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Original Research

Effects of different drying, extraction methods, and solvent polarity on the antioxidant properties of *Paeonia daurica* subsp. *mlokosewitschii*

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Abstract

The study presents the effect of drying methods (microwave-drying and freeze-drying), different solvents (80% methanol, 99% methanol, 80% ethanol), and different extraction methods on the antioxidant activity of *Paeonia daurica* subsp. *mlokosewitschii* leaves, estimated based on DPPH free radical scavenging activity. The highest antioxidant activity was revealed in the freeze-dried leaves. The drying method significantly influenced the antioxidant activity: the thermal microwave-drying resulted in lower antioxidant potential. The solvent polarity also played a significant role in the determination of the antioxidant activity of *Paeonia daurica* subsp. *mlokosewitschii* leaves: the lowest IC_{50} values (specific concentration of the sample required for 50% inhibition) were revealed for freeze-dried plants extracted with 80% methanol, followed by IC_{50} values obtained for the extraction with 80% ethanol, and the highest IC_{50} values were revealed for extracts of microwave-dried plants extracted with 99% methanol. The subsequent drying of freeze-dried plant extracts had no significant effect on the antioxidant activity of the extract. The subsequent drying of the methanol extract from microwave-dried plants at room temperature or 40°C significantly reduced IC_{50} value, however, the results were comparable with those, obtained for the methanol extracts of freeze-dried plants without subsequent drying of the extract. Thus, the optimal method of drying and extraction of Paeonia daurica subsp.

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mlokosewitschii leaves for preserving the antioxidant activities was established: freeze-drying of leaves followed by 24 h extraction with 80% methanol.

Keywords: *Paeonia daurica* subsp. *mlokosewitschii*, antioxidant activity, DPPH assay, solvents, drying methods.

Introduction

Research on anti-oxidants has become quite popular over the years as these substances are now considered potential therapeutic candidates to prevent free radical-induced damage to the human body [1]. Now synthetic antioxidants such as butylated hydroxytoluene are widely used, however, their negative effects, such as liver damage and a higher probability of carcinogenesis were demonstrated [2]. Therefore, the search for natural sources of antioxidants is increasingly important and medicinal plants are considered an easily available and potent source of antioxidants as they contain a mixture of different chemical compounds that may act individually or in synergy to cure disease and improve health.

Paeonia daurica subsp. *mlokosewitschii* is an endemic species of Georgian flora of narrow local distribution: it occurs only in Shida (Inner) Kakheti, Shiraki (Kashebi), and Dagestan (headstream of the Andis-Koisu River [3]. For the first time, the species was discovered in 1897 by Polish naturalist Ludwik Młokosiewicz [4]. The use of species of the genus Paeonia as traditional herbal remedies has been reported for dysmenorrhea, amenorrhea, epilepsy, spasms, and gastritis [5]. According to numerous phytochemical the treatment of hematemesis, blood stasis, investigations, 451 compounds, such as monoterpenoid glucosides, flavonoids, tannins, stilbenes, triterpenoids, steroids, and phenols have been isolated from Paeonia plants [6]. Studies of the pharmacological activities of these compounds have revealed their antioxidant, anti-inflammatory, antibacterial, antitumor, antiviral, and neuroprotective properties [7]. However, due to the narrow local distribution of *Paeonia daurica* subsp. *mlokosewitschii*, the phytochemical composition and medicinal properties of the species were not extensively investigated.

Different drying and extraction methods can alter the antioxidant activity in a sample and, subsequently, the possibility of its further use in the pharmaceutical industry [8]. This study aimed to determine the optimal drying and extraction method for *Paeonia daurica* subsp. *mlokosewitschii* leaves, preserving higher antioxidant activity, based on DPPH assay.

Materials and methods

Plant material

Leaves of *Paeonia daurica* subsp. *mlokosewitschii* were collected in July 2020 in the National Botanical Garden of Georgia

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(Tbilisi). Samples were placed in paper bags and transported to the laboratory within 1 h. Once in the laboratory, healthy leaves with uniform color, dimensions, and texture were detached from the plants. Samples of fresh plant material were frozen in liquid nitrogen and stored at –80 °C.

Drying processes

Leaves were dried using two different drying methods, i.e., freeze-drying and microwave-drying. For each drying method, 2 g of fresh leaves was used. Leaves were freeze-dried using a DW-10N freeze dryer in a vacuum flask of 500 mL at 10 Pa and a final condenser temperature of -55 °C until the plant material reached a constant weight, determined by measuring the dry weight (DW) of leaves. The dying process took 6 hours. Using microwave-drying, leaves were dried in a microwave oven (Beko; 800 W) until the plant material reached a constant DW. The drying process took 3 min.

Sample extraction

Extraction efficiencies of different solvents, namely, 80% methanol, 99% methanol, and 80% ethanol were tested. Leaves dried by different methods were extracted in a ratio of 1:10 with solvent, followed by continuous stirring for 24 h at room temperature using an orbital shaker at 270 rpm. Extracts were centrifuged at 5000g for 15 min and stored at -80 °C for further analysis.

For evaluation of the efficiency of different extraction methods, the supernatant was either used directly for the assay or dried at room temperature (in the hood) or at 40 °C (in the drying cabinet).

DPPH free radical scavenging activity assay

The free radical scavenging activity was measured using by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described earlier [9].

The stock solution was prepared by dissolving 24 mg DPPH with 100 ml methanol. The working solution was obtained by diluting the DPPH solution with methanol to attain an absorbance of about 0.98±0.02 at 517 nm using the spectrophotometer. A 3 ml aliquot of the working solution was mixed with 100 µl of the sample at various concentrations (10 - 500 µg/ml). The reaction mixture was shaken well and incubated in the dark for 30 min at room temperature. Then the absorbance was taken at 517 nm. A typical blank contained 3 ml of the working solution and the appropriate volume of the corresponding solvent and was incubated under the same conditions. Ascorbic acid was used as standard. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation:

$$\text{Scavenging effect (\%)} = [(\text{control absorbance} - \text{sample absorbance})/(\text{control absorbance})] \times 100$$

The concentrations of the sample required for 50 % inhibition (IC_{50}) were calculated to determine the concentration of the sample required to inhibit 50% of radical. The lower the IC_{50} value, the higher the antioxidant activity of the samples. IC_{50} was calculated by plotting the concentration of extract versus inhibition of DPPH (%) and data were fit with a straight line (linear

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regression). IC_{50} value was estimated using the fitted line, i.e.,

$Y = a * X + b$,

$IC_{50} = (50 - b)/a$

Statistical analysis

All the procedures for extraction and antioxidant studies were repeated in triplicate. The results were expressed as means± standard deviation of three parallel replicates. All data of the DPPH assay were analyzed statistically by one-way analysis of variance (ANOVA) using Microsoft Excel. A *p-value* of less than 0.05 was considered statistically significant.

Results and discussion

The DPPH method was introduced nearly 50 years ago by Blois [10] and now the method is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant capacity. DPPH is a stable free radical, which accepts an electron or hydrogen radical to become a stable, diamagnetic molecule, which has an absorbance in its oxidized form around 515-520 nm [11]. DPPH produces a purple solution in methanol and the solution becomes yellow when it reacts with antioxidant molecules which results in the scavenging of the radical by hydrogen donation, this change in the color can be monitored by the decrease in absorbance [12]. DPPH assay is a rapid and efficient method used for the evaluation of the free radical scavenging activity of medicinal plants [13]. For quantitative assessment of free radical scavenging activity by the DPPH assay, usually, parameter IC_{50} is determined. IC_{50} is the concentration of substrate that causes a 50% loss of the DPPH activity (manifested as the loss of color) and it is used for the interpretation of the results obtained by the DPPH method.

The goal of this study was the determination the optimal drying method and extractant for the preservation of the antioxidant activity in *P. daurica* subsp. *mlokosewitschii* plant material. The ability to retain antioxidants was estimated based on DPPH free radical scavenging activity and the percentage of DPPH free radical scavenging activity was calculated and compared with ascorbic acid as a standard (Fig. 1).

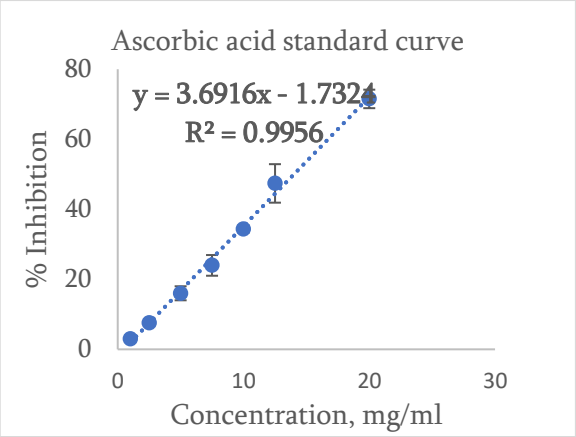

FIGURE 1. Ascorbic acid standard curve. Values are mean ± SD from three independent experiments.

Effect of different drying methods on DPPH free radical scavenging activity of extracts

The classical way for the start of isolation of natural compounds and the simplest and inexpensive methods to preserve the medicinal properties of plants is drying. However, it is well known that drying

temperature may affect the metabolic profile and antioxidant properties of the corresponding extracts [14]. Temperature and duration of treatment are the determining factors for the selection of the most efficient drying method to preserve antioxidant activity in plant materials [15]. Indeed, specific variations of temperature in the different drying methods may protect against the degradation of these components, leading to the maintenance or enhancement of the product quality of the analyzed plant material. Traditionally, drying at high temperatures, such as sun-drying, oven-drying, and microwave-drying are the most widely used and inexpensive drying methods. However, exposure of plant material to high temperatures for a prolonged period can affect the content of heat-labile bioactive compounds [16]. Previous studies have indicated that freeze-drying may avoid the loss of valuable chemical components when compared to other conventional methods [17].

In this study, we compared the effect of microwave-drying and freeze-drying on the antioxidant activity analyzed by DPPH assay in *P. daurica* subsp. *mlokosewitschii* leaves. The results of the study indicated that the extracts regardless of drying methods demonstrated a dose-dependent scavenging activity by reducing DPPH radical (Figs. 2 and 3). The antioxidant activity was higher in freeze-dried leaves than in microwave-dried leaves (Figs. 2 and 3).

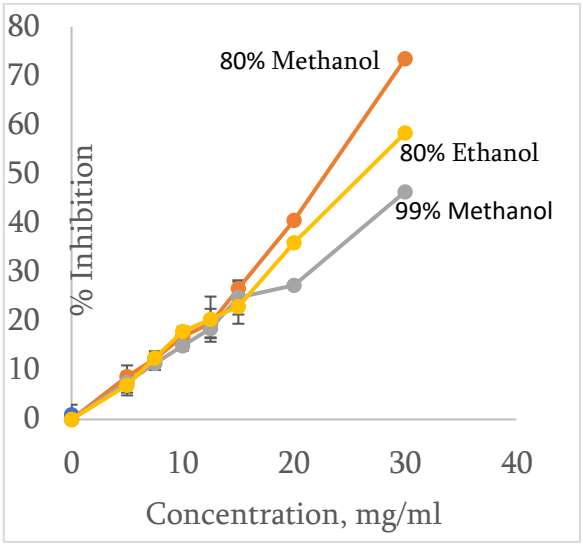

FIGURE 2. The radical scavenging activity, represented by the percentage of inhibition, of extracts of freeze-dried *P.daurica* subsp. *mlokosewitschii* leaves obtained using different solvents. Values are mean ± SD from three independent experiments.

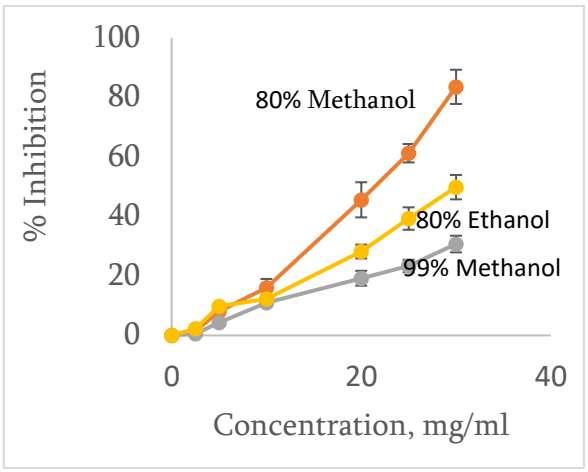

FIGURE 3. The radical scavenging activity, represented by the percentage of inhibition, of extracts of microwave-dried *P.daurica* subsp. *mlokosewitschii* leaves obtained using different solvents. Values are mean ± SD from three independent experiments.

By plotting the graph of extract concentrations against the scavenging

activity, IC_{50} values were calculated. Among treatments, freeze-drying and extraction with 80% methanol revealed the highest radical scavenging activity (the lowest IC_{50} = 17.99 μg/ml), while microwave- drying and extraction with 99% methanol Showed the lowest scavenging activity (IC_{50} = 58.29 μg/ml, Table 1). Interestingly, the IC_{50} for microwave-dried leaves extracted with 80% ethanol was higher, than the corresponding value for plants dried by freeze-drying. However, both values were still higher than the IC_{50} obtained for freeze-dried leaves extracted with 80% methanol.

Table 1. IC_{50} (DPPH assay) of extracts of freeze-dried and microwave-dried *P.daurica s*ubsp. *mlokosewitschii* leaves. Values are mean ± SD from three independent experiments. ∗p < 0.005, ns-nonsignificant, compared to 80% methanol extract.

Sample	Freeze-drying	Microwave-drying
Ascorbic acid	13.05 ±0.23	13.05 ±0.23
Methanol 80%	17.99 ± 1.00	21.30 ± 1.08
Ethanol 80%	24.81 ± 0.49*	20.31 ± 1.13^{ns}
Methanol 99%	32.71 ±2.13*	58.29±1.11*

In the next experiment, we investigated the effect of drying the extract and re-extraction of the pellet with the corresponding solvent (80% methanol, 99% methanol, or 80% ethanol, Table 2), since some studies recommend this method for DPPH assay [18]. Two methods of drying the extract: at room temperature (25 °C, drying in the hood) and drying at 40 °C (drying in the dying cabinet) were used to assess the effect of the temperature on the antioxidant content and DPPH free radical scavenging activity. Interestingly, the lowest IC_{50} value was obtained for extracts obtained by freeze-drying and dried at 40 °C, and extracts obtained by microwave-drying and dried at room temperature, extracted with 80% methanol (Table 2). The drying of the extracts did not affect significantly IC_{50} value for freeze-dried plants: a significant difference between values was not revealed for IC_{50} values of freeze-dried leaves extracted with 80% methanol (Table 1) and freeze-dried leaves extracted with 80% methanol followed by subsequent drying of the extract at the room temperature or 40 °C (Table 2). Interestingly, the opposite pattern was observed for leaves of microwave-dried plants: the drying of 80% methanol extracts at room temperature or 40 °C significantly reduced IC_{50} value (Table 2) in comparison with the IC_{50} obtained for 80% methanol extract (Table 1).

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Table 2. IC_{50} (DPPH assay) of extracts of *P.daurica* subsp. *mlokosewitschii* leaves subsequently dried at various temperatures. Values are mean ± SD from three independent experiments. ∗p < 0.005, ns-nonsignificant, compared to 80% methanol extract, obtained by either freeze or microwave-drying.

Sample	Freeze-drying		Microwave-drying	
	Room temperature	40 °C	Room temperature	40 °C
Ascorbic acid	13.05 ±0.23			
Methanol 80%	18.43 ± 0.87	17.46 ± 0.66^{ns}	16.67 ± 0.81	18.31± 1.21*
Ethanol 80%	24.50 ± 0.64*	23.29 ± 1.28*	20.01 ± 0.52*	24.25± 2.49*
Methanol 99%	27.02 ± 1.69*	21.31 ± 0.45*	31.17 ± 1.64*	32.98± 0.86*

Our results are consistent with the results of the study [19], demonstrating that low-temperature drying, such as freeze-drying and oven drying at 40 °C, produced products with higher antioxidant potential (total flavonoid content, total phenolic content, total antioxidant capacity, and radical scavenging activity) than drying at high temperatures. Also, the results of [20] showed that based on the antioxidant properties of maize waste, freeze-drying was more efficient than microwave-drying and spray-drying techniques. The highest recovery of maize waste phenolic compounds in this study was obtained using freeze-drying. Freeze-drying has high efficiency in moisture removal and maintains antioxidant compounds in plants [21]. During the freeze-drying process, ice crystals develop inside the tissue matrix and the removal of moisture content causes the tissue to become more brittle [22], which, in turn, causes the rupture of the cell structure, leading to the higher extraction efficiency of antioxidant compounds [23].

Effect of extraction solvents on DPPH free radical scavenging activity of extracts

In addition to the plant drying methodology, extraction methodology is also important in the antioxidant assay. The yield of the extract depends on the polarity of the solvent used for the extraction [24]. Obviously, the solubility of natural compounds and the choice of solvents can determine the yield of compounds. Now, polar solvents, such as methanol, ethanol, and acetone are major solvents used to extract some flavonols, alkaloids, polyphenols, and saponins [25].

The results of our experiments demonstrate that both, the polarity and the concentration of the solvent affect DPPH scavenging activity in *P.daurica* subsp. *mlokosewitschii* extracts. The highest DPPH scavenging activity was revealed in freeze-dried leaves, extracted with 80% methanol (Table 1 and Figs. 2 and 3). Interestingly, for microwave-dried leaves the DPPH scavenging activity was similar in extracts obtained using both 80% methanol and 80% ethanol, however,

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the use of 99% methanol decreased DPPH scavenging activity more than twice (Table 1). The same trend was observed for the extraction of freeze-dried leaves with different solvents, but differences between DPPH scavenging activities in different extracts were not so pronounced.

Similarly, to the first experiment, in the experiment with extract drying the lower IC_{50} values were revealed for freeze-and microwave-dried plants extracted with 80% methanol, followed by IC_{50} values obtained for the extraction with 80% ethanol, and the highest IC_{50} values were revealed for extracts of microwave-dried plants extracted with 99% methanol, regardless of the method of drying the extract (both at the room temperature or 40 °C, Table 2).

One-way analysis of variance (ANOVA) of the results obtained for the methanol and ethanol extracts showed that DPPH scavenging activity of *P.daurica* subsp. *mlokosewitschii* leaves were significantly influenced (p<0.05) by increasing the polarity of extracting solvent.

The polarity-dependent increase in extraction yield, antioxidant activity, reducing properties, and free radical scavenging activity of bean varieties was demonstrated [26]. The study of the effects of solvent type (ethanol, methanol, acetone, and water) and methanol concentration on extraction of total phenolic compound, total flavonoid compounds, and antioxidant capacity *Anacamptis collina* revealed that 95% methanol was the best extractant [27]. Such findings can be explained by the high affinity of antioxidant compounds towards more polar solvents. The results suggest the suitability of polar solvents for the extraction of antioxidant compounds from plant materials, particularly *P.daurica* subsp. *mlokosewitschii* leaves. Also, the obtained results suggest that phytochemical compounds extracted in polar solvents will be pharmaceutically more important due to comparatively higher values of antioxidant activity.

Conclusions

The results of the study demonstrated that drying methods (microwave-drying and freeze-drying), the polarity and the concentration of the solvent, and different extraction methods significantly affect the antioxidant activity of *Paeonia daurica* subsp. *mlokosewitschii* leaves. The highest antioxidant activity was revealed in the freeze-dried leaves, while the thermal drying resulted in lower antioxidant potential. The solvent polarity also played significant roles in the determination of antioxidant activity: the lowest IC_{50} values were revealed for freeze-dried plants extracted with 80% methanol, followed by IC_{50} values obtained for the extraction with 80% ethanol and the highest IC_{50} values were revealed for extracts of microwave-dried plants extracted with 99% methanol. The subsequent drying of the methanol extract of microwave-dried plants both at room temperature or 40 °C reduced IC_{50} value, however, this method is time-consuming and the IC_{50} values were

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comparable with those, obtained for the methanol extracts of freeze-dried plants without subsequent drying of the extract. As the result of the study the optimal method of drying and extraction of *Paeonia daurica* subsp. *mlokosewitschii* leaves for preserving the antioxidant activities was established: freeze-drying of leaves followed by 24 h extraction with 80% methanol.

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შრობის, ექსტრაქციის სხვადასხვა მეთოდების და საექსტრაქციო ხსნარების პოლარულობის გავლენა მცენარე *Paeonia daurica* subsp. *mlokosewitschii*-ის ანტიოქსიდანტურ აქტიურობაზე

ვალენტინა მიტოვა[1,2], მარინა ფირცხალავა[1], როზა ბიძინიშვილი[3], მალხაზ ვახანია[1,2], თორნიკე მინდიაშვილი[1,2], მარიამ კობიაშვილი[1,2]

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აბსტრაქტი

სტატიაში წარმოდგენილია შრობის მეთოდების (მოკლეტალღოვანი შრობა და ლიოფილიზაცია) გავლენა მცენარე Paeonia daurica subsp. mlokosewitschii-ის ფოთლების ექსტრაქტის ანტიოქსიდანტურ აქტიურობაზე. ანტიოქსიდანტური აქტიურობა შეფასდა ექსტრაქტის უნარით, შებოჭოს 2.2 დიფენილ-1-პიკრილჰიდრაზინის (DPPH) რადიკალები.

ყველაზე დიდი აქტიურობა გამოავლინა ლიოფილიზირებული ფოთლების ექსტრაქტმა. ექსტრაგირების პროცესში მნიშვნელოვანი აღმოჩნდა გამოყენებული ხსნარების პოლარულობა, რამაც განსაზღვრა მცენარის ფოთლების ექსტრაქტის ანტიოქსიდანტური აქტიურობა. IC_{50}-ის (ექსტრაქტის 50%-იანი ინჰიბირებისათვის აუცილებელი კონცენტრაცია) ყველაზე დაბალი მაჩვენებელი გამოვლინდა 80%-იანი მეთანოლის თანაობისას მომზადებულ ლიოფილიზირებულ ფოთლების ექსტრაქტში. მასთან შედარებით IC_{50}-ის მნიშვნელობა მეტი აღმოჩნდა 80%-იანი ეთანოლის პირობებში მიღებულ ექსტრაქტში, ხოლო IC_{50}-ის ყველაზე მაღალი მაჩვენებელი გამოავლინა მიკროტალღურ ღუმელში გამშრალმა მცენარის ფოთლებმა, ექსტრაქციის პროცესში გამოყენებული იყო 99%-იანი მეთანოლი.

მცენარის ექსტრაქტის შემდგომმა ლიოფილიზაციამ, არსებითი გავლენა არ იქონია ექსტრაქტის ანტიოქსიდანტურ აქტიურობაზე.

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მცენარის მეთანოლიანი ექსტრაქტის შემდგომმა შრობამ მიკროტალღურ ღუმელში 40°C-ს ტემპერატურაზე დააქვეითა IC_{50}-ის მნიშვნელობა. აღნიშნული შედეგები შედარებული იყო ლიოფილიზირებული ფოთლების მეთანოლიანი ექსტრაქტის მონაცემებთან, ექსტრაქტის მომდევნო შრობის გარეშე.

ამრიგად, დადგენილია *Paeonia daurica* subsp. *mlokosewitschii* -ის ფოთლების შრობის ოპტიმალური მეთოდი, რომელიც განაპირობებს მისი ანტიოქსიდანტური აქტიურობის შენარჩუნებას, რაც გულისხმობს ფოთლების ლიოფილიზაციას და 24 საათის განმავლობაში ექსტრაქტის მომზადებას 80%-იანი ეთანოლის გამოყენებით.

საკვანძო სიტყვები: Paeonia daurica subsp. mlokosewitschii, ანტიოქსიდანტური აქტიურობა, DPPH მეთოდი, ხსნარები, შრობის მეთოდები.

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