

Full Paper

Antiradical Activity of *Paulownia tomentosa* (Scrophulariaceae) Extracts

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Abstract: *Paulownia tomentosa* is a large deciduous tree planted mostly for its fast growing wood and decorative purposes. The tree is also used in traditional Chinese medicine. As a part of our study of natural polyphenols, the fruits of *Paulownia tomentosa* were extracted by EtOH and then subjected to liquid/liquid extraction. Fractions were analysed by TLC and HPLC to determine presence of phenolic substances. We identified and quantified acteoside (1) and isoacteoside (2) in the EtOAc and *n*-BuOH extracts; mimulone (3) and diplacone (4) in the MeOH extract. To determine the antiradical activity of extracts we used the anti DPPH and peroxy nitrite assays. The activity was expressed as Trolox C equivalents, IC₅₀ for DPPH scavenging and a time dependency course was established. The polyphenols content was determined; results were expressed as gallic acid equivalents. Using these methods we found the fractions of the *n*-BuOH, EtOAc and MeOH extracts that display antiradical activity, which could be exploited as potential pharmaceuticals.

Keywords: *Paulownia tomentosa*, antioxidant, DPPH, antiperoxy nitrite, phenolics

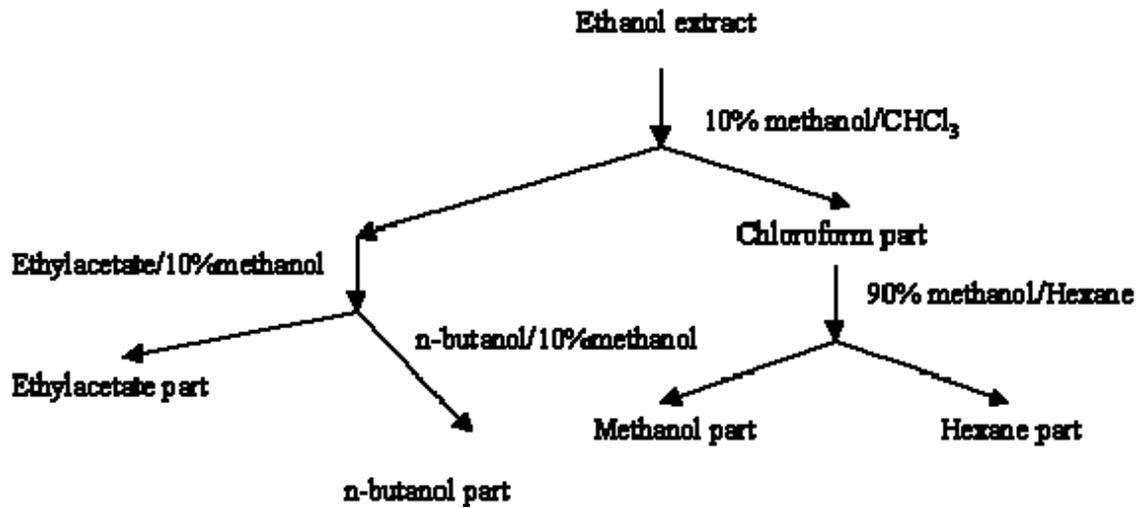
Introduction

Although with the development of chemical and pharmacological analysis methods we can study natural compounds more thoroughly, a great number of compounds and plants still remain unexplored. One such plant is *Paulownia tomentosa*. In this plant only a few compounds have been identified, mostly of polar character and divided into five groups: phenolic glycosides, furofuran lignanes, furanoquinones, iridoides and flavonoids [1-6]. A large group of essential oil substances has also been identified in the flowers [7]. These compounds and especially the flavonoids were identified from different species, where they probably serve as UV irradiation protectors. The increase in free radical species (corresponding with a high UV irradiation) is nowadays considered to be the true cause and effect of many metabolic disorders connected to such diseases as neurodegeneration, cancer or diabetes mellitus [8-10]. Nowadays a lot of different plant species are used as nutritional additives to add antioxidants to the organism to improve the immunity against these diseases [11]. As a part of our efforts to find new antioxidative and antiradical active compounds we have investigated in some depth the family Scrophulariaceae. A screening assay of *P. tomentosa* fruit extracts showed an antiradical effect; therefore we prepared a larger portion of EtOH extract from *P. tomentosa* fruits, which was separated into three parts by immiscible liquid/liquid extraction. The CHCl₃, EtOAc and *n*-BuOH fractions of the extract showed the presence of a large number of phenolic compounds after TLC and HPLC analyses, identified as prenylated or geranylated flavonoids on the basis of their UV spectra and retention times. We thus identified acteoside (**1**) and isoacteoside (**2**) in the *n*-BuOH and EtOAc extracts and mimulone (**3**) and diplacone (**4**) in MeOH extract and we quantified them. The antiradical activity of these extracts was confirmed by anti DPPH (diphenylpicrylhydrazyl) and peroxy nitrite scavenging assays. The total amounts of polyphenolics was also determined.

Results and Discussion.

Fruits of *P. tomentosa* were extracted with EtOH. The EtOH extract was then separated to fractions of similar polarity (Figure 1).

The separated fractions were analyzed by means of TLC and HPLC (Figure 2). These chromatographic analyses showed that thanks to the good choice of extraction solvents, a good separation of compounds of similar polarity was achieved. We identified the main components of the MeOH, *n*-BuOH and EtOAc extracts (by comparison with standards) and we quantified these compounds with help of HPLC. The components of the EtOAc and *n*-BuOH extracts are represented mainly by acteoside (**1**) and isoacteoside (**2**) (Figure 3; EtOAc extract **1** 43.1 % and **2** 24.3 % by dry weight; *n*-BuOH **1** 55.1 % and **2** 34.5 % of dry weight). The MeOH extract contained mimulone (**3**) and diplacone (**4**) (Figure 4, 4.3 % of **3** and 15.3 % of **4** in the dry extract, respectively). These compounds have been previously isolated from *P. tomentosa* [2,6]. Quantification was made by means of calibration curves derived from peak areas and the amounts of compound in the samples. The content of compounds in *P. tomentosa* extracts is summarized in Table 1.

Figure 1. Liquid/liquid fractionation of EtOH extract of fruits of *P.tomentosa*.**Table 1:** The content of compounds isolated from *P. tomentosa* fruit in prepared extracts. Data expressed as mols of compound per 100 g dry weight of extract.

| Extract | 1 | 2 | 3 | 4 |
|------------------------|------|------|------|------|
| MeOH extract | - | - | 0.01 | 0.04 |
| EtOAc extract | 0.07 | 0.04 | - | - |
| <i>n</i> -BuOH extract | 0.09 | 0.06 | - | - |

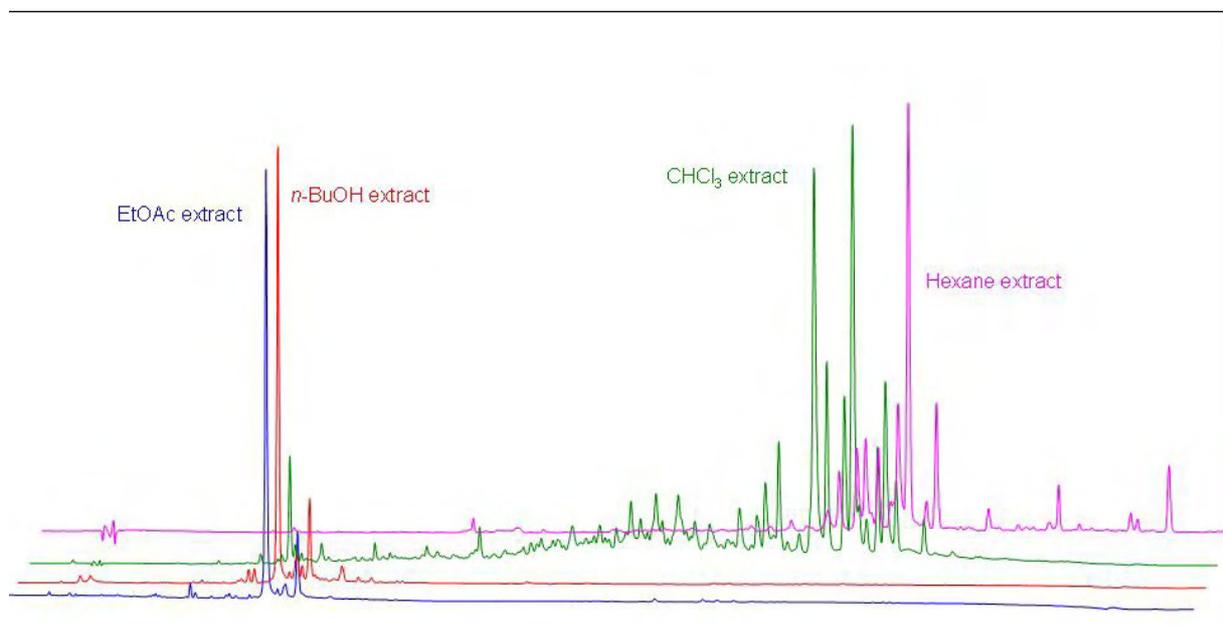
Figure 2: Comparison of the HPLC chromatograms of the MeOH, EtOAc, *n*-BuOH, and hexane extracts of *P. tomentosa* fruits, 1 g L⁻¹ (10 µL injection).

Figure 3: HPLC chromatograms of the EtOAc extract of *P. tomentosa* fruits, 1 g L⁻¹ (1 μL injection) showing acteoside (1) and isoacteoside (2).

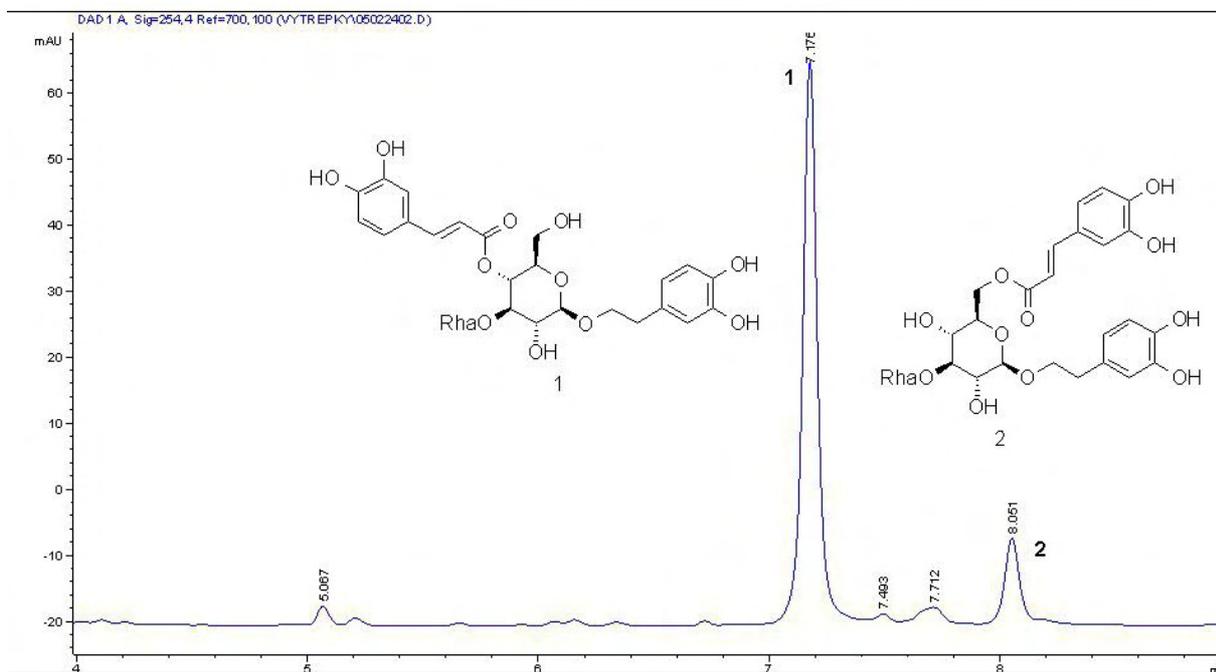
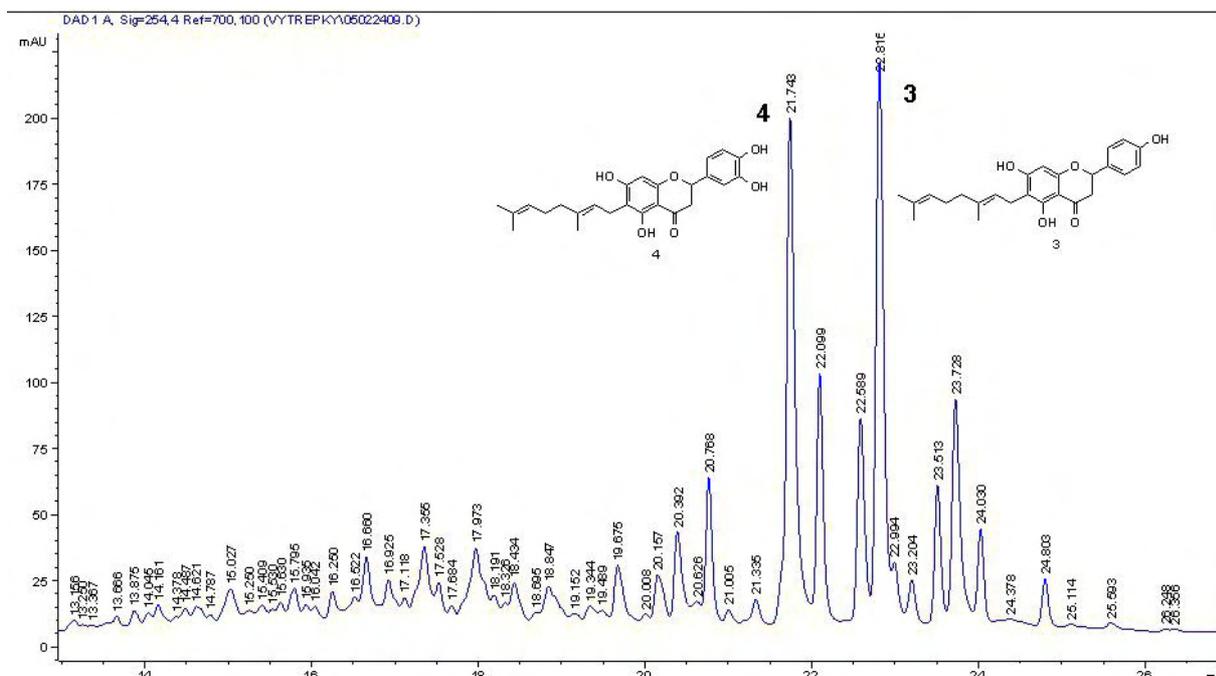


Figure 4: HPLC chromatograms of the MeOH extract of *P. tomentosa* fruits, 1 mg/mL (10 μL injection): mimulone (3), diplacone (4).



Separated fractions were tested for anti free radical activity. Both anti DPPH and antiperoxynitrite assays identified the promising activity of the CHCl₃, EtOAc and *n*-BuOH extracts. After separation of the CHCl₃-fraction only the MeOH one displayed antiradical activity. Antiperoxynitrite activity expressed as the percentage of inhibition of tyrosine nitration is shown in Table 2.

Table 2: Antioxidant activity and total phenolic content of tested extracts.

| Extract | Total Polyphenolics ^{a,c} | Antiperoxynitrite activity ^{a,b} | Anti DPPH activity EC ₅₀ ^d (mg/mL) | Anti DPPH activity ^b |
|-------------------|------------------------------------|---|--|---------------------------------|
| CHCl ₃ | 43.8±1.2 | 178±10 | 0.017 | 13.7 |
| EtOAc | 49.4±0.9 | 211±15 | 0.007 | 325.3 |
| <i>n</i> -BuOH | 47.3±0.8 | 190±1 | 0.008 | 284.7 |
| Hexane | 7.0±1.3 | 10±2 | 0.740 | 3.1 |
| MeOH | 31.3±1.1 | 203±4 | 0.032 | 72.1 |

^aValues are expressed as the means ± S.D. of three determinations.

^bData expressed as milimoles of Trolox equivalents per 100 g dry weight of extract.

^cData expressed as grams of gallic acid equivalents (GAE) per 100 g dry weight of extract.

^dTheoretical value calculated from calibration curve

Anti DPPH activity assays showed similar results, as also expressed in Table 2. With the assistance of measurements of samples of different concentrations we acquired graphs for obtaining the EC₅₀ values of extracts. At EC₅₀ we established a course of scavenging in 30 minutes. We expressed also anti-DPPH activity as TEAC.

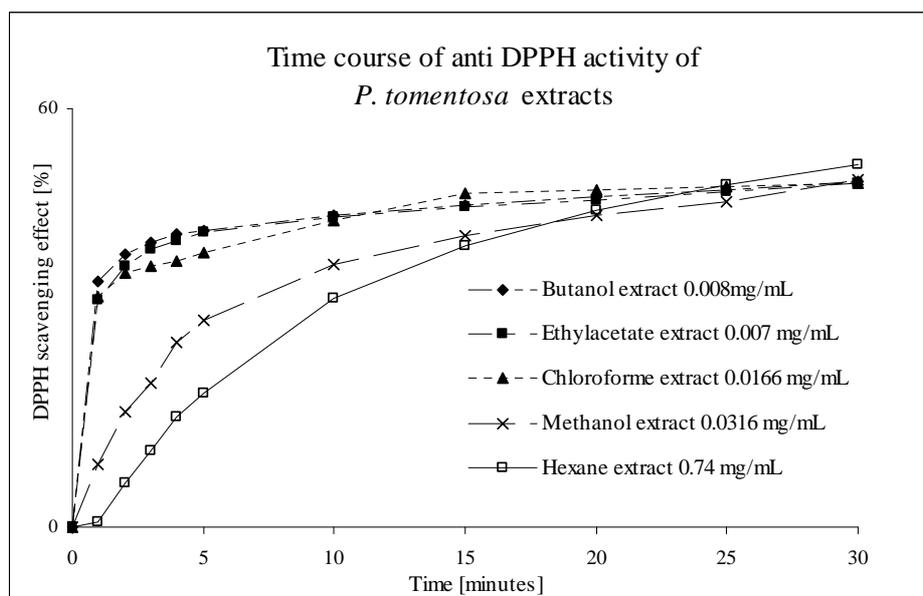
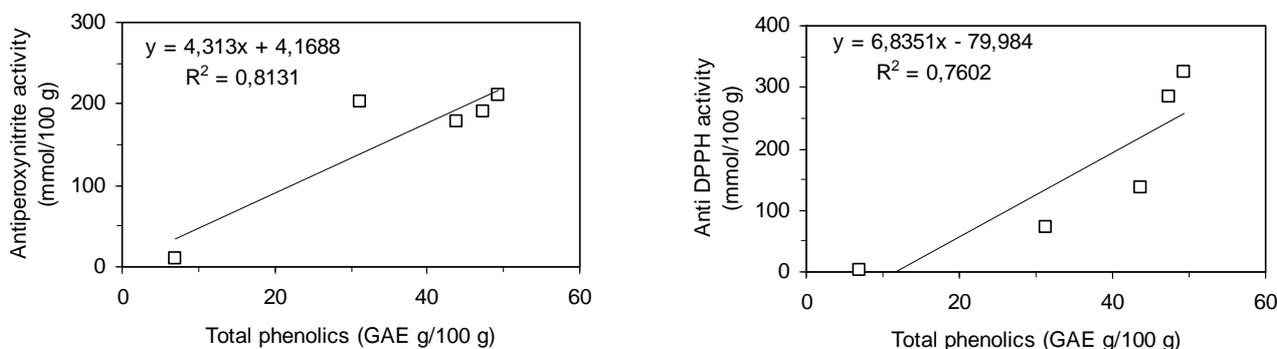
Figure 5: Time course of the anti DPPH activity of *P. tomentosa* extracts. EC₅₀ concentrations used, values determined from three parallel measurements.

Table 2 shows the values of EC_{50} and comparison of scavenging effect to effect of Trolox. C. EtOAc, *n*-BuOH and MeOH extracts are good scavengers in comparison to Trolox. C. Graph (Figure 5) presents the time course of reaction showing that tested extracts represent fast scavengers in the exception of hexane extract. Hexane extract is designated as slow scavenger.

Total amount of polyphenols in extracts was established by standard colorimetric methods. The results are presented as GAE in Table 1. The amount of polyphenolics was in good correspondence with the antiradical activity assay results, and hexane extraction removed nonactive and non-phenolic substances. The content of phenolic substances in chloroform (MeOH), EtOAc and *n*-BuOH extracts was comparable with those of the traditional Chinese medicine plant extracts *Rhus chinensis* and *Acacia catechu* [12]. Generally, the phenylpropanoid glycosides display a number of biological activities, for instance antiestrogenic [13], vasorelaxant [14] and anti-inflammatory [15]. The phenylpropanoid glycosides acteoside (**1**) and isoacteoside (**2**) are also known as excellent radical scavengers isolated from many plants [16]. Their antiradical potential was previously established with help of various methods including DPPH scavenging activity assays [17, 18]. In relation to the content of **1** and **2** in the *n*-BuOH and EtOAc extract this leads to the valid premise that **1** and **2** are the main compounds responsible for antiradical activity of these extracts. Mimulone (**3**) and diplacone (**4**) had also been previously isolated from *P. tomentosa* fruits and their anti DPPH activity was established [19, 20]. Compound **4** had been proved as antiradical active compound whereas activity of **3** was much lower due to the different substitution pattern of flavonoid B ring. The chromatographic analysis of MeOH portion of extract showed presence of variety of compounds, some of them could be more active in comparison with **4** and could be responsible for at least part of the antiradical activity of MeOH extract. The correlation between antiradical activity and content of phenolic compounds of extracts was stated. As shown in the Figure 6, there is a significant linear correlation ($r > 0.87$, $p \leq 0.05$). Presented results show the suitability of MeOH, *n*-BuOH and EtOAc extracts for use as radical scavenger sources after further work and confirm *P. tomentosa* fruits as a rich source of acteoside (**1**) and diplacone (**4**).

Figure 6: Correlation of antiperoxynitrite and anti DPPH activity of extracts and of total phenolics content.



Experimental

Plant material

Materials for extraction were fruits of *Paulownia tomentosa* Scrophulariaceae collected in autumn 2004 in the campus of VFU Brno. A voucher specimen (PT-02O) was deposited at the herbarium of the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic.

22.5 kg of fruits were collected, cleaned and then extracted by maceration in EtOH (3 × 20 L). The extract was filtered and the solution was evaporated *in vacuo*. The compounds **1-4** have been previously isolated from *P. tomentosa*.

Liquid/liquid extraction

The extraction flowchart is shown in Scheme 1. Dried EtOH extract was dissolved in 1:9 MeOH-H₂O (20 L) and extracted successively with CHCl₃ (3 × 20 L), EtOAc (3 × 15 L) and *n*-BuOH (3 × 20 L), with each extract being removed, dried with Na₂SO₄, filtered and the solvent was evaporated *in vacuo* before proceeding with the next extraction to give CHCl₃, EtOAc and *n*-BuOH fractions of 562 g, 78 g and 519 g, respectively. The CHCl₃ part was then extracted with a MeOH-H₂O-hexane system. Repeated treatment with hexane yielded 87 g of hexane fraction and 462 g of MeOH fraction. The presence of phenolics and different character of these extracts were verified by TLC and analytical HPLC.

TLC

Thin layer chromatography was performed on silica gel F₂₅₄ Merck (Germany). Mobile phase was a 95:5 (v/v) mixture of benzene-acetone. For visualization the chromatogram was sprayed with Neu reagent (diphenylethyl ester boric acid in MeOH, 1% solution) and inspected visually or using UV (254 and 356 nm).

Analytical HPLC

HPLC was done using an Agilent 1100 UV-VIS DAD (Agilent, Germany), a Supelcosil ABZ+Plus column (15 cm × 4.6 mm, 3 μm; Supelco, USA), and a gradient elution of MeCN:HCOOH (0.2%) from 10:90 (v/v) at 0 min up to 100:0 (v/v) after 28 min, then up to 100 % of MeCN after 32 min. Flow rate was 1 mL·min⁻¹, 40 °C. DAD (diode array detection) was performed at λ 254, 280 and 350 nm.

DPPH quenching assay

To quantify the DPPH quenching activity, the modified method of Braca *et al.* [21] was used. The MeOH DPPH (Sigma) solution was prepared at the concentration 22 g·L⁻¹. Tested extracts were

dissolved in MeOH at different concentrations. A volume of test solution (0.2 mL) was mixed with DPPH solution (1.8 mL) and the absorbance of the mixture at 517 nm was measured each minute during the first 5 minutes of experiment and then each 5 minutes for the next 25 minutes. Using the recorded data, scavenging effect r was calculated [$r = (1 - \text{sample absorbance/control absorbance}) \times 100$] and the EC_{50} of extracts and the time course of activity increase was drawn and the differences between the tested extracts were compared. The percentage of DPPH scavenging was compared to that of the calibrated Trolox standard. Results were expressed in terms of Trolox equivalent antioxidant capacity (mmol Trolox equivalents per 100 g dry weight of plant extract).

Antiperoxyinitrite assay

The measurement of inhibition of tyrosine nitration was performed in the following way [22]: a 10 mM solution of peroxyinitrite (8 μL) in 0.1 M NaOH was drawn and mixed up in HPLC injector with 1.0 mM solution of tyrosine in 0.05 M phosphate buffer pH 6.0 (42 μL) containing the sample (0.15 g L^{-1}) and DMSO in a 1:1 (v/v) ratio. The reaction mixture was then injected directly onto a HPLC column (Supelcosil ABZ+Plus, 25 cm \times 4.6 mm, 5 μm ; Supelco, USA); mobile phase was 90% 40 mM HCOOH: 10% MeCN (v/v) isocratic elution, flow rate 1 $\text{mL}\cdot\text{min}^{-1}$, detection at 276 nm. Inhibition of tyrosine nitration was calculated relative to peak area of 3-nitrotyrosine founded in the control measurement. The percentage of inhibition of tyrosine nitration was compared to that of the calibrated Trolox standard. Results were expressed in terms of Trolox equivalent antioxidant capacity (mmol Trolox equivalents per 100 g dry weight of plant extract).

Total amount of polyphenolics

The total amount of polyphenolics was determined by a colorimetric method. Appropriately diluted extract solution (700 μL) was oxidized by 0.2 N Folin-Ciocalteu reagent (400 μL) and then a solution of Na_2CO_3 (75 g L^{-1}) was added to a total volume of 10.0 mL. After 2 hours, the suspension was centrifuged (5000 rpm, 10 minutes) and the resulting absorption was measured ($\lambda=760$ nm). Quantification was made based on a gallic acid calibration curve. The results were expressed as gallic acid equivalents (GAE) per 100 g of dry weight of plant extract.

Acknowledgments

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Sample Availability: Samples of the compounds and extracts are available from authors.

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