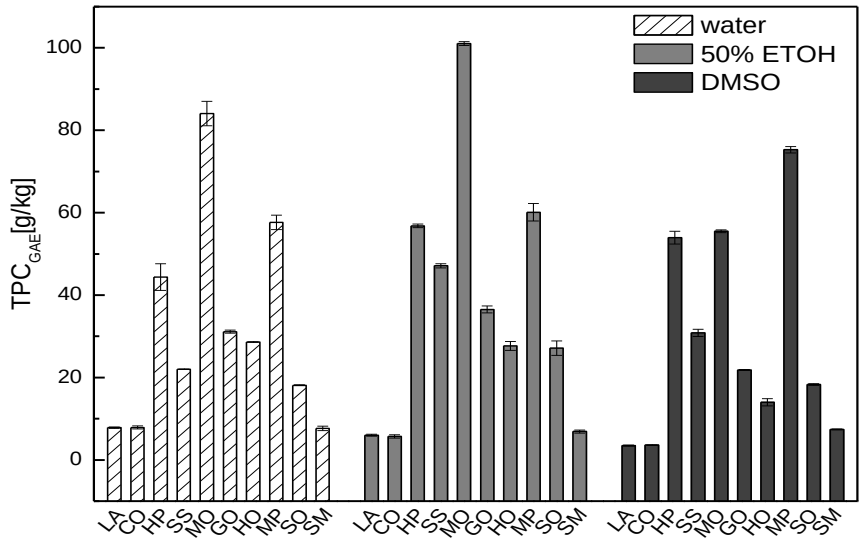
The results were evaluated and visualized using OriginPro v. 7.0 and are expressed as mean ± standard deviation (n = 4). The statistical analysis was performed using the statistical package Unistat v. 6.0. The analysis of variance using Tukey's HSD test was used for determination of differences among samples. A probability value of $P \leq 0.05$ was accepted for statistically significant results. Due to high number of experimental characteristics, the entire experimental dataset was

processed by multivariate statistical analysis canonical discrimination analysis (CDA) and k-th nearest neighbour discrimination.

RESULTS AND DISCUSSION

Obtained results confirmed expected dependence of all the monitored characteristics on solvent used, as previously also proved for isolation of phenolic compounds from basil leaves (Złotek et al. 2016). The total content of polyphenols and flavonoids, antioxidant properties, colour parameters as well as individual content of phenolic compounds of herbs under study are inversely proportional to the values of relative permittivity of the solvents, taken as a measure of polarity (Butorová et al. 2015). The observed tendency is demonstrated on Figure 1 proving the decrease in total phenolic compounds in the following order 50% ethanol > distilled water > DMSO. This trend was similarly observed for parameters TFC, TEAC, %RS as well as for individual phenolic compounds. Based on the results we can conclude that 50% ethanol was the most appropriate solvent for the extraction of phenolic compounds selected herbs. It allows obtain the maximum content of phenolic compounds and antioxidants from herbs and therefore it is suitable for routine analytical work and due to its harmlessness as well as for implementation into food industry (Tan et al. 2014).

Figure 1 Dependence of total phenolic content of medical herbs extracts on extraction solvent



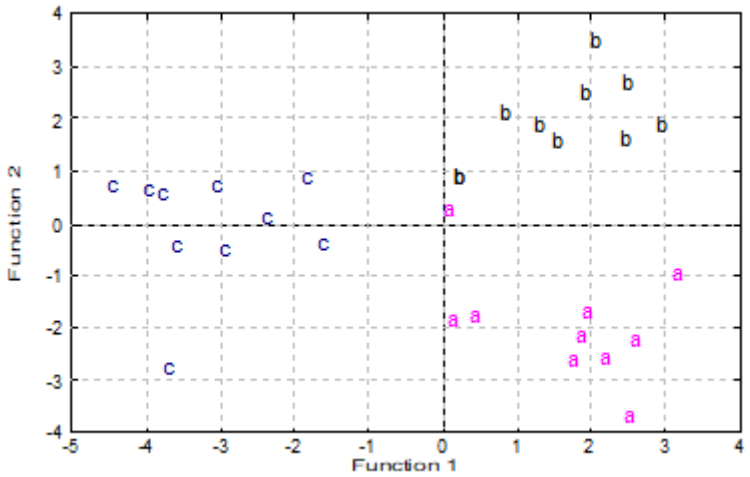
Legend: TPC_{GAE} – Total phenolic compounds content express as gallic acid equivalents, ETOH – ethanol, DMSO – dimethylsulfoxide, LA – Lavandula angustifolia, CO – Calendula officinalis, HP – Hypericum perforatum, SS – Salvia sclarea, MO – Melissa officinalis, GO – Galega officinalis, HO – Hyssopus officinalis, MP – Mentha piperita, SO – Salvia officinalis, SM – Silybum marianum

However, it can also be seen from Figure 1 that the differences in total phenolic content among the individual solvents at the first sight are not so high. Even the analysis of variance in the case of multiple comparison found statistically significant differences only in parameters a* and %RS from all studied parameters when the extraction solvent factor was used. In the case of ˙OH scavenging assay express as %RS statistically higher values were found in 50% ethanol extracts. Also two types of spin aducts (dominant DMPO-OH˙ adduct and minor by product DMPO-CH_2-CH_2-OH˙/ DMPO-CX˙) were detected in 50% ethanol extracts. On the other hand, only DMPO-OH˙ aduct was identified in water and DMSO herbal extracts. Further the negative values of %RS in DMSO and water extracts of individual herbs were found indicate pro-oxidative properties of the extracts, which can be caused by higher concentration of transition metals such as iron, copper and manganese in these extracts (Nasri and Rafieian-Kopaei 2014).

For the visualization of the differences among extraction systems canonical discrimination analysis was used. CDA results according solvent type constructed from all 20 experimental parameters (containing parameters TPC, TFC, TEAC, %RS, colour parameters L*, a*, b*, h°, C* and 11 individual phenolic compounds) show the differentiation of samples into three discrete zones.

From Figure 2 it is obvious that DMSO extracts (aprotic solvent) differ the most from water and ethanol extracts (protic solvents), although all three types of solvents are different from each other (Figure 2). As regards the influence of individual characteristics on discrimination function construction parameters C*, b*, TEAC and TFC are the most discriminating markers for distinguishing medical plants according extraction solvents. CDA classified samples according to extraction system with 96.7% correctness, also k-th neighbour analysis classified samples according to solvents with 100% correctness for k = 1, for k = 2 classification scores decrease to 60%.

Figure 2 Canonical discrimination analysis of medical plants according to extraction solvents. All 20 experimental parameters were used for discrimination function construction



Legend: a – distilled water, b – 50% ethanol, c – dimethylsulfoxide

CONCLUSION

The obtained results demonstrate high potential of combination of analytical techniques with multivariate analysis for differentiation and classification of herbal samples according to extraction systems and evaluation data in complex way. The differences in experimental characteristics were sufficient for successful differentiation and classification of medical plants according to extraction solvents. Based on the results, also 50% ethanol was chosen as the most suitable extraction system for the extraction of substances with antioxidant potential, which will be used in conjunction with industrial practice for further application into food products.

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