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Full Length Research Paper

Antioxidant activity of herb extracts from five medicinal plants from *Lamiaceae*, subfamily *Lamioideae*

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Five medicinal plants from the subfamily *Lamioideae* of the *Lamiaceae* were tested for antioxidant activity and screened for polyphenols content. Aerial parts of *Ballota nigra*, *Lamium maculatum*, *Leonurus cardiaca*, *Marrubium vulgare*, and *Galeopsis tetrahit* were extracted with methanol (MeOH) and subsequently partitioned by liquid-liquid extraction between petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EA) and n-butanol (BuOH). All 25 extracts and subfractions were assayed for $^{\bullet}$DPPH and HO$^{\bullet}$ scavenging and phosphomolybdenum reduction. Total polyphenols and free hydroxycinnamic acids were determined by spectrophotometric assays. Predictably, all species possess remarkable antioxidant capacity, but the relative differences between species and fractions depended on the method of testing. The LLE method allows the partitioning of most active polyphenols into the polar solvents. The BuOH fraction of *L. cardiaca* had a lowest EC_{50} for $^{\bullet}$DPPH scavenging - 4.45 µg/ml. The maximum inhibition of deoxyribose degradation of was demonstrated for *B. nigra* EA and BuOH fractions (79.32 ± 1.62% and 82.04 ± 2.28%, respectively). *B . nigra* EA, and *L. cardiaca* BuOH fractions had also the highest reducing capacity of 318.6 ± 14.7 mg/g and 271.4 ± 2.4 mg/g ascorbic acid equivalents. The studied plants can provide efficient antioxidant protection by complementary mechanisms, such as free radical scavenging and metal ions reduction. However, only polar fractions from *L. cardiaca* and *B. nigra* are the most potent.

Key words: *Lamioideae*, antioxidant, polyphenols, deoxyribose, *Ballota nigra*, *Galeopsis tetrahit*, *Lamium maculatum*, *Leonururs cardiaca*, *Marrubium vulgare*.

INTRODUCTION

The screening studies for antioxidant properties of medi-cinal and food plants have been performed increasingly for the last few decades in hope of finding an efficient remedy for several present-day diseases and means to delay aging symptoms (Halliwell, 2008) . The disorders related to excessive oxidation of cellular substrates (oxi-dative stress) include type II diabetes, neuro-degenera-tive diseases, or even some types of cancer. There is also a huge demand for natural antioxidants in food industry, for replacing the synthetic preservatives used to prevent fat rancidity or color loss.

Oxidizing agents may damage a number of biological molecules such as nucleic acids, membrane lipids, enzy-mes, or synovial fluid polysaccharides.

Secondary metabolites from medicinal plants function as small molecular weight antioxidants, but their parti-cular mechanisms of action are variable, and depend both on a structure and environment. Direct anti- radical, chain-breaking of the free radical propagation, as well as interaction with transition metals can play a role. Also, the inhibition of ROS-generating enzymes such as xanthine oxidase or inducible nitric oxide synthase is possible. One of the mechanisms *in vivo* is improving the endoge-nous cellular antioxidant mechanisms, such as up-regula-tion of the activity of superoxide dismutase – (SOD) (Halliwell, 2008).

The plants chosen for the present study belong to the large botanical family *Lamiaceae*. The family consists of

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Abbreviations: PE –petroleum ether, DCM – dichloromethane, EA – ethyl acetate, MeOH – methanol, BuOH – *n*-butanol, $^{\bullet}$DPPH – 1,1-diphenyl-2-picrylhydrazyl free radical.

several lower taxa such as subfamilies and tribes, differing also in their chemical properties. The largest subfamily is *Nepetoideae*, comprising most of the medicinal, spice, and aromatic herbs such as rosemary, sage, basil, lemon balm, marjoram, oregano, savory, mint, and many others. They contain rosmarinic acid and are frequently abundant in fragrant volatile terpenes (Wink, 2003). Most of the recognized sources of plant antioxidants belong to *Nepetoideae*. The second largest subfamily (of 3 to over 5 all, depending on the taxonomic system), *Lamioideae*, comprises less species, but some of them are also used in both official and folk phytomedicine. The absence of rosmarinic acid distinguishes it from *Nepetoideae* (Janicsak et al., 1999; Pedersen, 2000). *Lamioideae* contain a variety of iridoid glycosides, phenylpropanoid and phenylethanoid glycosides. Caffeic and chlorogenic acids represent the main free phenolic acids (Zgórka and Głowniak, 2001). Acylated flavone glycosides (esterified with coumaroyl moieties) are chemotaxonomic markers of this taxon along with acteoside-related phenyletha-noids (Pedersen, 2000).

Some species produce also a number of nitrogen-based metabolites such as pyrrholidine alkaloids (stachydrine, betonicine) or guanidines (leonurine) (Liu et al., 2007; Wink, 2003,).

Several popular medicinal plants belong to the genera: *Ballota*, *Galeopsis*, *Lamium*, *Leonurus* and *Marrubium*. In this paper, each genus is represented by one species: *Ballota nigra*, *Galeopsis tetrahit*, *Lamium maculatum*, *Leonurus cardiaca*, and *Marrubium vulgare*, all are common, wild growing in moderate climates, synanthropic weeds.

B. *nigra* L., black horehound, thrives along the roadsides, ruderal habitats, abandoned gardens on rich or poor soils, and is one of the most frequent *Lamiaceae* plant in some areas of Europe. It has been used medicinally in both folk herbalism and officially. It is, or used to be, listed in pharmacopoeias – French (Vrchovska et al., 2007) and Hungarian (Toth et al., 2007), for example. Its activities include sedative, neuroprotective, spasmolytic, antiinflammatory, antidiabetic (Nusier et al., 2007). A number of phytochemical data are available on this and several other *Ballota* species. The compounds include: phenylethanoid/phenylpropanoid glycosides (arenarioside, ballotetroside, forsythoside, martynoside, verbascoside), coumarylated flavonoids, and diterpenes (ballonigrine, ballotinone, and others) (Bertrand et al., 2000; Seidel et al., 2000; Toth et al., 2007; Vrchovska et al., 2007).

G. *tetrahit* L., hemp nettle, is one of the numerous species of *Galeopsis*, considered a stable hybrid of *G*. *pubescens* and *G*. *speciosa*, and a very common weed growing abundantly on roadsides and disturbed places. Like some other *Galeopsis* species it is also a noxious weed in Europe and North America. Several species are used medicinally, in particular a West European *G*. *segetum*, as expectorants, for complementary tuberculosis therapy, and other pulmonary afflictions (apparently due to the high silica content), as well as for treating wounds and insect bites. The phytochemicals include as in other members of the *Lamioideae*: phenylpropanoids, iridoids, stachydrine, flavonoids and tannins (Flamini et al., 2004; Tomas-Barberan et al., 1991). There are also informations about the toxicity of some species of this genus, especially for ruminant livestock or as a cause of coturnism, but it is attributed to compounds contained in the seeds. Therefore, care should be paid when collecting from wild to avoid plants at the fruiting period (Conn, 2001; Flamini et al., 2004).

L. *maculatum* L., spotted deadnettle, is a close relative to the popular European medicinal plant *L*. *album* (dead nettle). It occurs in rich and moist soils in gardens, parks, thickets, sometimes cultivated as an ornamental groundcover plant, spreading by rooting at nodes. It has not been recognized as a medicinal plant, but its resemblance to other related species determined its application in form of *Lamii herba*. It was used in folk medicine for preventing uterine bleeding, improving circulation, and in digestive problems.

The chemical composition, reported a few times, is typical for *Lamioideae*: phenylethanoid glycosides, several iridoid glycosides, flavonoids, diterpenes were reported (Akkol et al., 2008; Alipieva et al., 2007; Shuia et al., 2003).

L. *cardiaca* L., common motherwort, grows synanthropically in ruderal places, abandoned parks and along roadsides on rich soils, sometimes accompanies *B*. *nigra*.

It withdraws from many habitats in Europe as a result of their intensified land management and urbanization. However, as a valued medicinal plant, it is also cultivated as a source of crude drug for herbal industry. The aerial parts are used as *L*. *herba*. It was studied systematically as a medicinal plant several decades ago, but currently the reports on it are scarce (Matkowski and Piotrowska, 2006). Moreover, in the recent pharmacological and phytochemical literature, most of the published work on *L*. *herba* deals with one of the Oriental species *L*. *heterophyllus* or *L*. *sibiricus*, used by Traditional Chinese Medicine in blood circulation or menstruation problems, and during delivery. Alike the European species *L*. *cardiaca*, it has been also used in cardiovascular diseases (Liu et al., 2007). *L*. *cardiaca* has been listed in many pharmacopoeias, including the current European Pharmacopoeia (2008). Its traditional uses are as cardiac tonic, mild sedative and hypotensive infusions. The plant contains labdane diterpenes, flavonoid glycosides – rutin, hyperoside, several phenylpropanoid glycosides, pyrrholidine alkaloids – stachydrine, betonicine and iridoids. The pharmacological activity is apparently resulting from polyphenol and labdane diterpene content, although a presence of bufadienolides is also postulated (Knöss and Zapp, 1998; Miłkowska-Leyck et al., 2002).

M. *vulgare* L., white horehound, a sturdy plant capable of growing in poor soils in the vicinity of human settle-

ments, in waste and ruderal places. As the former species, it has become rare in some areas, and it is usually cultivated for medicinal purposes. The phytochemical composition of the *Marrubii herba* includes the labdane diterpenoids marrubiin, premarrubiin, marrubenol and others, polyphenols such as phenylpropanoid glycolsides, flavonoid glycosides of luteolin, apigenin, and quercetin, tannins, as well as pyrrholidine alkaloids – stachydrine, betonicine. The essential oil contains several sesqui- and monoterpenes (Knöss and Zapp, 1998; Sahpaz et al., 2002; Weel et al., 1999; Wolski et al., 2007). The herb is used mainly in cough as an expectorant, as well as stomachic, spasmolytic, and cardiac hypotensive drug. The therapeutic properties are usually ascribed to marrubiin and other diterpenes, and most of the research focuses on these compounds (Wolski et al., 2007).

The antioxidant activity of some of these plants have been also proved in various *in vitro* or *in vivo* studies, also as a part of screening assays of many medicinal plants (Katalinic et al., 2006; Mantle et al., 2000; Miliauskas et al., 2004; VanderJagt et al., 2002). How-ever, they have not been compared with a similar me-thods, and the material used varied from crude extracts/infusions to isolated compounds from different classes. For *G. tetrahit* and *L*. *maculatum*, the databases search returns no records to date (PubMed, Scopus, Scholar Google, Web of Science – accessed July 29th 2008).

In a previous paper from our laboratory, we reported the antioxidant activity of crude methanolic extracts of *L*. *cardiaca* and *M*. *vulgare*, together with other *Lamioideae* from the same genera (*Lamium*, *Galeopsis*) and *Stachys officinalis* (Matkowski and Piotrowska, 2006). The high anti-radical and peroxidation inhibiting activities prompted us to continue the study on *Lamioideae* plants as sources of antioxidant compounds. With an aim of elucidation of contribution of various polarity classes of the compounds to the antioxidant potential, we have performed a further fractioning of the crude extracts using liquid-liquid partitioning between solvents of increasing polarity. The preliminary screening for total polyphenols and total hydroxylcinnamic derivatives was performed to illustrate the distribution of these compounds into the different solvents.

MATERIAL AND METHODS

Plant material

L. *cardiaca* and *M*. *vulgare* were cultivated in the Medicinal Botanical Garden of the Department of Pharmaceutical Biology and Botany while the plants of *B*. *nigra*, *G*. *tetrahit*, and *L*. *maculatum* were collected from wild, synanthropic habitats in the surrounding area in the river valley of Odra, which constitutes part of the Central European Lowland. The aerial parts of all plants were harvested at full blooming, dried in a heated herb drier at 40°C, ground, weighed and submitted to solvent extraction.

Extraction

The weighed 50 g portions of herbal material were reflux-extracted three times in simmering 80% (v/v) aqueous methanol (500 ml each time). The raw extracts were pooled, filtered, and evaporated to dryness in a rotary vacuum evaporator. The dried crude MeOH extracts were weighed, and a 1 g portions of each were stored for further investigation, while the remaining parts were suspended in 250 ml 5% aqueous MeOH and subjected to sequential liquid-liquid extraction (LLE) with a solvent series of increasing polarity: petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EA) and *n*-butanol (BuOH). The partitioning was performed four times in glass separation funnels by mixing 100 ml of solvent with the aqueous phase and shaking with the rotary shaker for 15 min, and after standing, the removal of organic phase. The pooled fractions were evaporated to dryness, weighed, re-dissolved in MeOH and stored at -18°C for further assays.

Antioxidant testing

Free radical scavenging (FRS) assay

The ability of extracts to scavenge free radicals was assayed with use of a synthetic free radical compound - 2,2-diphenyl-1-picrylhydrazyl ($^{\bullet}$DPPH). The method of Brand-Williams et al. (1995) has been adapted. The extracts concentrations between 1 and 500 µg/mL was added to 100 µM methanolic $^{\bullet}$DPPH solution. The decrease in absorbance at 517 nm was recorded after 1, 5, 10, 20 and 30 min, using a Shimadzu 1601 UV/Vis spectrophotometer. The extract methanolic solution without $^{\bullet}$DPPH was used as a blank to be subtracted from all measurements.

The percentage of free radical scavenging was calculated according to the following equation: $FRS\% = (1-ABS_{extract}/ABS_{DPPH}) \times 100$.

The FRS% values were used for calculation of EC_{50} (defined as the concentration of the extract generating 50% of maximum dose response) estimated with dose-response curves calculated by nonlinear regression module of STATISTICA 8.0 (Statsoft, Poland). The quercetin methanolic solution was used as a reference antioxidant.

Deoxyribose degradation non-site specific inhibition assay

The protection against $^{\bullet}$OH dependent 2-deoxy-D-ribose degradation was estimated using the standard deoxyribose assay based on hydroxyl radical generated in Fenton reaction and measurement of the degradation product - malonyl dialdehyde (MDA) by chromogenic complexation with thiobarbituric acid (expressed as thiobarbituric acid reactive substances – TBARS). The method of Halliwell et al. (1987) was used with slight modification.

The incubation mixture contained 30 µl of the sample extracts dissolved in 8% MeOH in final concentrations in the mixture of 1 to 200 µg/ml; 2.8 mM 2-deoxy-D-ribose, 20 mM sodium phosphate buffer at pH 7.4, 100 mM of Fe^{+3}-EDTA complex, 1 mM of freshly prepared ascorbic acid and hydrogen peroxide, filled up to a total volume of 1.5 mL. The mixture was incubated for 60 min at 37°C in a water bath shaker. After that, 1.5 mL of ice-cold 10% solution of trichloroacetic acid (TCA) was added, followed by 2 mL of 1% solution of thiobarbituric acid (TBA). The TBA mixtures were heated at 95°C in a water bath for 30 min, cooled under cold tap water, and 2.5 mL of butanol was added. Each test tube was vortexed for 1 min and the absorbance was measured in the organic layer using the wavelength of 532 nm, against a blank. The results are expressed as the hydroxyl radical non-site specific scavenging second order rate constant, EC_{50} and maximum inhibition of TBARS formation.

Reducing power assay

Phosphomolybdenum (PMo) assay according to Prieto et al. (1998) was used to estimate the capability of the samples to reduce transition metal ions. The reagent solution contained ammonium molybdate (4 mM), sodium phosphate (28 mM), and sulfuric acid (600 mM) mixed with the samples diluted in methanol. The samples were incubated at 90°C for 90 min, cooled down to room temperature, and the absorbance of the green phosphomolybdenum complex was measured at 695 nm. The reducing capacity of extracts was calculated using the following equation:

$ABS_{final} = ABS_{sample} - ABS_{blank} - ABS_{extract}$

Where:

$ABS_{extract}$ = absorbance of sample where molybdate solution was replaced by water;
ABS_{blank} = absorbance of blank containing methanol (400 l) instead of extract sample.

For reference, the appropriate solutions of ascorbic acid have been used, and the reducing capacity of the analyzed extract was expressed as the ascorbic acid equivalent (AAE) per gram of sample dry weight.

Phytochemical screening

Total polyphenols

Total polyphenol content was estimated using the colorimetric method based on Folin–Ciocalteu reagent (Singleton and Rossi, 1965). All extracts (100 µl) were mixed with 200 µl of Folin–Ciocalteu reagent, and 3.16 ml of H_2O, and incubated at room temperature for 3 min. Following the addition of 600 µl of 20% (w/v) solution of anhydrous sodium carbonate to the mixture, total polyphenols were determined after 2 h of incubation at room temperature. The absorbance was measured at 765 nm. Quantification was done with respect to the standard calibration curve of gallic acid. The results were expressed as gallic acid gram equivalents (GAE) per gram of extracts.

Free hydroxycinnamic acids

The Polish Pharmacopoeia (6^{th} edition, 2005) protocol recommended in the dandelion leaf (*Taraxaci folium*) monograph was used. 1 ml of an extract stock solution was mixed sequentially in a test tube with 2 ml of 0.5 M hydrochloric acid, 2 ml of Arnow reagent (10% w/v aqueous solution of sodium nitrite and sodium molybdate), 2 ml of 8.5% w/v sodium hydroxide, and diluted to 10 ml with deionized water. The absorbance was read immediately at 490 nm and the percent of total hydroxycinnamic acids content was calculated according to the monograph using specific absorbance of caffeic acid.

Statistical processing

All quantitative assays were made in three or more repetitions (n 3) and repeated twice. The differences were tested for statistical significance with one way ANOVA followed by post hoc analysis by Tukey's test (Statistica 8.0 PL, Statsoft, Poland), assuming the differences to be statistically significant at $p < 0.05$. The correlation coefficients between antioxidant and phytochemical assays were calculated using linear regression function of Statistica 8.0PL.

Chemicals

All solvents, inorganic salts, and Folin-Ciocalteu reagent were from POCh (Gliwice, Poland), DPPH reagent from Sigma, thiobarbituric acid and 2-deoxy-D-ribose from Aldrich, gallic acid from Fluka (Sigma-Aldrich-Fluka Poland, Poznan), and trichloroacetic acid from Ubichem (Eastleigh, U.K.). All chemicals were of analytical grade.

RESULTS

Antioxidant activity

All five plants possess antioxidant potential, but discrepancies were noticed between the species and in the fractions of different polarity. The quantitative results of antioxidant assays are listed in Table 2. While the differrences between species and fractions are summarized below.

Free radical scavenging activity

The free radical scavenging assayed by $^{\bullet}$DPPH yellowing revealed the high activity of methanolic extracts from four species except *L*. *maculatum*, that was in terms of EC_{50}, three to five times less potent than the others. The non-polar solvent fractions PE and DCM were always weaker than MeOH extract as well as EA or BuOH fractions.

PE fraction was practically inactive within the tested concentrations

The DCM fraction was efficient in *B*. *nigra*, *L*. *cardiaca*, and *M*. *vulgare* with EC_{50} roughly two-fold higher than that of MeOH, but the maximum FRS barely surpassed 50%. On the other hand, in two remaining species, the EC_{50} was significantly higher, but the maximum scavenging exceeded 80%.

The polar fractions of EA and BuOH were potent with EC_{50} values significantly improved compared to MeOH and maximum effect around 95% exerted at low to medium concentrations (25 - 100 µg/ml), except BuOH from *G*. *tetrahit* that had surprisingly high EC_{50} of 173.22 ± 20.09 µg/ml and the maximum FRS over 90% only above 250 µg/ml. BuOH fraction was strongest in *L*. *cardiaca*, and it was the most efficient fraction of all tested samples.

The FRS had gradually increased during the 20 min of incubation, but most of the effect was achieved before the 5^{th} minute (Figure 1).

Transition metal reduction in P-Mo assay

The reducing power of the crude MeOH extracts was considerable, and the differences between species wer not as large as in other assays (roughly between 111 – 170 mg AAE per g of extract). The strongest MeOH extracts were from *L*. *cardiaca* and *G*. *tetrahit*, followed by

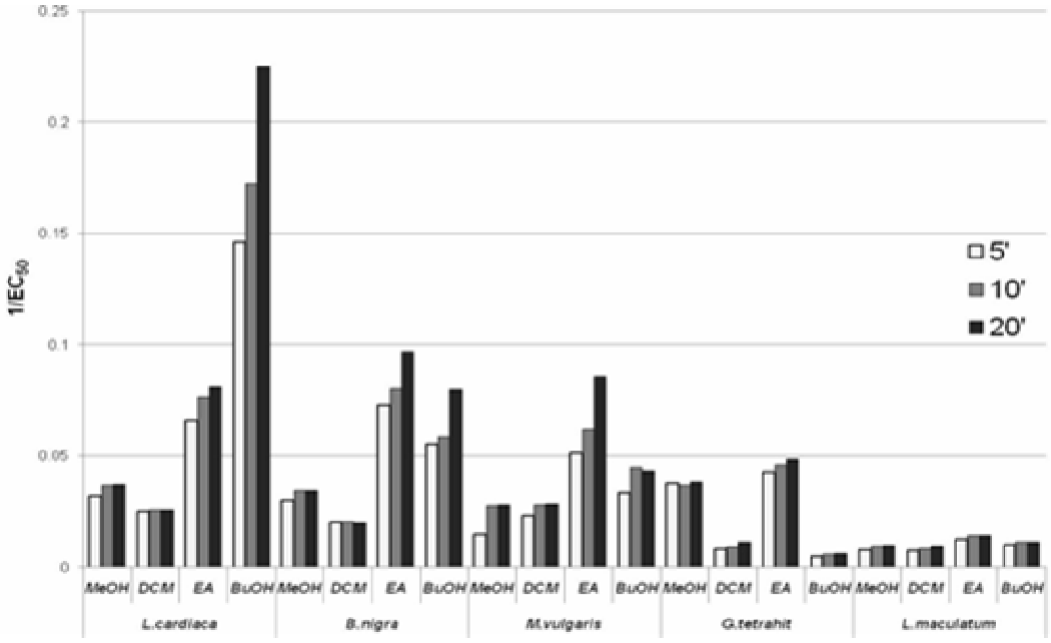

Figure 1. A comparison of free radical scavenging efficiency (shown as 1/EC50) during increased time of sample incubation with •DPPH solution – 5, 10 and 20 min.

by *B*. *nigra*, *L*. *maculatum*, and *M*. *vulgare*. The fractionation left a little activity in PE fraction, but in *B*. *nigra*, it was still 10 times higher than in the second strongest *G*. *tetrahit*.

Much larger interspecific differences were in DCM fraction. In *L*. *cardiaca* and *M*. *vulgare*, it either equaled (*L*. *c*.) or exceeded two-fold the MeOH extract values. In *B*. *nigra* it had approximately a quarter of the MeOH extract activity while in two remaining plants it was practically inactive. Unlike in the $^{\bullet}$DPPH assay, the polar fractions were not always the strongest (Table 1). The fractionation weakened the reducing power of all *L*. *maculatum* fractions, but significantly improved the activity of *B*. *nigra* polar fractions, *L*. *cardiaca* BuOH fraction only, and in *M*. *vulgare*, EA and DCM fractions.

The strongest of all samples were the EA from *B*. *nigra* and the BuOH from *L*. *cardiaca*.

Hydroxyl radical quenching and inhibition of deoxyribose degradation

All tested extracts and fractions, are targeted by hydroxyl radical and the reaction rates varied between species and fractions. However, in the PE fractions of *L*. *cardiaca* and *M*. *vulgare*, due to the pro-oxidant activity, the dose response was so disturbed that the rate constant was impossible to calculate reliably.

In comparison to standard $^{\bullet}$OH scavenger – (+)-epicatechin, the rate constants were about one order of magnitude lower ($1.44 \bullet 10^{13}$ for epicatechin, for the sample values see Table 2). The fastest reaction rate was exhibited by EA and BuOH fractions.

The efficacy of the inhibition of TBARS formation interms of EC_{50} was also better in polar fractions. In both mentioned aspects, the EA fractions of *B*. *nigra* and *L*. *maculatum* were among the strongest. When the maximum protection of deoxyribose from degradation is concerned, the results vary from that of rate constant or EC_{50}. The percent of inhibition reach a maximum of just below 70% in *G* . *tetrahit* and *M*. *vulgare* BuOH fractions to about 80% in *B*. *nigra* EA and BuOH fractions. Crude MeOH extract was stronger than any fraction only in *L*. *maculatum*. Unlike in other assays, some of the PE fractions demonstrated a substantial maximum inhibition percent. In PE and DCM fractions, the pro-oxidant effect was profound in lower and medium concentrations (from 1 to 5 µg/ml, depending on species and fraction), reaching, for example in *B*. *nigra* DCM fraction at 1 and 2 µg/ml the 80% increase in TBARS formation.

Estimation of phenolic compounds

The total polyphenols in methanol extracts subjected to LLE were distributed differently to the particular solvents depending on the species. The results are shown in Table 2. In some cases the Folin-Ciocalteu reactive compounds were concentrated above the level found in MeOH at least in one fraction – in *B*. *nigra*, *L*. *cardiaca*, and *M*. *vulgare*, while in *G*. *tetrahit* and *L*. *album*, the fractioning resulted in depletion of polyphenol concentration. Polyphenols were absent only from PE fractions, whereas in DCM fractions the differences were large between species: from 0.7 to 10% of fraction dry mass. In the polar fractions, the GAE concentration was greater in *B.*

Table 1. The results of antioxidant assays. Whenever applicable, the same superscript letters in a column indicate the lack of significant differences by Tukey test at $p < 0.05$, n 4. The values without a letter are significantly different to any other. (n.d. – not determined due to the irregular dose response).

Species	Extract/ fraction	Free radical scavenging by DPPH assay EC_{50} (µg/ml)±SE	Reducing power by PMo test (AAE mg/g) ±SD	$^{\bullet}OH$ scavenging rate constant $(l \bullet g^{-1} \bullet s^{-1}) \bullet 10^{12}$	Inhibition of deoxyribose degradation EC_{50} (µg/ml)±SE	Maximum inhibition % of deoxyribose degradation (±SD)
Leonurus cardiaca	MeOH	27.27 ±0.79	^{AF}173.1 ±36.9	0.856	0.79 ±0.11	59.89 ±1.86
	PE	>500	0	0	n.d.	n.d.
	DCM	39.14 ±5.95	^{AB}151.8 ±13.1	1.161	0.99 ±0.21	47.67 ±1.16
	EA	12.39 ±1.52	^{B}150.2 ±5.2	2.561	0.32 ±0.04	^{A}68.56 ±0.90
	BuOH	4.45 ±0.50	271.4 ±2.4	2.927	0.83 ±0.05	^{B}71.5 ±1.20
Marrubium vulgare	MeOH	36.69 ±8.13	111.2 ±5.3	0.906	3.62 ±0.33	49.99 ±0.30
	PE	>500	0	0	n.d.	n.d.
	DCM	35.51 ±1.19	^{CD}220.3 ±17.4	0.585	3.42 ±0.41	41.51 ±0.61
	EA	11.67 ±1.51	^{C}239.3 ±6.2	1.225	1.54 ±0.09	^{C}64.44 ±0.55
	BuOH	23.22 ±5.80	66.5 ±4.7	2.402	0.82 ±0.09	^{A}68.76 ±0.50
Ballota nigra	MeOH	29.06 ±4.01	^{B}150.2 ±0.5	1.502	0.51 ±0.05	^{A}68.65 ±1.29
	PE	>500	^{E}20.9 ±6.8	1.212	5.35 ±0.64	^{D}77.04 ±2.51
	DCM	49.91 ±6.13	36.5 ±9.4	1.316	8.06 ±0.25	^{AC}65.66 ±4.26
	EA	10.32 ±0.70	318.6 ±14.7	5.319	0.25 ±0.03	82.04 ±2.28
	BuOH	12.55 ±0.79	^{D}221.2 ±5.9	2.748	0.61 ±0.06	^{D}79.32 ±1.62
Lamium maculatum	MeOH	105.40 ±13.48	131.2 ±7.5	1.638	1.02 ±0.16	^{B}72.46 ±2.81
	PE	>500	1.2 ±0.0	0.629	5.53 ±1.01	57.71 ±9.62
	DCM	106.54 ±14.24	2.6 ±0.2	0.760	5.28 ±0.44	^{AC}63.69 ±4.54
	EA	70.59 ±12.11	85.6 ±0.8	4.548	0.35 ±0.01	^{C}65.27 ±1.57
	BuOH	91.24 ±11.33	73.6 ±0.7	1.489	1.36 ±0.38	^{C}63.08 ±1.42
Galeopsis tetrahit	MeOH	26.24 ±4.88	^{F}168.8 ±2.07	0.668	1.44 ±0.39	^{AC}66.14 ±1.85
	PE	>500	2.1 ±0.2	0.777	6.28 ±1.48	^{AC}64.19 ±2.66
	DCM	88.98 ± 7.99	5.7 ±0.0	0.928	>50	^{AC}67.18 ±3.94
	EA	20.66±1.77	^{F}172.4 ±0.4	1.852	0.46 ±0.04	^{AC}63.98 ±3.97
	BuOH	173.22 ±20.9	57.7 ±1.6	2.082	0.45 ±0.08	^{A}69.68 ±2.27

nigra, *G*. *tetrahit*, and *M*. *vulgare* EA fraction, and in BuOH fractions of *L*. *maculatum* and *L*. *cardiaca*.

The total hydroxycinnamic derivatives were more concentrated in the polar fractions than in MeOH extract. The highest increase (3 to 4-fold) was observed in *B*. *nigra* and *L*. *cardiaca* EA and BuOH fractions.

The correlation of phenolic compounds content to the antioxidant activity assayed by different methods is shown in Figure 2. The correlation coefficient *r*, exceed 0.9 only for PMo versus total polyphenol assay.

DISCUSSION

As a result of this study, the confirmation of considerable antioxidant potential in the *Lamiodeae* plants and the described differences between the species and solvent fractions provide information that extends the knowledge of possible mechanism that underlie their traditional uses. One has to remember that the contribution of polyphenolic antioxidants to the overall therapeutic properties of medicinal plants used for prevention of oxidative stress related disorders is still disputed (Halliwell, 2008). Moreover, the relevance of *in vitro* assays that are performed for screening can be different with respect to the physiological conditions. Hence, there is much more extraordinarily strong antioxidants reported for many herbal pro-ducts than data on their actual *in vivo* effects (Halliwell, 2008). Even though, the provision of thorough *in vitro* data for

Table 2. The results of extraction and spectrophotometric assays for polyphenol content. Whenever applicable, the same superscript letters indicate the lack of significant differences by Tukey test at $p < 0.05$. The values without a letter are significantly different to any other.

Species	Extract/fraction	Extraction yield g extract per 100 g of dried herb (g)	Total polyphenols (GAE mg/g ± SD)	Total hydroxycinnamic acids (% CAE ± SD)
Leonurus cardiaca	MeOH	23.2	150.1 ±8.9	9.03 ±0.03
	PE	0.72	0	0
	DCM	1.61	^{A}99.9 ±7.1	0.63 ±0.00
	EA	1.41	136.4 ±4.3	16.78 ±0.47
	BuOH	4.96	256.8 ±7.8	41.25 ±0.47
Marrubium vulgare	MeOH	26.6	^{B}63.4 ±1.7	3.23 ±0.24
	PE	0.76	0	0
	DCM	2.84	^{C}75.9 ±5.2	0.47 ±0.04
	EA	2.92	^{D}202.9 ±10.9	^{a}2.46 ±0.35
	BuOH	6.56	^{C}71.3 ±7.5	^{b}4.00 ±0.02
Ballota nigra	MeOH	27.1	114.7 ±3.0	^{c}11.72 ±0.07
	PE	5.31	0.0	^{d}1.04 ±0.68
	DCM	1.57	38.0 ±4.3	^{d}1.52 ±0.08
	EA	1.15	339.0 ±11.4	28.16 ±0.43
	BuOH	5.96	^{D}202.6 ±4.9	35.13 ±0.61
Lamium maculatum	MeOH	23.4	^{A}93.0 ±7.1	4.77 ±0.39
	PE	1.12	0	0
	DCM	0.617	7.0 ±1.9	^{a}2.46 ±0.36
	EA	1.62	^{C}70.8 ±8.8	8.30 ±0.37
	BuOH	10.9	^{A}86.3 ±7.3	5.94 ±0.18
Galeopsis tetrahit	MeOH	24.3	121.1±10.4	^{c}11.16 ±0.12
	PE	0.24	0	0
	DCM	1.32	17.5±4.3	^{b}3.88 ±0.15
	EA	2.58	^{A}85.6±5.9	17.73 ±0.32
	BuOH	7.04	81.5±1.6	5.45 ±0.08

less known medicinal plants can help in obtaining the complete prospect of the role of plant antioxidants in health of animal organisms, including that of humans. Among the tested 25 extracts and solvent fractions, several appeared to have a remarkable antioxidant potential, while others are only moderately active or inactive at all.

Also, the diversity of mechanisms tested by the chosen assays is the likely reason for the observed slight discrepancies between the results of different assays. This has been frequently reported in antioxidant studies (Dorman et al., 2003; Mantle et al., 2000; Matkowski and Piotrowska 2006). Therefore it is necessary not to relay on a single test or even the analyzed parameter (Aruoma, 2003). This is illustrated by the differences between the relative dose- dependent efficiency of antioxidant activity expressed as EC_{50} and the summed protection of oxidezable substrate revealed in deoxyribose assay. The lower EC_{50} does not always reflect the higher percent of TBARS inhibition, which is the ultimate measure of antioxidant capability. In this study, we have tested the complex matrices of both polyphenols and other compounds that could either add to the total antioxidant status, or diminish it as an inactive bulk. Apart from the typical phenolic antioxidants such as hydroxycinnamic acids, phenylpropanoid glycosides, flavonoids, tannins, a contribution from isoprenoids such as diterpenes or carotenoids is also likely. This should be explored by a de-

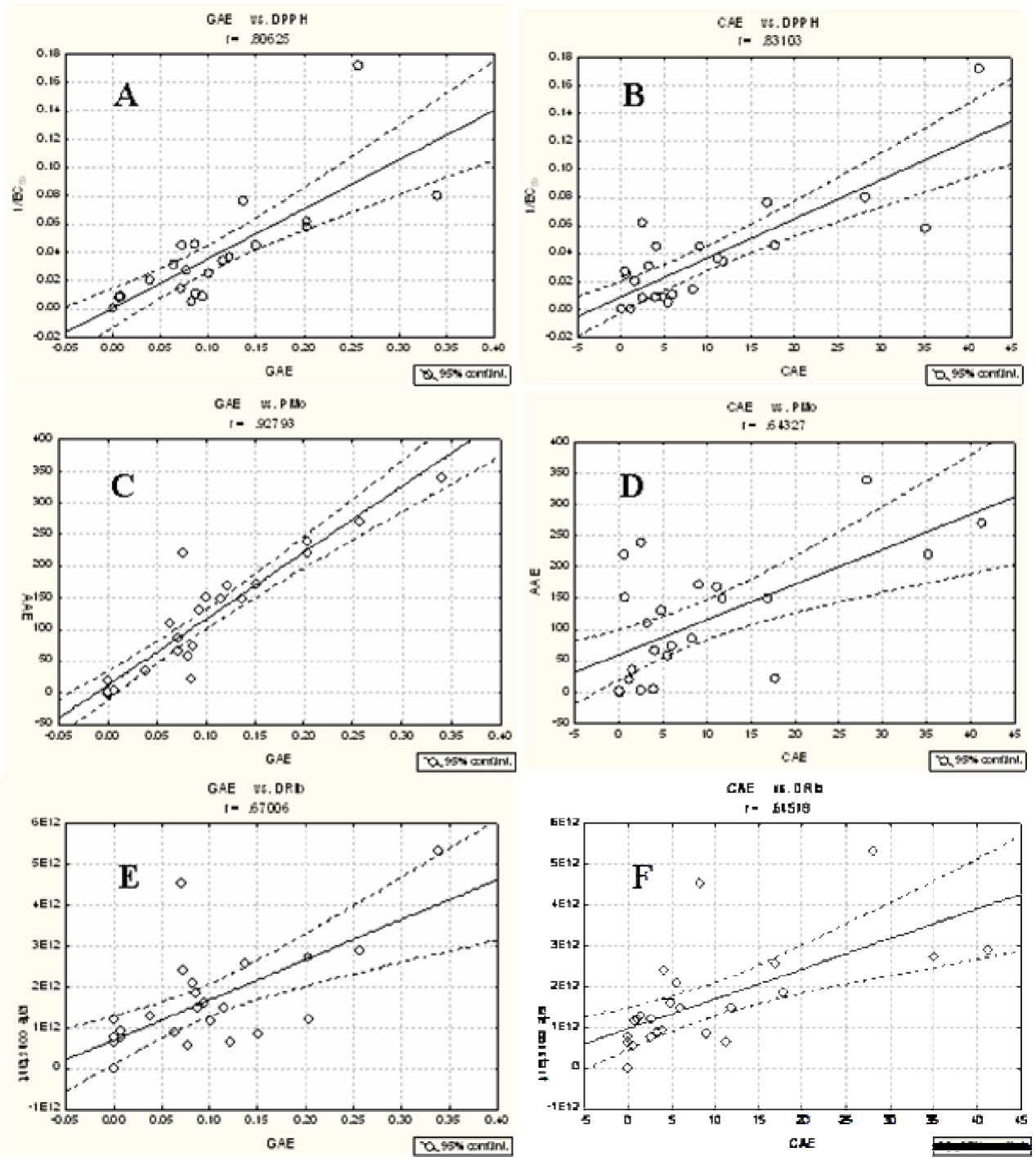

Figure 2. Correlations between the results of different antioxidant assays (Y axes) and estimation of phenolic compounds (X axes). A) $^{\bullet}$DPPH vs. total polyphenols, B) $^{\bullet}$DPPH vs hydroxycinnamic derivatives, C) PMo vs. total polyphenols, D) PMo vs. hydroxycinnamic derivatives, E)deoxyribose assay rate constant vs. total polyphenols, F) deoxyribose assay rate constant vs. hydroxycinnamic derivatives. The estimation of linear regression was performed at 95% confidence interval, using Statistica 8.0 PL.

tailed phytochemical study on purified non-polar fractions. The limited correlation of antioxidant assays results to the polyphenols content is also observed (Figure 2). The high correlation of PMo and total polyphenol assay can be explained by related chemical mechanisms involving molybdenum ions (Prieto et al., 1998; Singleton and Rossi, 1965). However, there is also a significant correlation of total hydroxycinnamic derivatives to the FRS

activity.

In the previous paper, we reported the antioxidant activity of methanolic extracts of several *Lamioideae* herbs, including *L*. *cardiaca* and *M*. *vulgare*. A species related to *G*. *tetrahit* - *G*. *speciosa* was also studied. *L*. *cardiaca* was found to be one of the most potent free radical scavengers, whereas *M*. *vulgare* was more efficient in reducing power and lipid peroxidation assays. These plants have been rarely studied in this respect, and the data on their antioxidant potential are scarce (Mantle et al., 2000; Matkowski and Piotrowska, 2006; VanderJagt et al., 2002; Weel et al., 1999). In our results, the high activity of *L*. *cardiaca* BuOH fraction should suggest the contribution of antioxidant mechanism to the cardiovascular system protecting properties, analogously to the related, more studied Oriental motherwort species (Liu et al., 2007; Sun et al., 2005). Interestingly, in the present paper, the extract of *M*. *vulgare* was weaker than most other species. The variation in drug quality may account for this observation. However, the LLE of the crude extract yielded highly active polar fractions, and the non- polar DCM fraction. The activity of DCM fractions of both *M*. *vulgare* and *L*. *cardiaca* could be attributed to labdane diterpenoids such as marrubenol (Knöss and Zapp, 1998; Wolski et al., 2007). However, this hypothesis should be confirmed by bioactivity guided isolation of active compounds from this fraction. The high pro-oxidant activity of DCM fractions also undermines the potential benefits. The polar fraction of a close species *M*. *globosum* subsp. *globosum*, was also stronger than non-polar in three antioxidant assays (Sarikurkcu et al., 2008).

A related genus – *Ballota*, contains several species that have been investigated by several groups with respect to antioxidant polyphenols. *B*. *nigra* is the most popular plant that has been confirmed to contain a number of antioxidant phenylpropanoids (Seidel et al., 2000). In our study, *B*. *nigra* was highly potent in every assay. The EA fraction was the strongest among all five species. The hydroxycinnamic derivatives, but not total polyphenols was also highest. Therefore, the antioxidant properties of black horehound are likely to result from the abundance in phenylpropanoids.

As for the other two species, there are no detailed literature data regarding their antioxidant properties, but several papers reports the activity of other *Lamium sp*. (Budzianowski and Budzianowska, 2006; Yalcin et al., 2007) . *L*. *maculatum* contains phenylpropanoid glycolsides and several flavonoids that could contribute to its activity (Shuia et al., 2003; Yalcin et al., 2007). However, in none of the three used assay this species was as efficient as any of the others. Only EA fraction showed a considerable activity in deoxyribose test and MeOH extract, but none of fractions, in PMo reduction.

Galeopsis speciosa that had been studied before (Matkowski and Piotrowska, 2006), is closely related to *G*. *tetrahit*, and due to the similarity in morphology and habitats of *Galeopsis* sp. they are likely to be used for the same purposes without distinguishing the particular species. In comparison to other tested species, MeOH extract of *G*. *speciosa* was rather weak, despite the high total polyphenols content. Here, both MeOH extract and EA fractions of *G*. *tetrahit* were comparable to the three strongest species, and significantly stronger than *L*. *maculatum*. Whether this discrepancy reflects a constant interspecific difference or variation in drug quality remains to be verified by broader comparative research on several hemp-nettle species.

Conclusion

In conclusion, the medicinal herbs belonging to the subfamily *Lamioideae* are rich sources of antioxidants, in addition to the more popular and widely described *Nepetoideae*. The crude extracts can be fractionated in order to obtain highly efficient concentrated antioxidant mixtures that can be used for further purification or provide antioxidant protection from oxidative stress and prevent deterioration of food ingredients. The LLE with polar solvents such as EA or BuOH can significantly improve the antioxidant potential in some cases and provides hints for using certain solvents for herbal preparations. The traditional use of these, sometimes neglected herbs justifies their including in official medicinal product listings such as pharmacopoeias, as it has been done for *L*. *cardiaca* and *B*. *nigra*. However, the detailed understanding of the role of particular compounds in the differ-rent antioxidant mechanisms requires thorough phytochemical and pharmacological investigations.

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