Chromatographic and spectrophotometric analyses of the DPPH free radical scavenging activity of the fractionated extracts from *Lamium album* L., *Lamium purpureum* L. and *Viscum album* L.

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S u m m a r y

Chloroform, butanolic and water fractions from the methanolic extracts of *Lamium album* L. and *L. purpureum* L. flowers and different organs of mistletoe (*Viscum album* L.), namely leaves, stalks and fruits were investigated for the free radical scavenging properties by using colour free radical DPPH$^{\bullet}$ as a stain reagent for dot-blot assay on a TLC plate and two-dimensional thin-layer chromatography (2D-TLC) analysis (2D-TLC-DPPH test) as well as a dye reagent for the spectrophotometric assay. For each plant material, butanolic fractions showed the strongest activity, of which those of the *Lamium* species were nearly equal to that of the known antioxidant - BHA. According to 2D-TLC chromatography, the phenolic compounds present were responsible for the antiradical activity of the fractions.

Key words: Lamium album, Lamium purpureum, Viscum album, TLC, DPPH, antiradical activity

Lamium album L. and *Lamium purpureum* L. (*Lamiaceae*) grow wild in Poland, but only the former is used in phytotherapy. It provides a medicinal material - *Flos Lamii albi*, which is used for treatment of inflammatory conditions of mucous membranes, painful and excessive menstruation, as well as infections of the upper respiratory tract [1].

Viscum album L. (*Loranthaceae*), a semi-parasite of the leafy and coniferous trees, provides a medicinal material, *Herba Visci*, with very wide spectrum of pharmacological activity. The most important in therapy is its anti-neoplastic and blood pressure lowering activity [1, 2, 3]. Moreover, it is also applied as an anti-arteriosclerotic, an analgesic and a calmative remedy [2].

The flowers of *L. album* L., *L. purpureum* L. and shoots of *Viscum album* L. contain a variety of phenolic compounds expected to posses antioxidative properties [2, 4-8]. In the family *Lamiaceae* the antiradical properties were already found in other species due to the presence of rosmarinic acid, derivatives of hydroxycynammic acids and other phenolic compounds [9].

It is generally accepted that antioxidative properties of the plant constituents represent an important factor of their pharmacological activity.

The aim of this work was investigation of the antioxidative properties of the fractionated extracts from *Lamium album* and *Lamium purpureum* flowers and *Viscum album* leaves, stalks and fruits, by analysis of their ability to scavenge a synthetic free radical DPPH$^{\bullet}$ of intensive violet colour by means of chromatography and spectrophotometry.

MATERIAL AND METHODS

Plant material

The flowers (exactly corollas with adhered stamens) of *L. album* L. and *L. purpureum* L. were collected directly from intact plants in 2002 in Jerzykowo (Poland). The herb of *V. album* L. (parasiting on black poplar *Populus nigra* L.) was harvested in 2003 near Nowe Miasto nad Wartą (Poland) and divided into leaves, stalks and fruits. All the material was air-dried at room temperature except for fruits, which were dried at 40°C.

Extraction

Flowers of *L. album* (4.80g) and *L. purpureum* (0.95g), and leaves (37.60g), stalks (89.40g) and fruits (4.80g) of *V. album* were extracted separately three times with MeOH. The concentrated extracts were partitioned into chloroform, 1-butanol and water soluble fractions.

Chromatographic analysis for phenolics

Samples of each fraction were dissolved in an appropriate solvent - chloroform (chloroform fractions), methanol (butanolic fractions) or 50% ethanol (water fractions) at the concentration 0.1g/ml and analysed for presence of phenolic compounds by 2D-TLC on pre-coated cellulose plates (Merck) in 1-butanol-acetic acid-water 4:1:5 (v/v/v) (first dimension) and acetic acid-water 15:85 (v/v) (second dimension) as mobile phases. The developed chromatograms were visualised under $UV_{365\ nm}$ irradiation before and after spraying with 1% $AlCl_3$ in ethanol followed by heating or with 0.1% 2-aminoethanol diphenylborate (NA) in ethanol. The brown, yellow or blue spots

changing to yellow fluorescence with $AlCl_3$ were considered to correspond to flavonoids, whereas blue spots were considered to be those of phenolic acids derivatives (e.g. caffeic acid conjugates) or other phenolics [4].

TLC-DPPH tests

The significantly modified methods described by Choi et al. [13] were employed. 2D-TLC chromatograms prepared as described above, with marked spots detected under UV, (2D-TLC-DPPH test) or silica gel plates (dot-blot TLC plate test) with spotted methanolic solutions (concentration 1.0 mg/ml) in volumes corresponding to the amounts of 1, 2, 5 and 10 μg of each fraction and reference compounds like *tert*-butylhydroxyanisole (BHA) and quercetin, were sprayed with $DPPH^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl radical) (Aldrich) 600 μM methanolic solution and observed after 30 minutes in day light. The compounds with antiradical activity appeared as yellow spots against the purple-blue background.

DPPH spectrophotometric assay

The method used to determine the free radical scavenging activity was adapted from that described by Gao et al. [10]. 0.2 ml samples of solutions of investigated fractions in a suitable solvent (chloroform fractions in chloroform, butanol fractions in methanol, water fractions in 50% ethanol) or BHA (in methanol) (as a positive control) with concentrations (20, 40, 60, 80, 100, 250, 500 and 1000 μg/ml) or solvent itself (as a negative control) were mixed with 2.8 ml of $DPPH^{\bullet}$ 100 μM solution in methanol. After 30 min. the absorbance was measured at the wavelength 517 nm on a Specord M-40 spectrophotometer (Zeiss, Jena, Germany). Every measurement was repeated three times. The free radical $DPPH^{\bullet}$ scavenging (i.e. reduction) activity was calculated from the equation: Activity [% of DPPH reduction] = $[(A-A_x) / A] \times 100\%$, where A – absorbance of $DPPH^{\bullet}$ solution with methanol, A_x - absorbance of a DPPH solution with a tested fraction solution (test) or BHA (positive control) solution. The antiradical activity SC_{50}, defined as the concentration of a sample showing 50% DPPH radical scavenging activity, was determined from a graph in which concentration and reduction activity were plotted against each other (Fig. 1) considering the 15-fold dilution of the tested fraction sample in a cuvette.

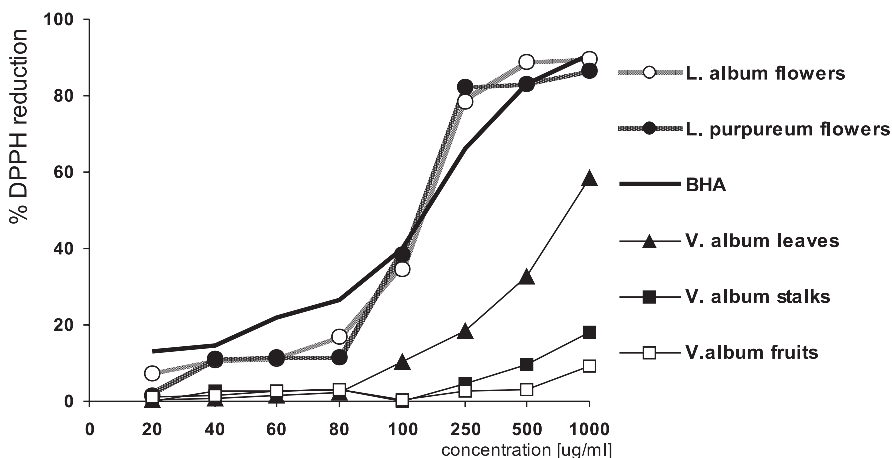

Figure 1. DPPH scavenging activity spectrophotometric assay of various concentrations of butanolic fractions from *L. album*, *L. purpureum* and *V. album*.

RESULTS

The concentrated methanolic extracts were partitioned into chloroform, butanol and water soluble fractions. Each fraction was examined by 2D-TLC on cellulose using UV light visualization before and after spraying with $AlCl_3$ or aminoethanol diphenylborate (NA) reagents to determine the complexity of phenolic compounds present as well as a free radical DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl radical) spray reagent to detect spots of components with antiradical activity.

The butanolic fractions from each plant material were found to be the richest in phenolic compounds. In turn, water fractions showed poor presence of such constituents, while chloroform fractions were deprived of them. From the appearance of spots in $UV_{365\ nm}$ light before and after spraying with reagents ($AlCl_3$ and NA) one may suggest that a majority of them responded to flavonoids and cinnamic acids derivatives [4], which otherwise are well known for the antiradical properties [8, 13].

When 2D TLC chromatograms were sprayed with DPPH$^{\bullet}$ solution, the most compounds reacting with DPPH$^{\bullet}$ radical (discolouration of violet colour) were found in the butanolic fractions from both *Lamium* species as well as stems or leaves of *Viscum*. In a case of water fractions the antiradical active spots were found for *L. album* and weaker ones for *L. purpureum* and *V. album*. Chloroform fractions did not contain active compounds with an exception of chloroform fractions from leaves and fruits of *Viscum*, however, with rather weak antiradical response – not higher than 24%.

The 2D-TLC-DPPH tests suggested that phenolic compounds like flavonoids and caffeic acid derivatives are responsible for the antiradical properties of the butanol fractions being the most active from each plant material studied.

Semi-quantitative determination of the antiradical activity of fractions was carried out by the dot-blot assay on the TLC plates (TLC-DPPH test). The known amounts of fractions and reference compounds (BHA and quercetin) were spotted onto silica gel plates, which were sprayed with a DPPH• solution. TLC-DPPH test on silica gel plates also confirmed the strongest antioxidative activity in the case of the butanolic fractions from each species studied.

The DPPH-scavenging activity of each fraction was measured quantitatively by spectrophotometric determination of reduction of DPPH• absorption at 517 nm in the presence of tested plant fractions. In the investigated range of concentrations (20-1000 μg/ml), the significant DPPH• antiradical activity was shown by butanolic fractions (Fig. 1, Table 1 and 2) especially those from *L.album* (7.4% - 89.6%) and *L.purpureum* (1.6% - 86.6%), which was comparable to that of the known antioxidant - BHA (12.9 - 90,7%). The respective SC_{50} values (see Table 2) were 9.9, 9.8 and 9.1μg/ml. In turn, water fractions exerted low activity (e.g. *L. album* – maximum 17.7%, *L.purpureum* – maximum 12.2%), while chloroform fractions appeared to be inactive. Also butanolic fractions from various organs of *V.album* showed noticeable, although weaker than BHA, anti-DPPH• activity, which decreased in an order: stems (1.73% - 69.7%), leaves (0.5% - 58.3%) and fruits (0% - 26.4%). Water and chloroform fractions of *V.album* were much less active; the highest respective values -18.1% and 24.0% were found for fractions from stems.

Table 1

DPPH• scaveging activity (in %)* of butanolic fractions from *L.album*, *L.purpureum* and *V. album* (graphically presented in fig.1).

substance or fraction	concentration (μg/ml)							
	20	40	60	80	100	250	500	1000
Lamium album								
flowers	7.47 ±0.10	10.91 ±0.24	11.03 ±0.42	17.09 ±0.07	34.48 ±0.02	78.48 ±0.14	88.75 ±0.01	89.62 ±0.02
Lamium purpureum								
flowers	1.67 ±0.20	11.25 ±0.08	11.73 ±0.45	11.48 ±0.07	38.60 ±0.13	82.29 ±0.78	83.02 ±0.01	86.60 ±0.12
Viscum album								
leaves	0.50 ±0.18	0.81 ±0.09	1.71 ±0.06	2.42 ±0.27	10.21 ±0.07	18.43 ±0.01	32.65 ±0.15	58.33 ±0.10
stalks	1.73 ±0.40	6.10 ±0.24	6.50 ±0.32	11.02 ±0.24	11.25 ±0.15	19.48 ±0.16	39.74 ±0.07	69.75 ±0.11
fruits	-1.41 ±0.26	0.95 ±0.13	10.03 ±0.63	7.41 ±0.35	5.83 ±0.04	8.10 ±0.07	19.06 ±0.07	26.44 ±0.08
BHA (reference)	12.95 +0.05	14.48 ±0.05	21.86 ±0.07	26.60 ±0.07	40.15 ±0.11	66.31 ±0.06	83.20 ±0.06	90.76 ±0.17

* mean values ± standard deviation of triplicate measurements

Table 2

Concentrations of butanolic fractions and a reference antioxidant BHA sufficient to obtain 50% of DPPH reduction (SC_{50} values).

fraction or substance	SC_{50} (μg/ml) *	(fraction : DPPH mass ratio)
Lamium album		
flowers	9.9	(0.160:1)
Lamium purpureum		
flowers	9.8	(0.158:1)
Viscum album		
leaves	> 66	(1.065:1)
stalks	> 66	(1.065:1)
fruits	> 66	(1.065:1)
BHA (reference)	9.1	(0.147:1)

* 15-fold dilution in a cuvette was considered

DISSCUSION

Synthetic stable free radical DPPH• has intensive violet colour which vanishes upon acceptance of an electron from other compounds thus considered to have radical scavenging properties. Thanks to these properties DPPH• has been very frequently applied for antioxidative determination of plant extracts and isolated metabolites by chromatographic and spectrophotometric methods for many years [12]. Among investigated plant species the highest antiradical effect was exerted by *L. album* but that of *L. purpureum* was only slightly lower and for both species it was almost equal to that of the known antioxidant BHA. In turn, *Viscum album* exhibited rather low antiradical properties. Previous investigations of plant extracts showed that their power of antioxidative activity correlates with the level of total phenolic compounds [10, 11]. No doubt, such phenol compounds like flavonoids and caffeic acid esters have significant antioxidative properties as has been already shown with the pure isolated compounds [9, 12, 13].

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CHROMATOGRAFICZNE I SPEKTROFOTOMETRYCZNE ANALIZY AKTYWNOŚCI ZMIATANIA WOLNEGO RODNIKA DPPH PRZEZ FRAKCJONOWANE EKSTRAKTY Z *LAMIUM ALBUM* L., *LAMIUM PURPUREUM* L. I *VISCUM ALBUM* L.

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S t r e s z c z e n i e

Frakcje chloroformowe, butanolowe i wodne ekstraktów metanolowych z kwiatów *Lamium album* L. i *Lamium purpureum* L. oraz różnych organów *Viscum album* L. (liście, łodygi, owoce) badano pod względem właściwości zmiatania wolnych rodników, stosując barwny wolny rodnik DPPH$^{\bullet}$ jako odczynnik do testów plamowych na płytkach TLC, analizy metodą dwukierunkowej chromatografii cienkowarstwowej (2D-TLC) oraz oznaczeń spektrofotometrycznych. W wypadku każdego materiału roślinnego najsilniejszą aktywność wykazały frakcje butanolowe, z gatunków *Lamium* prawie równą aktywności znanego antyoksydantu – BHA. Z rezultatów chromatografii cienkowarstwowej dwukierunkowej 2D-TLC wynika, że za aktywność przeciwrodnikową frakcji odpowiedzialne są obecne w nich związki fenolowe.

Słowa kluczowe: Lamium album, Lamium purpureum, Viscum album, TLC, DPPH, aktywność przeciwrodnikowa