The antioxidant activities (AOA) of crude methanolic extracts (CME) and its ethyl acetate (EAF), butanol (BF), petroleum ether (PEF), chloroform (CF) and water fractions (WF) of eight Bulgarian medicinal plants were analyzed using the ABTS cation radical decolorization assay. The presence of compounds possessing antioxidant activity was identified in three fractions: in EAF of Fragaria vesca, Rheum officinale and Melissa officinalis, in BF of Cynodia vulgaris, Hypericum perforatum and Origanum vulgare and in CME of Arctostaphylos uva-ursi. Total phenolic content (TPC) was also determined for each extract/fractions, EAF contained the highest TPC as compared to the other fractions for all species followed by the BF for Arctostaphylos uva-ursi, Fragaria vesca, Hypericum perforatum, Melissa officinalis and Origanum vulgare and by CME for Alchemilla vulgaris, Cynodia vulgaris and Rheum officinale. AOA correlated positively to TPC of CME, BF and EAF, all of them showing a potential value as a source of natural antioxidants.

Key words: Bulgarian medicinal plants, antioxidant activity, total polyphenol content, fractions

Since ancient times plants have been used to prepare teas and beverages, and spices and herbs have been added to different type of food to improve quality, taste and flavor. Recently many plants and plant products have been recognized for their antioxidant properties (Kähkönen et al., 1999; Choi et al., 2002; Cai et al., 2004; Djordjic et al., 2006; Pourmorad et al., 2006; Wong et al., 2006). Plant phenolic compounds (flavonoids, phenolic acids and tannins) are the substances thought to contribute to a great extend to the antioxidant potential of plants (Njoveldt et al., 2001; Higdon et al., 2003; Scalbert et al., 2005). The antioxidant activity (AOA) and total phenolic content (TPC) of aqueous and aqueous-alcoholic extracts of over 50 Bulgarian medicinal plants have been extensively studied (Ivanov 2007; Ivanova et al., 2005, Ivanova et al., 2009, Kiseleva et al., 2004; Kiseleva et al., 2006), and plants with high TPC correlating to their antioxidant potential were identified. However, there are no relevant studies on the fractions containing highest amount of antioxidants from these plants. The aim of this work was to examine phenolic compounds of extracts of eight Bulgarian plants and their fractions and to evaluate their AOA.

Material and methods

Plant material

Plant material was collected in different regions in Bulgaria. The species were identified and voucher specimens were deposited at the Department of Biology and Pharmaceutical Sciences, Faculty of Pharmacy, Medical University of Varna.

Extraction and fractionation

5 g powdered dry material was extracted for 30 min with 100 ml methanol at room temperature in ultrasound chamber. The extract was filtered and the plant material was extracted another two times using the same procedure. All filtrates were combined and the crude methanolic extract was evaporated to

![Diagram of extraction and fractionation procedure](image_url)
dryness under vacuum. The residual was dissolved in 50 ml distilled water and the solution was further consecutively extracted with petroleum ether, chloroform, ethyl acetate and butanol (fig. 1).

For measuring the AOA and the TPC of fractions 10 mg fractionated dry material was dissolved in 1 ml of appropriate solvent: chloroform for petroleum ether (PEF) and chloroform (CF) fractions; absolute ethanol for crude methanolic extract (CME), ethyl acetate (EAF) and butanol (BF) fractions and distilled water for water fraction (WF).

**Antioxidant activity** was measured using the ABTS (2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) cations radical decolorization assay (Re et al., 1999). The method is based on the consumption of preformed in the presence of potassium persulfate ABTS radical (ABTS+). Addition of antioxidants to ABTS+ reduces it to ABTS. Absorption was measured at 734 nm. Uric acid was used as a standard. The antioxidant activity is presented as mmol/L Uric Acid Equivalents (UAЕ). The results are presented as means ± S.D. Each measurement was performed at least in triplicate on Synergy 2 plate reader.

**Total phenolic content** was measured using the Folin-Ciocalteu reagent as described by Singleton and Rossi (1965). Absorption was measured at 760 nm. TPC was expressed as mmol/L Quercetin Equivalents (QE). Results are presented as means ± S.D. Each measurement was performed at least in triplicate on Synergy 2 plate reader.

**Statistical analysis**

All results are presented as means ± standard deviation of three determinations and all were averaged. Statistical analysis was performed by employing GraphPad Prizm 3.0 statistical software. TPC was plotted against AOA and the correlations were analyzed by calculating the correlation coefficient.

**Results and discussion**

Eight Bulgarian medicinal plants (Alchemilla vulgaris, Arctostaphylos uva-ursi, Cynonia vulgaris, Fragaria vesca, Hypericum perforatum, Melissa officinalis, Origanum vulgare and Rheim officinale) were selected for fractioning based on their AOA and TPC of water and water-alcoholic extracts AOA and TPC of each fraction from all eight plants were measured (fig. 2).

The highest AOA for the CME was measured for Arctostaphylos uva-ursi (63.23±2.97 mmol/L), for the PEF – for Cynonia vulgaris (23.75±0.1 mmol/L), for CF – for Hypericum perforatum (6.51±0.01 mmol/L) and Arctostaphylos uva-ursi (6.02±0.4 mmol/L), for EAF – for Fragaria vesca (98.42±7.43 mmol/L), for BF – for Fragaria vesca (60.27±0.93 mmol/L) and Arctostaphylos uva-ursi (59.91±1.23 mmol/L) and for WF – for Fragaria vesca (22.49±0.15 mmol/L).

Highest TPC for the CME was determined for Alchemilla vulgaris (12.26±0.29 mmol/L), for the PEF and CF – for Rheum officinale (2.38±0.05 mmol/L, and 2.91±0.04 mmol/L, respectively), for EAF – for Fragaria vesca (16.78±0.47 mmol/L), for BF – for Hypericum perforatum (9.76±0.13 mmol/L and for the WF – for Melissa officinalis (6.3±0.11 mmol/L). Fragaria vesca (5.63±0.22 mmol/L) and Origanum vulgare (5.6±0.31 mmol/L).

With the aim of establishing a quantitative relationship between the AOA and the content of phenolic compounds, correlation study was carried out (Fig. 3). A high positive correlation (r = 0.87) was established for the EAF; (r = 0.83) for BF and for the CME (r = 0.71). The other fractions did not exhibit this quantitative relationship – the r values or the PEF CF and WF were r = 0.19, r = -0.13 and r = 0.21, respectively. These results indicate that the phenolic compounds were extracted...
Figure 3  Correlation between antioxidant activity and total phenolic content in extracts/fractions
Obrázok 3  Korelácia antioxi达dznou aktivitu a celkovým obsahom fenolov v extraktoch/frackách

predominantly into the CME and in the EAF and BF fractions. The absence of significant correlation indicates that the low but still present AOA measured in PEF, CF and WF could be contributed to other extracted compounds different from phenolics. A correlation between AOA and TPC has been reported for methanolic extracts from different plants (Jeetendra et al., 2011; Alali et al., 2007).

AOA analyses of plant fractions indicated that Alchemilla vulgaris, Fragaria vesca, Rheum officinale and Melissa officinalis exhibited highest AOA of their EAF; Cytisus vulgaris, Hypericum perforatum and Oreganum vulgare – of their BF. Only Arctostaphylos uva-ursi had most active CME against the pre-formed ABTS radical.

Highest TPC was measured for all species in the EAF followed by the BF for Arctostaphylos uva-ursi, Fragaria vesca, Hypericum perforatum, Melissa officinalis and Oreganum vulgare and further by CME for Alchemilla vulgaris, Cotinus coggygria, Cytisus vulgaris and Rheum officinale.

Comparison between the extract/fractions indicated that EAF generally had higher AOA and concentration of total polyphenols followed by the BF and CME, while the WF, PEF and CF exhibited low AOA and total phenolics concentration (fig. 4).

Conclusions

This investigation identified the presence of compounds possessing antioxidant activity in three fractions: in EAF of F. vesca, R. officinale, and M. officinalis, in BF of C. vulgaris, H. perforatum and O. vulgare and in CME of A. uva-ursi.
The TPC of EAF was highest as compared to the other fractions for all species followed by the BF for A. uva-ursi, F. vesca, H. perforatum, M. officinalis and O. vulgare and by CME for A. vulgaris, C. vulgaris and R. officinale. AOA correlated positively to TPC of CME, BF and EAF, all of them showing a potential value as a source of natural antioxidants. These results represent a good basis for further analyses of selected plants to the discovery of new natural food additives.

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References

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