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Antioxidant Activity of Hydrolyzed and Non-Hydrolyzed Extracts of the Inflorescence of Linden (*Tiliae inflorescentia*)*

Aktywność przeciwutleniająca hydrolizowanych i niehydrolizowanych wyciągów z kwiatostanów lipy (*Tiliae inflorescentia*)

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Abstract

Background. Dry extracts of linden tree inflorescence modified by alkaline degradation (L3) and enzymatic hydrolysis of the glycosidic moiety (L4) as well as unmodified ethyl acetate (L2) and crude methanol (L1) extracts were investigated for their phenolic compound content and antioxidant activity.
Objectives. The aim was to examine whether modification of extracts of the inflorescence of linden by alkaline degradation or enzymatic hydrolysis influences the antioxidant properties of the extracts and the phenolic compounds present in them. An original method for calculating antioxidant activity was used with a defined antioxidant activity unit.
Material and Methods. The amount of phenolic compounds in the extracts was determined by liquid chromatography (HPLC). The antioxidant activity of the extracts was investigated by comparing the level of thiobarbituric acid-reactive substances in the presence of extract and in a control sample without extract.
Results. The highest number of antioxidant units ($TAU_{535/mg}$) per 1 mg of extract was observed for the non-hydrolyzed ethyl acetate extract (L2) and lowest for the crude methanol (L1) extract. The highest number of $TAU_{535/ph}$ units per 1 mg of phenolic compounds was in the extract hydrolyzed under alkaline conditions (L3) and the lowest in the enzymatically hydrolyzed extract (L4).
Conclusions. Alkaline degradation of glycosides (but not enzymatic hydrolysis) leading to an increased concentration of aglycones enhanced the antioxidant activity of the extracts and the antioxidant efficiency of phenolic compounds (**Adv Clin Exp Med 2009, 18, 4, 329–335**).

Key words: *Tiliae inflorescentia*, linden extracts, lime extracts, antioxidant activity, phenolic compounds.

Streszczenie

Wprowadzenie. Suche wyciągi: zmodyfikowany za pomocą degradacji alkalicznej (L3) i hydrolizy enzymatycznej części cukrowej (L4), a także niezmodyfikowany wyciąg do octanu etylu (wyciąg octanowy) (L2) i wyjściowy wyciąg metanolowy (L1), otrzymane z kwiatostanów lipy, zostały zbadane pod względem zawartości związków fenolowych i aktywności przeciwutleniającej.
Cel pracy. Określenie, czy modyfikacja wyciągów z kwiatostanów lipy za pomocą degradacji alkalicznej lub hydrolizy enzymatycznej wpłynie na przeciwutleniające właściwości wyciągów oraz związków fenolowych zawartych w tych wyciągach. W pracy zastosowano oryginalny sposób obliczania aktywności przeciwutleniającej za pomocą zdefiniowanej jednostki aktywności przeciwutleniającej.
Materiał i metody. Ilość związków fenolowych w wyciągach oznaczono metodą chromatografii cieczowej HPLC. Aktywność przeciwutleniającą wyciągów badano, porównując stężenie substancji reagujących z kwasem tiobarbiturowym w obecności wyciągów i w próbach kontrolnych (bez wyciągów).
Wyniki. Najwięcej jednostek ($TAU_{535/mg}$) obliczono dla 1 mg niehydrolizowanego wyciągu octanowego (L2), a najmniej dla 1 mg wyjściowego wyciągu metanolowego (L1). Najwięcej jednostek $TAU_{535/ph}$ obliczono dla 1 mg związków fenolowych w wyciągu octanowym poddanym degradacji alkalicznej (L3), a najmniej dla 1 mg związków fenolowych w wyciągu octanowym poddanym hydrolizie enzymatycznej (L4).

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Wnioski. Można stwierdzić, że alkaliczna degradacja glikozydów (nie enzymatyczna hydroliza) prowadząca do wzrostu stężenia aglikonów prowadzi do zwiększenia przeciwutleniającej aktywności wyciągów i przeciwutleniającej skuteczności związków fenolowych (**Adv Clin Exp Med 2009, 18, 4, 329–335**).

Słowa kluczowe: *Tiliae inflorescentia*, wyciągi z lipy, aktywność przeciwutleniająca, związki fenolowe.

The inflorescence of the linden (*Tiliae flos* Ph. Eur.), originating from two strains, i.e. the small-leaved (*Tilia cordata* Miller) and the large-leaved linden (*Tilia platyphyllos* Scop), was used. The medical use of this material is to relieve cough during inflammation of the respiratory tract resulting from mucilage coating the mucosa. A diaphoretic action was also noted, but no specific constituents have been found responsible for this activity. In folk medicine, the linden flower is used as a diuretic, stomachic, antispasmodic, and sedative agent [1]. The inflorescence of the linden contains between 3 and 10% polysaccharides which are mucilaginous. Furthermore, condensed tannins [2] (strong antioxidants), such as dimers of procyanidin (B-2), were identified in this raw material. Other phenolic compounds identified in linden flowers are flavonoids, mainly quercetin glycosides (rutin, quercitrin and isoquercitrin), kaempferol glycosides [3], and phenolic acids [4] (caffeic, *p*-coumaric, and chlorogenic acids).

Free radicals and reactive oxygen species (ROS) have been implicated in many human pathological conditions, including rheumatoid arthritis [5], hemorrhagic shock [6], cardiomyopathy [7], cystic fibrosis [8], and gastrointestinal ischemia [9–11]. A high concentration of ROS leads to the destruction of cell membranes [12], proteins, and nucleic acids [13, 14], which is dangerous because it leads to carcinoma formation [15]. Plant extracts rich in phenolic compounds with antiradical activity [16, 17], such as extracts from the linden, could be used as medicines or preventive agents protecting humans and other animals from the destructive action of free radicals and ROS.

In this study a methanol extract from linden tree inflorescences was obtained. Part of the extract was subjected to modification of the phenolic fraction by means of enzymatic hydrolysis or alkaline degradation. The modified and unmodified extracts were further investigated for their phenolic compound content and antioxidant activity.

The main aim was to observe whether modification of the phenolic fraction leads to an increase in the antioxidant properties of the extracts.

Material and Methods

Raw Material

The inflorescence of linden (*Tiliae inflorescentia* from two species: *Tilia cordata* Mill. and *Tilia platyphyllos* Scop., *Tiliaceae*) was obtained from "Herbapol" Lublin S.A. Poland. A voucher specimen (Herbarium No. ZS-9) was deposited at the Department of Pharmacognosy, Wroclaw Medical University, Wrocław, Poland

Reagents

Acetonitrile, methanol, ethyl acetate, and rutin (quercetin 3-O-rutinoside) were from Merck, Germany, HCl and acetic acid from Polish Chemical Reagents, Poland, Tris (tris(hydroxymethyl)aminomethan), TBA (thiobarbituric acid) from Loba Feinchemie, Austria, Tween 40 (polyoxyethylenesorbitan monopalmitate) from Sigma-Aldrich, USA, rapeseed oil from the Industry of Fats EWICO Sp. z o.o., Poland, tannin from Serva Feinbiochemica, Germany, trichloroacetic acid (TCA) from Ubichem, UK, protocatechuic (3,4-dihydroxybenzoic) acid, quercitrin (quercetin 3-O-rhamnoside), isoquercitrin (quercetin 3-O-glucoside), kaempferol, kaempferol 3-O-glucoside, and kaempferol 3-O-rutinoside from Extrasenthese, France, quercetin and β-glucosidase from almonds from Fluka, Switzerland, and sodium borohydride ($NaBH_4$) from BDH Chemicals Ltd, UK. The compound isolated and identified in this department was quercetin 3,7-*O*-dirhamnoside.

Preparation of Extracts

Dried linden tree inflorescence (80 g) was exhaustively extracted with 1600 ml of methanol in a Soxhlet apparatus at 70°C. Then the methanol was evaporated at 40°C under reduced pressure and the dry extract was dissolved in 1600 ml of hot water. The aqueous solution was stored for 2 days at 4°C, then filtered with a Filtrak 388 filter and the precipitate discarded. The solution was divided into equal 400-ml portions. The first was dried under reduced pressure to obtain extract L1 (3.34 g). The second was extracted with ethyl acetate (6 × 100 ml) and then the ethyl acetate extract was dried under reduced pressure to obtain L2 (0.396 g).

The third was subjected to alkaline degradation of the glycosidic part under reducing conditions (pH 11, 15 min., 50°C, 1 g $NaBH_4$). After degradation, the solution was adjusted to pH 7 (HCl) and extracted (7 × 100 ml) with ethyl acetate. The ethyl acetate extract was then evaporated to dryness under reduced pressure to obtain L3 (0.319 g). The fourth portion was subjected to enzymatic hydrolysis (pH 5, β-glucosidase from almonds: 6 units/mg, Fluka, Switzerland, 6 h, 35°C). One unit of β-glucosidase activity corresponds to the amount of enzyme which liberates 1 µmol of glucose per minute at pH 5 and 35°C. To stop the reaction, the solution was heated at 100°C for 5 min (enzyme denaturation). After cooling, the solution was extracted with ethyl acetate (7 × 100 ml). The ethyl acetate extract was concentrated to dryness under reduced pressure to obtain L4 (0.371 g). All the extracts (L1, L2, L3, and L4) were analyzed for their phenolic compound content (by HPLC) and antioxidant activity.

High-Performance Liquid Chromatography Analysis of the Phenolic Compounds

Extracts L1, L2, L3, and L4 were dissolved in HPLC gradient-grade methanol at 2 mg/ml and filtered through membrane filters (Millipore, 0.22 mm). A high-performance liquid chromatograph (Knauer, Berlin, Germany) was equipped with type 64 HPLC pumps and a UV-VIS detector (254 nm). A LiChrospher 100, RP-18 (5 µm) column (250 × 4 mm) (Merck) was used. The flow rate was 1 ml/min and the injection volume 20 µl. The analyses of the phenolic compounds were carried out at room temperature with a gradient of acetonitrile (A) in 2% acetic acid in water (B) according to Oszmiański et al. [18], i.e. from minutes 0 to 10 from 3 to 10% (A) in (B), minutes 10 to 24 from 10 to 14% (A) in (B), minutes 25 to 44 from 14 to 40% (A) in (B), and minutes 44–45 from 40 to 100% (A) in (B). The identification and quantitative analyses were done during extract analysis by measuring and comparing the areas and retention times of the identified peaks with those of the peaks of standards.

Measurement of Antioxidant Activity

Antioxidant activity was measured according to Buege and Aust [19]. The oxidation process was started with irradiation by a bactericidal UV lamp of wavelength 254 nm (LB-301.1, BakMed, Łódź) with a light intensity of 2.1 mW/cm^2.

TBA Reagent

The TBA reagent was prepared as follows: A) 375 mg of TBA was dissolved in 30 ml of hot water. After cooling, 40 mg of tannin was added. B) 15 g of TCA was dissolved in 68 ml of water. Then 2 ml of 10 M HCl was added. Before the experiment, 30 ml of A was added to 70 ml of B.

Preparation of the Emulsion

Ninety µl of rapeseed oil was emulsified in 35 ml of Tween 40 (4 mg/ml) in 0.2 M Tris-HCl buffer, pH 7.4.

Sample Preparation

Four 8-ml samples were prepared as follows: The control samples K_0 and K_1 were prepared to control oil stability without and with the extract, respectively, in the absence of an oxidizing agent. K_0 is the non-irradiated sample without extract and K_1 the non-irradiated sample containing 0.15 mg/ml (0.5 mg/ml for L1) extract. S_0 is the irradiated sample without extract and S_1 the irradiated sample containing 0.15 mg/ml (0.5 mg/ml for L1) extract.

Measurement of Antioxidant Activity

Oxidation was started by UV 254 nm irradiation. The samples were incubated at 25°C for 60 min. Aliquots (0.5 ml) of the samples were taken at time 0 (start of the reaction) and after 60 minutes of irradiation and added to 2 ml of TBA reagent. Then these samples were heated at 100°C (boiling water bath) for 20 min. After cooling, the samples were centrifuged (1000 × *g*, 5 min) and the absorbance was measured in a 1-cm glass cuvette at 535 nm (Carl Zeiss Jena, Spekol 11). The antioxidant activity was given in the units defined below.

One unit of antioxidant activity (TAU_{535}) corresponds to the amount of substance in 1 ml of sample which causes a decrease in the absorbance (A_{535}) of 1 in comparison with the control sample (without substance) under the test conditions described in this study. The number of antioxidant units was calculated per mg of extract ($TAU_{535/mg}$) and mg of phenolic compounds ($TAU_{535/ph}$). One can interpret the $TAU_{535/ph}$ value as the antioxidant efficiency of phenols.

$$TAU_{535/mg} = \frac{A_0 - A_e}{m_e},$$

where $TAU_{535/mg}$ is the number of antioxidant units

in 1 mg of extract, A_0 the difference between the absorbance of sample S_0 and that of the control sample K_0, A_e the same difference between sample S_1 and control sample K_1, and m_e the weight of extract [mg] in 1 ml of sample.

$$TAU_{535/mg} = \frac{TAU_{535/mg}}{m_{ph}} ,$$

where $TAU_{535/ph}$ is the number of antioxidant units calculated per mg of phenolic compounds, $TAU_{535/mg}$ the number of antioxidant units calculated per mg of extract, and m_{ph} the amount of phenolic compounds [mg] in 1 mg of extract.

Results and Discussion

The quantitative and qualitative analyses of the phenolic compounds were done by HPLC. Identification was made on the basis of the retention times of the compounds in comparison with standards, whereas the amounts of the compounds were calculated on the basis of the peak areas and compared with those of the standards. The results are shown in Tab. 1 and Figs. 1 and 2. The number of antioxidant units calculated per mg of extract ($TAU_{535/mg}$) and mg of phenolic compounds ($TAU_{535/ph}$) are given in Figures 3 and 4 and Table 2. The inflorescence of the linden is a raw material with high antioxidant activity, as was observed earlier during a screening test in this laboratory [20].

The aim was to develop techniques to modify the glycosidic moiety of flavonoids identified in linden flower extracts and to study the influence of these modifications on the antioxidant potential of the extracts. Two techniques were chosen: 1) a mild enzymatic (β-glycosidase) hydrolysis method and 2) a more invasive method, leading, under alkaline conditions, to complete degradation of the glycosides. Extract L1 contained a small amount of phenolic compounds compared with the other extracts (Tab. 1, Fig. 1a). The dominant fraction was glycosidic derivatives of kaempferol, such as kaempferol 3-*O*-rutoside (4.5% w/w) and kaempferol 3-*O*-glucoside (2.1% w/w); the concentrations of rutin and isoquercitrin were 1.6% (w/w) and 1.0% (w/w), respectively. The total phenolic content of L1 (Tab. 1) was the lowest of all the extracts (9.6% w/w). Extract L2 was the ethyl acetate extract which was not subjected to alkaline or enzymatic modification. The total phenol content was 55.6% (w/w) (Tab. 1). The glycoside content was 50% (w/w) and that of aglycones (kaempferol and quercetin) 3% (w/w).

L3 and L4 were ethyl acetate extracts which were subjected to modification of the gylcosidic moiety, under severe conditions in the case of L3 and mild conditions in the case of L4. The total phenol content (Tab. 1) in L3 (26% w/w) was much lower than in L2 (55.6% w/w), the glycoside

Table 1. The amount of phenolic compounds measured with HPLC expressed in percentages in extracts L1, L2, L3 and L4 calculated per weight of extract

Tabela 1. Ilość związków fenolowych oznaczonych metodą HPLC przeliczona na masę wyciągów L1, L2, L3 i L4

Phenolic compound (Związek fenolowy)	Amount of phenolic compounds calculated per weight of extract (Ilość związków fenolowych przeliczonych na wagę wyciągu) [m_{ph} × 100]			
	L1	L2	L3	L4
1. Protocatechuic acid	0.3	2.1	2.1	1.9
2. Rutin	1.6	2.2	0.5	4.8
3. Isoquercitrin	1.0	5.9	1.5	5.2
4. Quercetin 3.7-*O*-dirhamnoside	0.076	0.25	–	0.20
5. Kaempferol 3-*O*-rutoside	4.5	28.3	2.5	29.0
6. Kaempferol 3-*O*-glucoside	2.1	13.4	2.4	14.0
7. Quercitrin	0.07	0.41	–	0.44
8. Quercetin	–	1.28	6.0	1.3
9. Kaempferol	–	1.71	11.0	3.3
Total (Suma)	9.6	55.6	26.0	60.1

– traces amount.

– ilości śladowe.

Table 2. Antioxidant activity of extracts from flowers of linden demonstrated as number of units TAU_{535}. Antioxidant activity was measured as ability of extracts for decreasing rapeseed oil peroxidation. The UV 254 nm was used as factor to initiation of oil peroxidation. $TAU_{535/mg}$ is the number of antioxidant units calculated per 1 mg of extracts; $TAU_{535/ph}$ is the number antioxidant units calculated per 1 mg of phenolic compounds

Tabela 2. Przeciwutleniająca aktywność wyciągów z kwiatostanów lipy przedstawiona jako liczba jednostek TAU_{535}. Aktywność przeciwutleniająca została określona jako zdolność wyciągów do zmniejszenia peroksydacji oleju rzepakowego. Do zainicjowania peroksydacji oleju zastosowano światło ultrafioletowe UV 254 nm. $TAU_{535/mg}$ jest ilością jednostek przeciwutleniających przeliczonych na 1 mg wyciągów; $TAU_{535/ph}$ jest ilością jednostek przeciwutleniających przeliczonych na 1 mg związków fenolowych

Antioxidant activity (Aktywność przeciwutleniająca)		
Extract (Wyciąg)	$TAU_{535/mg}$	$TAU_{535/ph}$
L1	0.350 ± 0.047	3.398 ± 0.460
L2	1.155 ± 0.262	2.078 ± 0.472
L3	1.222 ± 0.154	4.968 ± 0.625
L4	1.089 ± 0.147	1.812 ± 0.244

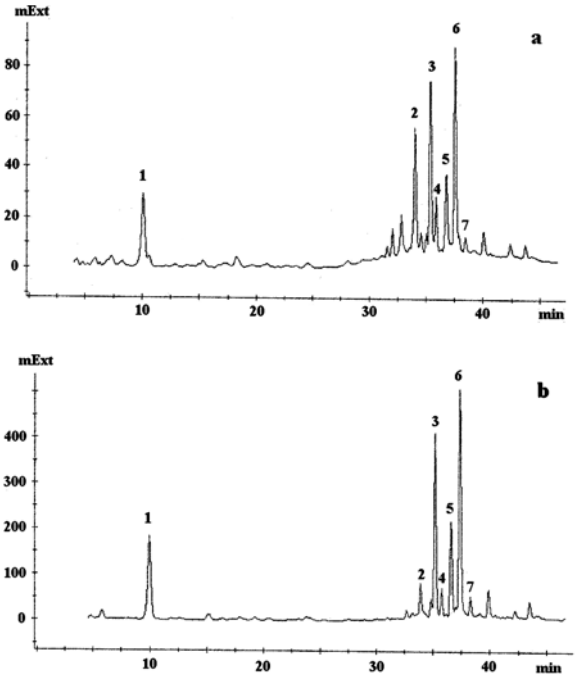

Fig. 1. The HPLC chromatogram analysis of phenolic compounds in extracts L1 (a) and L2 (b) obtained from inflorescence of linden (*Tiliae inflorescentia*) carried out at 254 nm. Identified compounds are marked with the following numbers: 1 – protocatechuic acid, 2 – rutin, 3 – isoquercitrin, 4 – quercetin 3,7-dirhamnoside, 5 – kaempferol 3-rutinoside, 6 – kaempferol 3-glucoside, 7 – quercitrin

Ryc. 1. Chromatogram HPLC analizy związków fenolowych w wyciągach L1 (a) i L2 (b) otrzymanych z kwiatostanów lipy (*Tiliae inflorescentia*) wykonany przy długości fali 254 nm. Zidentyfikowane substancje zostały zaznaczone następującymi numerami: 1 – kwas protokatechowy, 2 – rutyna, 3 – izokwercetyna, 4 – 3,7-diramnozyd kwercetyny, 5 – 3-rutozyd kemferolu, 6 – 3-glukozyd kemferolu, 7 – kwercytryna

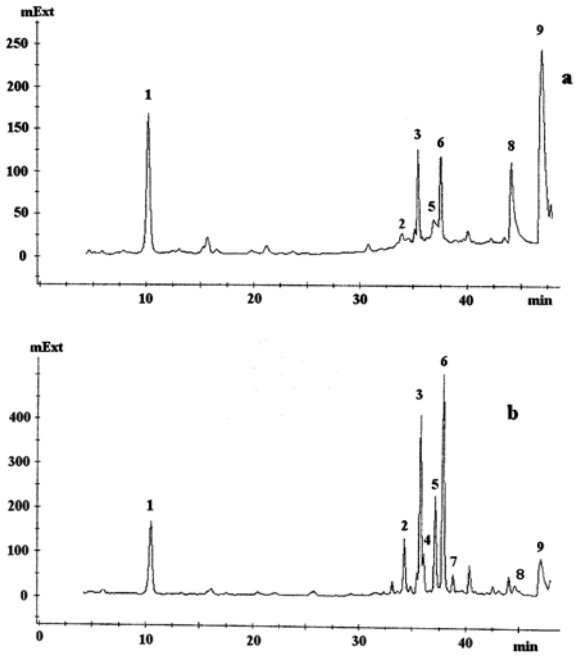

Fig. 2. The HPLC chromatogram (at 254 nm) analysis of phenolic compounds present in extracts L3 (a) and L4 (b) obtained from inflorescence of linden (*Tiliae inflorescentia*) Identified substances are marked with the following numbers: 1 – protocatechuic acid, 2 – rutin, 3 – isoquercitrin, 4 – quercetin, 3,7-dirhamnoside 5 – kaempferol 3-rutinoside, 6 – kaempferol 3-glucoside, 7 – quercitrin 8 – quercetin, 9 – kaempferol

Ryc. 2. Chromatogram HPLC (254 nm) analizy związków fenolowych obecnych w wyciągach L3 (a) i L4 (b) otrzymanych z kwiatostanów lipy (*Tiliae inflorescentia*). Zidentyfikowane związki zostały zaznaczone następującymi numerami: 1 – kwas protokatechowy, 2 – rutyna, 3 – izokwercetyna, 4 – 3,7-diramnozyd kwercetyny, 5 – 3-rutynozyd kemferolu, 6 – 3-glukozyd kemferolu, 7 – kwercytryna, 8 – kwercetyna, 9 – kemferol

content being 6.9% (w/w) and aglycone 17% (w/w). L4 contained 60.1% (w/w) total phenols: 54% (w/w) glycosides and 4.6% (w/w) aglycones. The concentrations of protocatechuic acid (a strong antioxidant) were 0.3, 2.1, 2.1, and 1.9% in L1, L2, L3, and L4, respectively.

The highest number of antioxidant units calculated per mg of extract ($TAU_{535/mg}$) was observed in extract L3 (1.222 ± 0.154) and lower numbers in extracts L2, L4, and L1 (1.155 ± 0.262, 1.089 ± 0.147, and 0.35 ± 0.047, respectively). When the number of antioxidant units was calculated per mg of phenolic compounds ($TAU_{535/ph}$), the highest value was calculated for L3 (4.968 ± 0.625), the others, in decreasing order, being L1 (3.398 ± ± 0.460), L2 (2.078 ± 0.472), and L4 (1.812 ± ± 0.244).

Alkaline hydrolysis, leading to the large increase in aglycone content in extract L3, increases the antioxidant activity of this extract. Some authors found stronger antioxidant activity of flavonoid aglycones than of glycosides [21, 22]. The present authors' earlier study [23] showed increased antioxidant activity of extracts obtained from *Helichrysum arenarium* after alkaline hydrolysis. The conclusion was that this was caused by an increase in caffeic acid concentration after hydrolysis. In this study the increase in the antioxidant efficiency ($TAU_{535/ph}$) of the alkaline hydrolyzed extract L3 was caused by an increase in the concentration of flavonoid aglycones, which are stronger antioxidants than glycosides. The relative proportion of phenolic glycosides and aglycones does not change after enzymatic hydrolysis. The amounts of glycosides and aglycones in extracts L2 and L4 are very similar. The antioxidant features of these extracts are also similar.

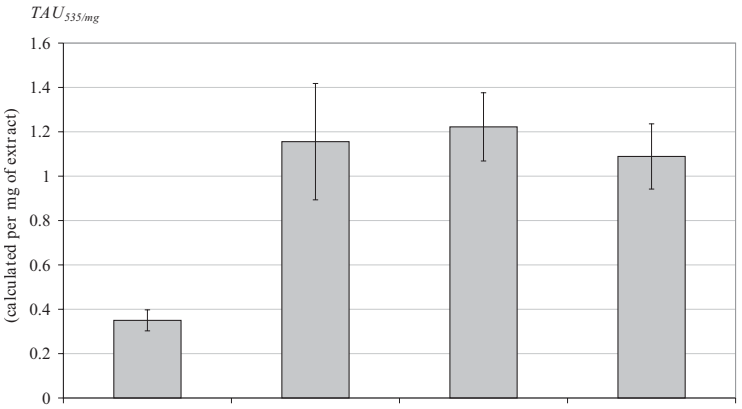

Fig. 3. Antioxidant activity ($TAU_{535/mg}$) of extracts L1, L2, L3 and L4 from flowers of linden calculated per mg of extracts

Ryc. 3. Aktywność przeciwutleniająca ($TAU_{535/mg}$) wyciągów L1, L2, L3 i L4 z kwiatów lipy przeliczonych na mg wyciągów

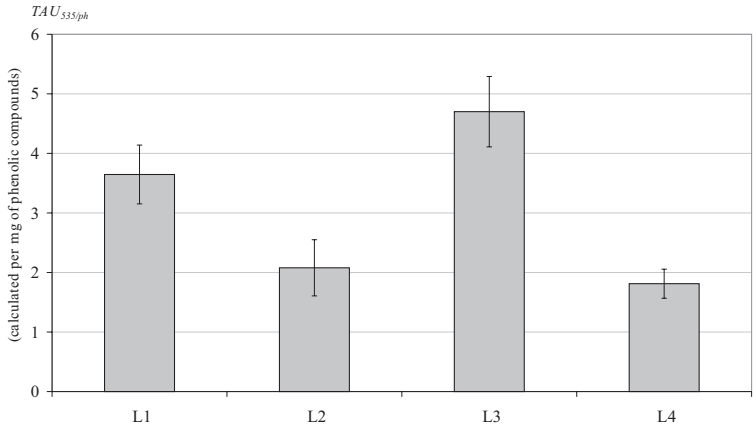

Fig. 4. Antioxidant activity ($TAU_{535/mg}$) of extracts L1, L2, L3 and L4 from flowers of linden calculated per mg of phenolic compounds

Ryc. 4. Aktywność przeciwutleniająca ($TAU_{535/mg}$) wyciągów L1, L2, L3 i L4 z kwiatów lipy przeliczonych na mg związków fenolowych

In conclusion, it can be stated that: 1) alkaline hydrolysis appears to be advantageous for antioxidant activity thanks to an increased concentration of aglycones; 2) enzymatic hydrolysis was ineffective and does not cause a substantial change in the phenol pattern or in the antioxidant activity of the extracts; 3) the use of the antioxidant units presented here is useful because it enables the measurement and comparison of antioxidant activity calculated per mass of extract or phenols or also per mass of raw material [24]; the use of antioxidant units also enables estimation of the yield of isolation of substances possessing antioxidant activity.

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