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Antioxidant Activity of Extracts and Different Solvent Fractions of *Glechoma hederacea* L. and *Orthosiphon stamineus* (Benth.) Kudo

Aktywność przeciwutleniająca ekstraktów i frakcji *Glechoma hederacea* L. i *Orthosiphon stamineus* (Benth.) Kudo

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Abstract

Background. *Orthosiphon stamineus* is a popular medicinal plant from tropical Asia consumed in the form of infusions. *Glechoma hederacea*, once popular as a medicinal plant and as a bitter beer supplement, is a ubiquitous weed growing wild in moderate climates of Eurasia. Both plants belong to the *Nepetoideae* subfamily and are rich in phenolic antioxidants such as rosmarinic acid and flavonoids. Antioxidant activity can contribute to their traditional uses in phytotherapy as well as to their preventive value in degenerative and life-style diseases.
Objectives. Two medicinal *Lamiaceae* species were chosen to test their antioxidant potential using three complementary assays. Preliminary screening for total polyphenols and phenolic acids was also performed to find correlations with the activity. Fractionation of the crude extract was carried out to reveal the contributions of compounds of different polarity to total antioxidant status. Interspecific variation was also analyzed.
Material and Methods. Aqueous methanol extracts were prepared by reflux extraction from *Orthosiphonis folium* and *Glechomae herba*. The extracts were fractionated using liquid-liquid extraction (LLE) with petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EA), and *n*-butanol (BuOH). All extracts and fractions were studied for their antioxidant activity using spectrophotometric assays: $^{\cdot}$DPPH scavenging, phosphomolybdenum reduction, and deoxyribose degradation assay. Polyphenol content was studied by the Folin-Ciocalteu method, Arnow reaction, and Lopez-Arnaldos assay.
Results. Predictably, both species possess remarkable antioxidant capacity, but the relative differences between the species and fractions depended on the applied assay. Varying correlation of total polyphenol content and the amounts of various chemical classes of polyphenols was also observed depending on the mechanism of antioxidant testing. In *O. stamineus*, partitioning the crude extract led to distribution of the initially very high activity among the DCM, EA, and butanol fractions, whereas in *G. hederacea* the moderate activity of the methanol extract was greatly enhanced in the EA and butanol fractions, which were the strongest in all assays.
Conclusions. These plants can provide efficient antioxidant protection by complementary mechanisms such as free radical scavenging and metal ion reduction. However, *Orthosiphonis herba* is more effective as an integral extract due to the synergistic action of its components, while *Glechomae herba* needs fortification by fractioning the crude extract into polar solvents to increase the proportion of rosmarinic acid, the most active agent in this herb (**Adv Clin Exp Med 2008, 17, 6, 615–624**).

Key words: *Orthosiphon stamineus*, *Glechoma hederacea*, antioxidant, rosmarinic acid.

Streszczenie

Wprowadzenie. *Orthosiphon stamineus* to popularna roślina lecznicza pochodząca z Azji tropikalnej i spożywana w postaci naparów, a *Glechoma hederacea* jest pospolitym i wszędobylskim chwastem strefy umiarkowanej Eurazji, kiedyś popularny jako ziołolecznicze oraz dodatek do piwa. Rośliny te, należące do podrodziny *Nepetoideae*, są szczególnie bogate w kwas rozmarynowy oraz glikozydy flawonoidowe. Ich aktywność antyoksydacyjna może wpływać na właściwości terapeutyczne w chorobach cywilizacyjnych, a także uzasadniać ich tradycyjne zastosowanie w ziołolecznictwie.
Cel pracy. Zbadanie właściwości antyoksydacyjnych oraz zawartości całkowitych polifenoli, kwasów fenolowych oraz kwasu rozmarynowego w obu gatunkach rodziny *Lamiaceae*, a także zbadanie wpływu frakcjonowania rozpuszczalnikami o wzrastającej polarności na dystrybucję związków antyoksydacyjnych.

Materiał i metody. Surowce zielarskie *Orthosiphonis folium* i *Glechomae herba* poddano ekstrakcji 80% metanolem oraz frakcjonowaniu przez wytrząsanie z eterem naftowym (PE), chlorkiem metylenu (DCM), octanem etylu (EA) i butanolem (BuOH). Właściwości antyrodnikowe i antyoksydacyjne zostały zbadane metodami spektrofotometrycznymi: testem $^{\bullet}$DPPH, redukcji molibdenianu i ochrony deoksyrybozy przed degradacją oksydacyjną. Wstępna analiza fitochemiczna pod względem związków fenolowych została przeprowadzona metodą Folin-Ciocalteu dla całkowitych polifenoli, Arnowa dla pochodnych hydroksycynamonowych oraz Lopez-Arnaldos dla kwasu rozmarynowego.

Wyniki. Obie badane rośliny mają właściwości antyoksydacyjne *in vitro*, różnice międzygatunkowe jednak są znaczące, zależnie od badanej frakcji oraz metody analizy. Stwierdzono też różnice w korelacji aktywności do różnych klas polifenoli, w zależności od mechanizmu działania antyoksydacyjnego. U *O. stamineus* frakcjonowanie ekstraktu metanolowego prowadzi do rozdzielenia pierwotnie bardzo dużej aktywności między frakcje dichlorometanową, octanu etylu i butanolową. U *G. hederacea* początkowa mniejsza aktywność była znacznie wzmocniona we frakcjach octanu etylu i butanolowej, najsilniejszych we wszystkich testach.

Wnioski. Badane gatunki mogą przyczynić się do ochrony antyoksydacyjnej na drodze uzupełniających się mechanizmów: zmiatania wolnych rodników i redukcji metali przejściowych. *Orthosiphonis herba* jest aktywniejszy jako cały ekstrakt dzięki synergistycznemu działaniu jego składników, a *Glechomae herba* wymaga wzmocnienia przez frakcjonowanie ekstraktu metanolowego do polarnych rozpuszczalników w celu zwiększenia udziału kwasu rozmarynowego, który jest najaktywniejszym związkiem czynnym tej rośliny (**Adv Clin Exp Med 2008, 17, 6, 615–624**).

Słowa kluczowe: *Orthosiphon stamineus*, *Glechoma hederacea*, antyoksydant, kwas rozmarynowy.

The *Lamiaceae* mint family is a large taxon of several thousand species which includes numerous popular and less known herbs with pronounced therapeutic properties. Most of the fragrant spices, such as sage, oregano, thyme, rosemary, marjoram, mint, and others, are also valued as medicinal and sometimes pharmacopoeia-listed plants. This large botanical family is taxonomically divided into several subfamilies, one of the largest being the *Nepetoideae*, which also comprises species that are most important as a source of antioxidants [1]. Their antioxidative properties have been studied and published in countless reports. The subfamily is distinguished by a high content of rosmarinic acid as the main phenolic compound as well as by numerous flavonoid glycosides. Rosmarinic acid (RA, α-O-caffeoyl-3,4-dihydroxyphenyllactic acid, Fig. 1) is a potent antioxidant and possesses valuable pharmacological properties. RA shows anti-inflammatory, antimicrobial, antimutagenic, and antiviral activity. Examples of plant sources of RA are *Coleus blumei*, *Melissa officinalis*, *Ocimum basilicum*, *Salvia sp.* (*Lamiaceae*), *Anchusa officinalis, Eritrichum sericeum*, and *Lithospermum officinale* (*Boraginaceae*), as well as hornworts (*Anthoceros sp.*) and ferns [2, 3].

The two species chosen for the present study are, from the phytotherapeutic point of view, quite far away from each other. *Orthosiphon stamineus* is a tropical species growing and cultivated in South-East Asia and is known in Western herbalism as Java Tea. It has also been included in the European Pharmacopoeia under this name, with the leaves used for crude drug (*Orthosiphonis folium*) [4]. The plant is a popular herb in Malaysia, Indonesia, Thailand, Vietnam, and neighboring countries. Its applications include urinary system diseases such as kidney and bladder inflammation and lithiasis due to its mild diuretic and anti-inflammatory action. It is also claimed to be an efficient drug for fever, rheumatism, hypertension, and even gonorrhea and syphilis. It is used to prepare a herbal tea drunk for renal complaints [4, 5]. *O. stamineus* contains a number of potentially bioactive compounds, chiefly from the versatile polyphenolic group. Rosmarinic acid is one of the major compounds, accompanied by several lipophilic flavones with highly methoxylated substitution patterns, such as eupatorin, sinensetin, teramethylscutellarein, and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone [5–7]. It also contains diterpenoids such as isopimarane and staminane skeleton-based compounds demonstrating cytotoxic activities [9] as well as triterpenoid acids and phytosterols. The anti-inflammatory activity of the mentioned polymethoxylated flavones was confirmed by inhibition of 15-lipoxygenase [10].

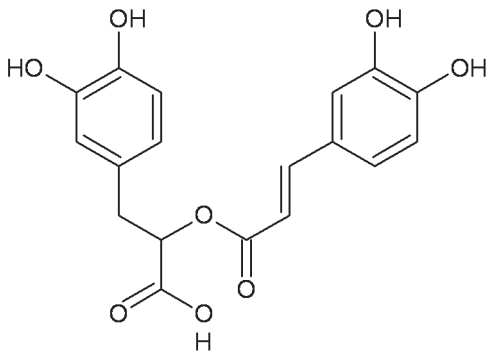

Fig. 1. The structure of rosmarinic acid

Ryc. 1. Kwas rozmarynowy

Glechoma hederacea, or ground ivy, is a herbaceous perennial creeper. The plant grows abundantly on rich soils in moderate climate in the Northern Hemisphere in both natural and synanthropic habitats. As a common and familiar plant, it has been consumed in some countries as a spicy addition to salads or soups. It spreads easily by stolons and can sometimes become an annoying weed. Therefore its medicinal uses are based on collection from the wild. *Glechoma hederacea* leaves or flowering herb have been mainly applied in inflammations, common colds, and congestions and also as a tonic and a diuretic. Some other uses include the treatment of arthritis, diabetes, and scurvy [11, 12]. It is available in herbal stores throughout Europe. It has not been placed in pharmacopoeias, but it is mentioned in other herbalist references [13]. The chemical composition of ground ivy includes alkaloids, sesquiterpenoids, triterpenoids, flavonoid glycosides, and rosmarinic acid [11, 12, 14–17]. Pharmacological and clinical studies of this species are rather scarce compared with other, more popular *Nepetoideae*, although some published results indicate its anti-inflammatory and antibacterial properties [11, 18].

Antioxidant activity in *Orthosiphon stamineus* has already been reported in a few papers [6–9]. However, different antioxidant assays and extract types were used each time and no comparisons with any related species were performed. For *Glechoma hederacea*, only one reference deals specifically with antioxidant testing using the DPPH assay [11].

As can be seen from the descriptions of the two species, they have quite similar traditional uses but come from distant regions and climates and their current positions in phytotherapy are also different. The aim of the present study was to compare both plants as potential sources of antioxidant polyphenols.

Antioxidants have been studied extensively for decades in the hope of finding compounds protecting against a number of diseases attributed to free radical-induced damage and oxidative stress. This subject has been thoroughly reviewed in a number of publications, one of the key references being the recent edition of Halliwell and Gutteridge's book [20]. The initially enthusiastic and numerous studies involving screening for plant antioxidants are now being questioned with respect to their *in vivo* relevance [21]. Nevertheless, there are still many gaps in the data for a number of species, especially when *in vitro* antioxidant activity has to be compared between different species. This is partially due to the inconsistent methodology, but also to the widespread over-interpretation of crude extract data with little attention to the interspecific chemical variability of plants.

In the present paper, antioxidant activity assayed with three tests based on complementary mechanisms is described. Besides crude aqueous-methanolic extracts, several liquid-liquid (LLE) fractions were examined for their antioxidant activity. The phytochemical composition of several polyphenol classes using spectrophotometric assays was also studied to determine correlation with antioxidant properties.

Material and Methods

Plant Material

Dried commercial samples of both species purchased from a herbal store were used. *Orthosiphonis folium* was from Flos, Poland, and *Glechomae herba* from Kawon, Gostyń, Poland. Both drugs were in 50-g packages and the expiration date was at least six months after the conducted experiments.

Extraction

Weighed portions of the herbs (50 g each) were extracted under reflux in round-bottomed flasks with 500 ml of simmering 80% (v/v) aqueous MeOH for 6 hours. The extraction was repeated three times with a fresh 500-ml portion of the solvent. The three portions of crude extracts were pooled, filtered, and evaporated to dryness *in vacuo* using a rotary evaporator. The dried extract was weighed to determine extraction efficiency, then a weighed portion of the dry mass was taken for further testing while the remaining, major part was suspended in 100 ml of 5% (v/v) aqueous MeOH for sequential liquid-liquid extraction (LLE) with four solvents of increasing polarity: petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EA), and 1-butanol (BuOH). The crude extract suspension was mixed with 50 ml of LLE solvent in a 300-ml separation funnel and, after shaking for 15 minutes and removing the organic layer, the procedure was repeated five times. The solvent fractions were evaporated to dryness and weighed. All extracts and fractions were stored at –18°C until use.

Antioxidant Testing

Free Radical Scavenging (FRS) Assay

The ability of the extracts to scavenge free radicals was assayed using the stable, synthetic, nitrogen-based, free radical compound 2,2-diphenyl-1-picrylhydrazyl (•DPPH). The method of Brand-Williams et al. [22] was adapted as in a previous

study by the present author [23]. The extract was dissolved in MeOH at concentrations between 1 and 500 μg/ml and mixed with an equal volume of a 200 μM methanolic ·DPPH solution. The sample was incubated in the dark at room temperature and the decrease in absorbance was recorded after 1, 5, 10, 20, and 30 minutes. The measurement wavelength was set at 517 nm. Methanolic extract solution without ·DPPH was used as the blank to be subtracted from all measurements. The percentage of free radical scavenging was calculated according to the equation: FRS% = $= (1\text{-}ABS_{extract}/ABS_{DPPH}) \times 100$. The FRS% values were plotted against the sample concentration to calculate EC_{50} (the concentration of extract generating 50% of the maximum dose response) estimated from dose-response curves calculated by the nonlinear regression module of STATISTICA 8.0 (Statsoft, Poland). A quercetin methanolic solution was used as the reference antioxidant.

Deoxyribose Degradation Non-Site-Specific Inhibition Assay

The protection against ·OH-dependent 2-deoxy-D-ribose degradation was estimated using the standard deoxyribose assay based on hydroxyl radical generated in the Fenton reaction and measurement of the degradation product malonyl dialdehyde (MDA) by chromogenic complexation with thiobarbituric acid (expressed as thiobarbituric acid-reactive substances, or TBARS). The method of Halliwell et al. [24] was used with slight modification.

The incubation mixture contained 30 μl of the sample extracts dissolved in 8% MeOH at final concentrations in the mixture of 1 to 200 μg/ml, 2.8 mM 2-deoxy-D-ribose (Aldrich), 20 mM sodium phosphate buffer (pH 7.4), 100 mM of Fe^{+3}-EDTA complex, 1 mM of freshly prepared ascorbic acid, and hydrogen peroxide filled to a total volume of 1.5 ml. The mixture was incubated for 60 minutes at 37°C in a water bath shaker. Then 1.5 ml of an ice-cold 10% solution of trichloroacetic acid (TCA) was added followed by 2 ml of a 1% solution of thiobarbituric acid (Fluka) (TBA). The TBA mixtures were heated at 95°C in a water bath for 30 minutes, cooled under cold tap water, and 2.5 ml of butanol was added. Each test tube was vortexed for 1 minute and, after the separation of layers, the absorbance was measured in the organic (upper) layer using a wavelength of 532 nm against a blank containing the reagents without deoxyribose. The results were expressed as the hydroxyl radical non-site-specific scavenging rate constant, EC_{50}, and maximum inhibition of TBARS formation.

Reducing Power Assay

The phosphomolybdenum assay according to Prieto et al. [25] 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 to room temperature, and the absorbance of the green phosphomolybdenum complex was measured at 695 nm. The reducing capacity of the extracts was calculated using the equation: $ABS_{final} = ABS_{sample} - ABS_{blank} - ABS_{extract}$, where $ABS_{extract}$ is the absorbance of the sample in which molybdate solution was replaced by water and ABS_{blank} the absorbance of the blank containing methanol (400 μl) instead of the extract sample. For reference, appropriate solutions of ascorbic acid were used and the reducing capacity of the analyzed extract was expressed as the ascorbic acid equivalents (AAE) per gram of sample dry weight.

Phytochemical Screening

Total Polyphenols

Total polyphenol content was measured using the Folin-Ciocalteu colorimetric method described by Singleton and Rossi [26]. All extracts (40 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 a 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 extract.

Free Hydroxycinnamic Acids

The European Pharmacopoeia (edition 6.2) [5] protocol recommended in the *Melissae folium* monograph was used. One ml of 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), and 2 ml of 8.5% w/v sodium hydroxide and diluted to 10 ml with deionized water. The absorbance was read immediately at 505 nm and the percentage of total phenolic acid content was calculated according to PhEur [5] using the specific absorbance of rosmarinic acid.

Rosmarinic Acid

The spectrophotometric method by Lopez-Arnaldos et al. [27] enables selective quantitation of rosmarinic acid in plant extracts by the formation of a blue-colored complex with ferrous ions. In this assay, extract at a concentration of 1 mg/ml in 0.2 M sodium acetate buffer (pH 6.0) was added with $FeSO_4$ to a final concentration of ferrous ions of 1 mM. The dye reaction was developed for 60 min. at 25°C and the absorbance was read at 572 nm. The concentration of rosmarinic acid was calculated using a molar absorption coefficient of $\varepsilon = 3.82 \times 10^3$ [27].

Statistical Analysis

All assays were done with three or more repetitions ($n \geq 3$) and repeated twice. The means were tested for statistical significance of differences with one-way ANOVA followed by *post hoc* analysis by Duncan's multiple range test (Statistica 8.0 PL, Statsoft, Poland), assuming differences to be statistically significant at $p < 0.05$. The coefficients of determination (R^2) between the antioxidant and phytochemical assays were calculated using the multiple linear regression function of Statistica.

Results

Antioxidant Activity

Free Radical Scavenging Activity

The free radical scavenging assayed by DPPH discoloration revealed a high activity of the methanolic extracts of *O. stamineus*, significantly stronger than those of *G. hederacea* (EC_{50} 8.77 ± 0.71 and 29.95 ± 4.33 μg/ml, respectively). The subsequent liquid-liquid extraction showed different distributions of activity between the solvents in both species. The *Orthosiphonis folium* MeOH extracts had significantly higher activity than the four fractions. However, three of them (DCM, EA, and BuOH) had $EC_{50} < 50$ μg/ml, with EA being the strongest (11.79 ± 1.04 μ/ml), followed by BuOH (20.76 ± 1.89 μ/ml) and DCM (41.28 ± 6.04 μ/ml). The PE fraction was slightly active (maximum 50% scavenging) only above 100 μg/ml, so EC_{50} could not be estimated within the tested range of concentrations. In contrast, for *Glechomae herba* the activity of the primary MeOH extract was fortified in the EA and BuOH fractions, for which EC_{50} was < 10 μg/ml, whereas the non-polar PE and DCM fractions were practically inactive, with a complete lack of scavenging by PE and up to 25% by DCM. Fig. 2A shows the differences expressed as $1/EC_{50}$.

P-Mo Assay

In contrast to FRS activity, the differences between the MeOH extracts in their reducing ability towards molybdenum ions were insignificant in this assay (*O. stamineus* 171.80 ± 0.54 and *G. hederacea* 170.55 ± 6.56 mg AAE/g extract). On the other hand, the liquid-liquid fractioning of *G. hederacea* resulted in similar relationships between the antioxidant activities of the fractions as in the DPPH FRS assay, with the exception that the DCM fraction was relatively active, yielding about 2/3 of the reducing power of the MeOH extract. For *Orthosiphon* there were more differences in the DPPH assay results; the EA fraction was stronger than the non-fractionated MeOH and the DCM and BuOH extracts did not differ significantly (Fig. 2B).

Inhibition of Deoxyribose Degradation

The methanol extracts of both species inhibited non-site-specific deoxyribose degradation by over 50%. The *Orthosiphon stamineus* extracts and fractions were more efficient in terms of maximum inhibition percentage, except for the BuOH fraction, which was stronger in *G. hederacea*. However, with regard to the calculated rate constant and EC_{50}, the DCM fraction from *G. hederacea* was also more efficient, despite having the lowest maximum inhibition (Fig. 2 C, D). Pro-oxidant activity was also prominent in the non-polar fractions, especially at lower concentrations of the *O. stamineus* DCM and *G. hederacea* PE fractions. In contrast, in the MeOH extracts and polar subextracts, the slightly pro-oxidant activity at the highest concentrations resulted in lowered inhibition percentage (Fig. 3). In this assay, similarly to the other two, *O. stamineus* was highly efficient in the MeOH extract (EC_{50} 0.40 μg/ml) but weaker after fractioning, except for the EA fraction, which was even stronger, reaching 84.33 ± 2.41% inhibition and an EC_{50} of 0.37 μg/ml. On the other hand, the polar fractions of *G. hederacea* were significantly stronger than the MeOH extract, reaching the remarkable EC_{50} of 0.28 μg/ml and over 70% of maximum inhibition in the BuOH fraction.

Polyphenol Screening

The content of the different polyphenol classes of all the extracts are shown in Table 1. Both species are rich in total polyphenols, total phenolic

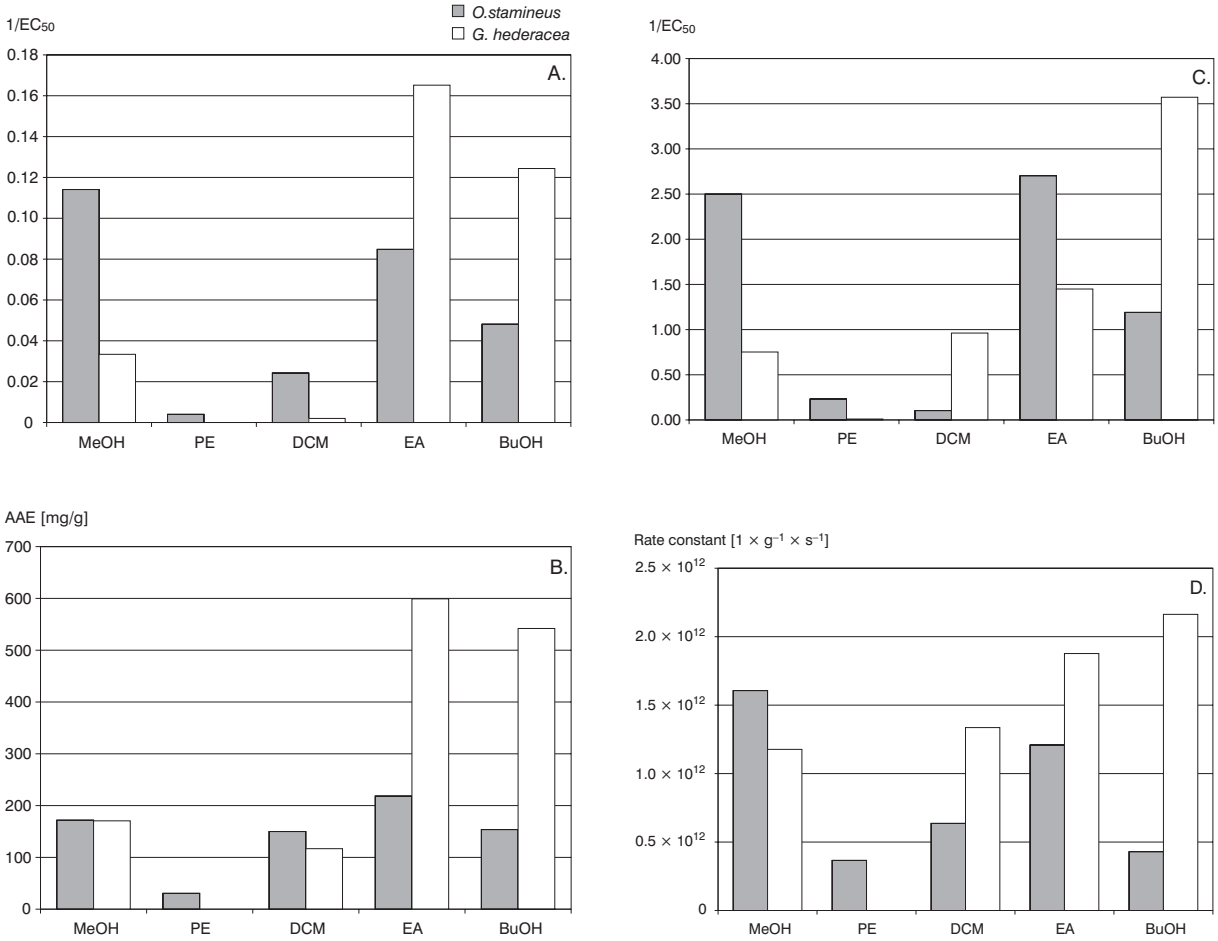

Fig. 2. The results of antioxidant assays: A) free radical scavenging activity tested by DPPH assay, expressed as $1/EC_{50}$, B) reducing power assayed by P-Mo test expressed as ascorbic acid equivalents [mg AAE/g extract], C) inhibition of deoxyribose degradation expressed as $1/EC_{50}$, D) comparison of $^{\bullet}OH$ scavenging rate constants for extracts and fractions as calculated from the deoxyribose assay dose-response curves

Ryc. 2. Wyniki testów antyoksydacyjnych: A) zmiatania wolnych rodników w teście DPPH, wyrażone jako odwrotność EC_{50}, B) zdolność redukująca badana metodą P-Mo, wyrażona jako ekwiwalenty kwasu askorbinowego [mg AAE/g ekstraktu], C) zahamowanie degradacji dezoksyrybozy wyrażone jako odwrotność EC_{50}, D) porównanie stałych szybkości reakcji zmiatania rodnika hydroksylowego dla ekstraktów i frakcji wyliczone z krzywych zależności od dawki w teście z dezoksyrybozą

acids, and rosmarinic acid. The compounds were distributed among the different solvents in various amounts. The most abundant were the polar fractions of *G. hederacea*, in which total polyphenols predominated in the fractions dry mass (60.3% in the EA and 56.6% in the BuOH fractions). The content in the MeOH extract of *O. stamineus* was higher than in *G. hederacea*, but the fractions were all less abundant in gallic acid equivalents. Moreover, the content of the non-polar solvent fractions was also substantial (3% in PE and 13% in DCM fractions). With respect to the phenolic acids, they were more abundant in the *G. hederacea* extracts than in the *O. stamineus*, but in both species, extraction with EA and BuOH significantly increased the caffeic acid equivalent content. The spectrophotometric assay for rosmarinic acid demonstrated high content of RA in both species, but it was two times higher in the *O. stamineus* MeOH extract than in the *G. hederacea*. However, LLE of *G. hederacea* enriched the EA fraction very efficiently (up to over 40% of RA in dry mass), while in *O. stamineus* the increase in RA was only about 3% (from 19.58% to 22.74%) and substantial amounts were still present in the BuOH fraction (11.42%, in *Glechoma* 4.84%).

The correlations calculated as R^2 coefficients of determination for the antioxidant versus polyphenol assays revealed differences in the contributions of each class of polyphenols with the response of the antioxidant assays (Fig. 4). The consistently highest correlation was between GAE and all three assays. On the other hand, rosmarinic acid content did not correlate with any of the antioxidant tests. Total phenolic acids correlated reasonably only with the reducing power assay (P-Mo).

Discussion

The *in vitro* antioxidant assays used in the present investigation are based on various mechanisms. A certain degree of discrepancy between the results of different tests is therefore expected. Nonetheless, both plants possess significant antioxidant potential, confirmed by all three tests.

The protection of a biomolecule against oxidative damage is of utmost importance for antioxidant defense [20, 21]. In the non-site-specific deoxyribose assay, as performed in this study, this is achieved by competitive scavenging of the hydroxyl radical, one of the most detrimental forms of ROS [20]. The generation of $^{\bullet}OH$ by the Fenton system can also be suppressed by the sequestration of iron by strong chelators, provided they are competitive to EDTA. However, the ability of the antioxidants to reduce transition metal ions, as demonstrated here by the P-Mo assay, can contribute to Fe^{2+} recycling, which accelerates $^{\bullet}OH$ production. Hence this is the likely reason for the observed pro-oxidant activity resulting either in increased TBARS formation above that of the control (e.g. in the *O. stamineus* DCM fraction) or in the inversed-U--shaped dose-response curves (Fig. 3).

The correlation analysis revealed that the results of all three assays best correlated with the gallic acid equivalent levels estimated by the Folin-Ciocalteu method. Less clear is the correlation with total hydroxycinnamic acids, which was significantly high only for the P-Mo assay. Both the Folin-Ciocalteu and the Arnow methods involve molybdenum ions, and even if the particular mechanisms of the reactions vary, one can expect correlation with the P-Mo results.

The lowest coefficients for rosmarinic acid content are obvious, since there are at least two classes of polyphenols that act as antioxidants in each species. RA determines the activity only in ethyl acetate fractions. In crude extract, the activity of RA can be either masked by less active compounds in *G. hederacea* or contribute partially to the total antioxidant status in concert with methoxyflavones in *O. stamineus*.

In general, there was no clear correlation of any of the phytochemical assays with any of the measures of deoxyribose assay, but the highest coefficient of determination was observed between GAE and the calculated rate constant of $^{\bullet}OH$ scavenging. The disturbed dose response in this assay reflects the involvement of complex mechanisms of free radical scavenging, iron reduction, or chelation. The response is additionally complicated by differences in the reaction rates in the mixtures of compounds present in the extracts.

Akowuah et al. [7–9] reported on the antioxidant activity of *O. stamineus*. The high activity of this herb extracted with water, acetone, methanol, and chloroform corroborates our results. The slightly decreased activity of LLE fractions compared with the primary MeOH extract suggests a sy-

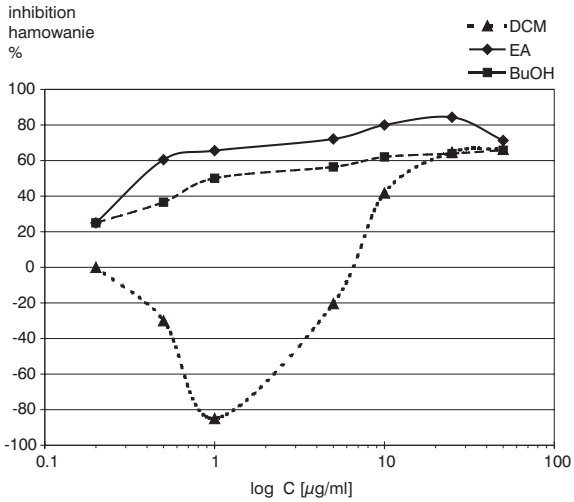

Fig. 3. An example of three different typical dose-response curves (% of TBARS inhibition) in the deoxyribose assay obtained with the DCM, EA, and BuOH fractions of *Orthposiphon stamineus*

Ryc. 3. Przykłady trzech typowych krzywych zależności od dawki (% zahamowania tworzenia TBARS) w teście z dezoksyrybozą dla frakcji DCM, EA, BuOH *Orthosiphon stamineus*

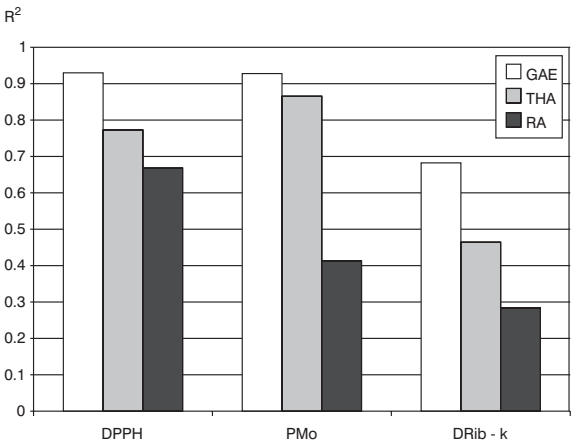

Fig. 4. A comparison of the coefficients of determination (R^2) between the antioxidant and phytochemical assays. GAE – total polyphenols by the Folin-Ciocalteu method, THA – total hydroxycinnamic acids, RA – rosmarinic acid, DRibk – deoxyribose assay $^{\bullet}OH$ scavenging rate constant

Ryc. 4. Porównanie współczynników determinacji (R^2) między testami antyoksydacyjnymi i oznaczeniami polifenoli. GAE – całkowite fenole metodą Folin--Ciocalteu, THA – całkowite pochodne hydroksycynamonowe, RA – kwas rozmarynowy, DRibk – test z dezoksyrybozą – stała szybkości reakcji z $^{\bullet}OH$

Table 1. The results of extraction and spectrophotometric assays for polyphenol content. Wherever applicable, the same superscript letters indicate a lack of significant differences by Duncan's test at $p < 0.05$. Values without a letter are significantly different from all others

Tabela 1. Wyniki ekstrakcji i spektrofotometrycznych oznaczeń zawartości polifenoli. Te same litery w indeksie górnym wskazują brak statystycznie istotnych różnic według testu Duncana przy p < 0,05. Wartości bez liter różnią się w sposób istotny statystycznie od wszystkich pozostałych

Extract/fraction (Ekstrakt/frakcja)	Species (Gatunek)	Extraction yield from 50 g of dried herb (Wydajność ekstrakcji z 50 g s.m.) [g]	Total polyphenols (Całkowite polifenole) [GAE mg/g ± *SD*]	Total hydroxycinnamic acids (Całkowite pochodne hydroksycynamonowe) [mg/g ± *SD*]	Rosmarinic acid (Kwas rozmarynowy) [mg/g ± *SD*]
Methanol (Ekstrakt metanolowy)	*Orthosiphon stamineus*	10.24	304.2 ± 2.6	190.1 ± 1.1	195.8 ± 0.9
	Glechoma hederacea	8.50	^{A}131.3 ± 5.2	^{D}123.5 ± 12.9	92.7 ± 2.0
Petroleum ether (Frakcja eteru naftowego)	*Orthosiphon stamineus*	0.40	^{C}30.3 ± 2.0	1.2 ± 1.1	N.D.
	Glechoma hederacea	0.10	N.D.	N.D.	N.D.
Dichloromethane (Frakcja chlorku metylenu)	*Orthosiphon stamineus*	4.60	^{A}129.0 ± 2.0	12.6 ± 1.2	1.4 ± 0.6
	Glechoma hederacea	1.60	^{C}25.7 ± 7.8	0.0 ± 0.4	18.8 ± 0.6
Ethyl acetate (Frakcja octanu etylu)	*Orthosiphon stamineus*	1.76	272.8 ± 8.8	^{D}121.9 ± 5.4	227.4 ± 1.5
	Glechoma hederacea	2.60	^{B}603.3 ± 36.1	378.7 ± 5.9	418.8 ± 9.4
Butanol (Frakcja butanolowa)	*Orthosiphon stamineus*	2.80	150.1 ± 3.6	167.5 ± 3.6	114.2 ± 0.1
	Glechoma hederacea	3.04	^{B}565.6 ± 28.4	267.2 ± 22.7	48.4 ± 1.9

nergistic or cumulative action of compounds differing in polarity. Indeed, depending on the solvent, either rosmarinic acid or lipophilic methoxylated flavones prevail, as confirmed by Akowuah et al. [8], who found the latter compounds predominating in $CHCl_3$ extract in which RA was absent. In the present study the considerable activity of the DCM fraction likely results from the content of lipophilic flavones such as sinensetin, eupatorin, and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone [8]. This has not been observed in the other studied species, where hydrophilic compounds play a major role in determining antioxidant capacity, as illustrated by the enormous increase in activity following partitioning to EA and BuOH. In the EA extract of both plants, rosmarinic acid was the major antioxidant, but only in the *G. hederacea* EA extract did it seem to be the single determinant of activity, while in the *G. hederacea* BuOH fraction the activity depended on other polyphenols reacting with Folin-Ciocalteu and Arnow reagents. Butanol is the preferred solvent for some tannoid compounds or phenolic glycosides. Interestingly, Kumarasamy et al. [11] reports the highest $^{\bullet}$DPPH scavenging activity in n-hexane extract, followed by DCM and MeOH, quite contradictory to the present study in which the *G. hederacea* PE and DCM fractions were inactive. This could be explained by the different extraction approaches, since in the mentioned paper the solvent extracts seemed to have been prepared independently, hence the possibility of efficient extraction of some isoprenoid antioxidants such as xanthophylls with nonpolar solvents. However, the usually low extraction yield obtained with hydrocarbon solvents suggests the minor role of such compounds in the total antioxidant capacity of the herb.

Rosmarinic acid is a characteristic compound in the *Nepetoideae* subfamily and is therefore a major antioxidant in the plants under study. In *G. hederacea*, partitioning with EA enables enrichment of its content and could be used as the initial step for RA purification for use as an antioxidant. The composition of the equally active BuOH extract requires elucidation by further phytochemical study. Yamauchi et al. [16] reported the isolation of luteolin and apigenin glucosides in addition to several other phenol glycosides from the

n-butanol fraction of MeOH extract. Luteolin glycosides from various *Lamiaceae* species are known for their antioxidant activity [28].

The author concluded that the two studied Nepetoideae plants are rich in polyphenolic antioxidants. One fundamental difference between them is the relationship between the various classes of compounds, as revealed by liquid-liquid extraction of the methanolic extract. In *O. stamineus* there are two putative groups, separated into non-polar and polar solvents, determining the total antioxidant status. These are the polar depside rosmarinic acid and highly methoxylated lipophilic flavones, the activities of which are added in the crude extract, which should be recommended for use instead of purified fractions. On the other hand, the *G. hederacea* methanolic extract has relatively lower activity than the other species, but fractioning into ethyl acetate and n-butanol enhances the antioxidant potential enormously. The excellent separation of rosmarinic acid into an EA fraction and other polyphenols into BuOH provides an easily available source of different natural antioxidants to be used as food additives or chemopreventive supplements. These properties should encourage more studies to confirm the benefits of using this somehow neglected but widespread and valuable medicinal plant.

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