



Phytochemical Evaluation, Antioxidant Activity and Toxicity of *Paeonia daurica* ssp. *macrophylla* Root

Seyde Nargess Sadati Lamardi¹, Nilofar Taleb Kashefi², Narguess Yassa^{3*}

¹Department of Traditional Pharmacy, School of Traditional Medicine, Tehran University of Medical Sciences, Tehran, Iran.

²Department of Phytochemistry, Islamic Azad University, Pharmaceutical Sciences Branch, (IAUPS), Tehran, Iran.

³Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Abstract

Background and objectives: *Paeonia daurica* ssp. *macrophylla*, is an herbaceous and perennial plant which belongs to Paeoniaceae family. Two species of this plant grow in northern parts of Iran. The roots in powder form have been used in Persian traditional medicine for treatment of epilepsy, nightmares and gynecological diseases. Several biological activities such as antioxidant and anti-tumor effects of *Paeonia* species have been reported. **Methods:** methanol-water (80-20) extract (total extract) was fractionated using n-hexane, chloroform and ethyl acetate. Antioxidant activity of the total extract and fractions were evaluated using DPPH and FRAP assays. Total phenolics content of the extracts was determined by Folin-Ciocalteu method. In addition, cytotoxic activity of the fractions was determined against brine shrimp larvae. Column chromatography with normal phase silica gel and preparative TLC were also used for the isolation and purification of compounds. **Results:** Evaluation of the results indicated that the ethyl acetate and chloroform fractions with IC₅₀ values of 16.55, 23.9 µg/mL, respectively showed potent radical scavenging activity. As well, the ethyl acetate and chloroform fractions indicated the highest antioxidant power by FRAP assay. Due to the potent antioxidant activity, the chloroform fraction was chosen for further investigations. Three compounds were identified as benzoic acid, veratric acid and oleanolic acid by different spectroscopic methods. **Conclusion:** According to our findings in this study, the root of *Paeonia daurica* ssp. *macrophylla* has beneficial antioxidant activity without toxicity and the therapeutic use of this plant in traditional medicine can be somewhat justifiable.

Keywords: antioxidant; benzoic acid; brine shrimp lethality; oleanolic acid; *Paeonia daurica* ssp. *macrophylla*; veratric acid

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Introduction

Paeonia daurica ssp. *macrophylla* (Albov) D.Y. Hong (Synonym *Paeonia wittmannianna*) is an herbaceous and perennial plant, which is the single genus in Paeoniaceae family. Plants which belong to *Paeonia* genus have been known for beautiful appearance. They grow in Asia, including China and Japan, southern Europe and North America and also in Iran. *Paeonia daurica* ssp. *macrophylla* is one of the two species growing in the north parts of Iran [1,2]. The genus *Paeonia*, known as “Ood-e-saleeb” in

Persian traditional medicine, has been used for treatment epilepsy, nightmare, tremors, paralysis and other brain disorders, in addition to uterine problems, stomach and kidney pains [3]. Peony species have also been used as medicinal plants in some countries like China as an analgesic, sedative, anti-inflammatory agent, as a remedy for female genital diseases and for blood stagnation [4].

Paeonia species are rich sources of several bioactive compounds comprising

* Corresponding author: yasa@tums.ac.ir

monoterpenoids, triterpenoids, flavonoids, phenols, and tannins [5,6]. There are several reports on anti-oxidative effect of Chinese *Paeonia* species, *P. lactiflora* Pall. and *P. suffruticosa* Andr. with high antioxidant capacity and free radical scavenging activity. Another study on three species of *Paeonia* has shown that the petal extracts were potent antioxidants found by ABTS-DPPH methods, also there was a strong relationship between the antioxidant activity and the polyphenol content of the petals extract [7,8]. Picerno et al. have reported that the methanol and butanol extracts and polyphenolic compounds such as gallic acid and paeoniflorin from *Paeonia Rockii* roots which is one of the varieties grown in China are able to scavenge free radicals and inhibit the growth of *Candida albicans* [9]. Furthermore, 4-hydroxybenzoic acid and gallic acid were isolated from the fruits methanol extract of *Paeonia emodii*, grown in northern areas of Pakistan, which have shown strong inhibitory effect on enzyme lipoxygenase and the ABTS radical cation (ABTS⁺) [10].

Because of such biological importance and the traditional uses of *Paeonia* species especially the root powder, this study was aimed to evaluate the phytochemicals and the antioxidant effects and toxicity of *Paeonia daurica* ssp. *macrophylla* root extract.

Material and Methods

Instruments and chemicals

¹H and ¹³C NMR spectra were measured in CDCl₃ and TMS as internal standard with Bruker Avance spectrometer (500 MHz). Melting points were recorded on a Reichert-Jung apparatus. Column chromatography was performed on silica gel 60 (230-400 mesh, Merck, Germany). Pre-coated Silica gel GF254 sheets (Merck, Germany) were applied for the thin layer chromatography. Solvents: methanol, chloroform, ethyl acetate and hexane were purchased from Pars chemistry (Iran). DPPH, Trolox, buffer acetate, hydrochloric acid, gallic acid, sodium bicarbonate and TPTZ (2,4,6-Tris (2-pyridyl)-s-triazine) were obtained from Merck Company (Germany). Artemia brine shrimp eggs were obtained from Sigma-Aldrich (Germany) and Ocean nutrition (Belgium) Companies.

Plant material

The roots of *Paeonia daurica* ssp. *macrophylla* were collected from Alborz Moutains (2700-2900

m) Mazandaran Province, Iran in July 2016 and were identified by Prof. GH. Amin. A voucher specimen (6620-TEH) was deposited at the Herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

Preparation of the extract and fractions

The root parts of the plant were cut and dried in shade. Two kilograms of the samples were powdered with a grinder and macerated in 80% methanol for three days at room temperature. The excessive solvent was evaporated with a rotary vacuum evaporator at 40 °C to give a brown extract (crude sap 545.5 g). The crude sap was extracted using n-hexane, then the solvent was evaporated to dryness (hexane fraction 14.66 g), the remainder was extracted first by chloroform and then with ethyl acetate same as hexane solvent and the solvents were evaporated separately (chloroform fraction 21.14 g, ethyl acetate fraction 5.60 g). At the end, the residue that was soluble in methanol was labeled methanol fraction (504 g).

Isolation of substances

The chloroform fraction was selected for phytochemical studies because of higher amounts, good antioxidant activity and lack of toxicity. Twelve g of the chloroform fraction was chromatographed on silica gel (mesh 230-400) column (60×5 cm) eluting with a gradient of hexane-ethyl acetate and methanol to afford different fractions. After examining the obtained fractions with TLC, the hexane-ethyl acetate 60:40 fraction was subjected to preparative TLC by solvents ethyl acetate-methanol (80-20% and 70-30%): to give compound **1** (3.22% dry wt) and compound **2** (2.53% dry wt) and compound **3** (4.19% dry wt). The 3 substances were identified by using different spectroscopic methods (¹H and ¹³C-NMR) and confirmation with published data. [11-15].

Benzoic acid (compound **1**) was a colorless crystalline solid, m.p. 118 °C, veratric acid (3, 4-dimethoxy benzoic acid) (compound **2**) was a white amorphous powder, m.p. 174 °C and oleanolic acid (compound **3**) was a white amorphous powder, m.p. >300 °C.

Free radical scavenging assay

The free radical scavenging activity of the total extract and fractions were evaluated using 2-2 diphenyl-1 picrylhydrazyl (DPPH) method [16,17]. Trolox was used as the standard (8, 4, 2

µg/mL). One mL of different concentrations of the samples (10, 20, 50, 100, 200 µg) and Trolox were added to 2 mL of DPPH (4×10^{-5} g/mL), 1 mL of methanol in 2 mL DPPH and 1 mL of each samples in 2 mL of methanol were used as the control and blank, respectively. All samples were allowed to stand for 30 min in a dark place at Laboratory temperature, and the absorption was recorded at 517 nm. The test was done in triplicate. IC₅₀ values (the concentration of sample that reduced 50% of DPPH free radicals) were reported as means±SD. The free radical inhibition was calculated by the following equation:

$$\text{Inhibition (\%)} = [A \text{ control} - A \text{ sample} / A \text{ sample}] \times 100$$

A control= Absorbance of control solution, A sample= Absorbance of various concentrations of samples.

FRAP value

Ferric reducing antioxidant power assay is based on reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants and was performed according to previous studies [18,19].

Total phenolics content

Total Phenolics was determined colorimetrically using Folin-Ciocalteu reagent as described by Velioglu et al. 1998; 200 µL from each sample was added to 1.5 mL of Folin-Ciocalteu reagent that was 10 times diluted with distilled water and stored at 20 °C for 5 min. Then 1.5 mL of sodium bicarbonate solution (60 g/L) was added to the mixture. After 90 min at 22 °C, absorbance was measured at 765 nm by spectrophotometer; the experiments were repeated three times. The phenolics content calibration curves was drawn by measuring the absorption of certain concentrations (25-150 mg/L) of gallic acid as the standard and the results were stated as milligrams of gallic acid equivalents (GAE) per gram of dry matter (total extracts and fractions) as means±SD [20,21].

Brine shrimp lethality assay

The toxicity of methanol extract and hexane, chloroform, and ethyl acetate fractions on brine shrimp was evaluated according to the former studies [17,22,23].

Statistical analyses

Analyses of samples were done in triplicates and the values were reported as mean±SD. IC₅₀ and LC₅₀ were measured with Curve Expert 1.3.

Results and Discussion

Free radical scavenging effects of the total extract and different fractions of *P. daurica* ssp. *macrophylla* root were assessed with 2, 2-diphenyl-1-picryl-hydrazyl (DPPH). IC₅₀ values were displayed in table 1. Evaluation of the results indicated that the ethyl acetate (IC₅₀=16.55µg/mL) and chloroform fractions (IC₅₀=23.9µg/mL) were more potent than other samples.

The results for antioxidant power of different fractions of the root extract using FRAP method, were reported based on mmol FeII/g of the extract. Ferric reducing antioxidant power of the extracts was calculated using the calibration curve and regression equation of ferrous sulfate ($R^2 = 0.9959$, $y = 0.0008x - 0.029$). According to table 1, the antioxidant power of all samples increased concentration dependently. Among the samples, the ethyl acetate and chloroform fractions showed the highest antioxidant power.

Total phenolics content (mg of GAE/g of sample) varied from 5.83 to 72.7 mg using the standard curve of gallic acid ($R^2 = 0.9958$, $y = 0.0064x + 0.0027$) (table 1).

According to the results of toxicity assay, it was revealed that the LC₅₀s of the all samples were greater than the standard toxicity (potassium dichromate [24] LC₅₀=27.29 µg/mL), so all the tested extracts were considered to be nontoxic (table 1).

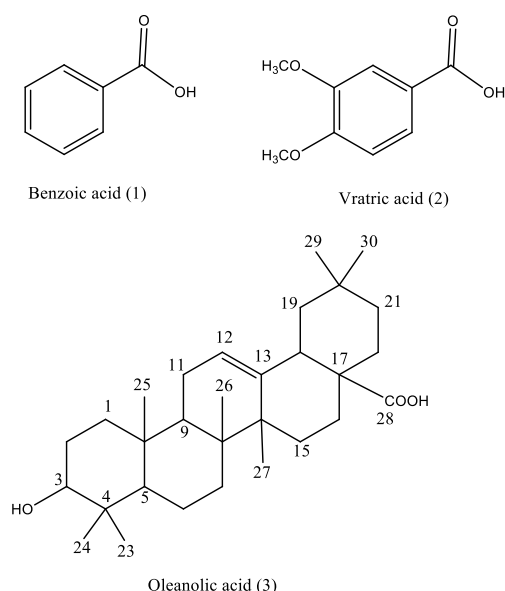
The chemical structures of the isolated compounds **1-3** from the chloroform fraction of *P. daurica* ssp. *macrophylla* root were elucidated by comparison of their NMR (¹H, ¹³C-NMR data with those reported in the literature [11-15]. Mass spectra results of substances **1**, **2** and **3** were: EIMS, *m/e* 182(M⁺), 167(M-CH₃), 152(167-CH₃), 137(M-COOH), 107(152-COOH); EIMS, *m/e* 122(M⁺), 105 (M-OH), 77 (M-COOH); EIMS, *m/e* 456 (M⁺), 438 (M-H₂O), 411 (M- COOH), 248, 233, 220, 207, 189, 175, respectively. The chemical structures of the isolated compounds have been shown in figure 1. Compound **1** was identified as benzoic acid, a simple aromatic carboxylic acid, which occurs naturally in many herbs. Compound **2** was characterized as 3, 4-dimethoxybenzoic acid (veratric acid).

Table 1. DPPH radical scavenging activity, total phenolics content (TPC), brine shrimp lethality and FRAP results of different fractions from *Paeonia daurica* ssp. *macrophylla* root

| Samples | DPPH | TPC | Brine shrimp lethality | FRAP |
|------------------------|--------------------------|-------------------------|--------------------------|--------------------|
| | IC ₅₀ (µg/mL) | mg gallic acid/g sample | LC ₅₀ (µg/mL) | mmol FeII/g sample |
| Hexane fraction | 73.91 | 34.40±95 | 679.50 | 0.31±0.02 |
| Chloroform fraction | 23.90 | 22.70±0.73 | 401.40 | 1.33±0.03 |
| Ethyl acetate fraction | 16.55 | 72.70±1.60 | 879.60 | 2.44±0.10 |
| Methanol fraction | - | - | 827.80 | 0.52±0.06 |
| Total extract | 133.81 | 5.83±0.35 | - | 0.85±0.08 |
| Trolox | 3.30 | - | - | - |

- Not examined

Benzoic acid derivatives like paeoniflorin, a benzoyl monoterpene glycoside, are the major active constituents in many species of the genus *Paeonia* [5]. Veratric acid in *Paeonia* species has not been previously reported. The compound **3** was identified as oleanolic acid, a pentacyclic triterpenoid compound, which has been reported from many medicinal plant species including *P. lactiflora* [14].

**Figure 1.** Chemical structures of isolated compounds **1**, **2**, and **3**

The total extract and some fractions of *P. daurica* ssp. *macrophylla* root, which was collected from north of Iran, were considered for evaluation of antioxidant effects using DPPH and FRAP assay and also for total phenolics contents and phytochemical investigations. According to the results, the ethyl acetate and chloroform fractions showed potent radical scavenging activity as well as antioxidant power using DPPH and FRAP assay.

Many studies have confirmed the antioxidant effects of *Paeonia* species that are associated

with the presence of phenolic compounds existing in these medicinal plants [23,25]. Lee SE et al. have reported that *Paeonia suffruticosa* root extract along with some other extracts has shown high dose-dependent DPPH radical scavenging activity with IC₅₀ value of 5.9 µg/mL [7]. Strong dose-dependent scavenging abilities on 2, 2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), hydroxyls, superoxide anions, and DPPH radicals from the flavonoid-rich flower extracts of a Chinese medicinal plant *P. ostii*, have been reported by Zhang H et al. [26]. Five main flavonoids, dihydrokaempferol, apigenin-7-O-β-D-glucoside, apigenin-7-O-β-D-neohesperidoside, kaempferol-7-O-β-D-glucopyranoside, and kaempferol-3-O-β-D-glucopyranosyl-7-O-β-D-glucopyranoside, have also been identified [4].

The methanol extract of *Paeonia rockii* root (EC₅₀=13.3 µg/mL) and n-BuOH soluble fraction (EC₅₀=6.5 µg/mL) have shown significant DPPH free-radical scavenging effects due to their high polyphenol contents [9]. The methanol extract of *P. suffruticosa* showed strong radical scavenging activity. The antioxidant activity of ethyl acetate soluble fraction was stronger than that of the other fractions with IC₅₀=1.2 µg/mL [8].

Three pure compounds, benzoic acid, veratric acid and oleanolic acid were isolated from the chloroform fraction and were identified by using ¹³C, ¹H-NMR and Mass spectroscopy methods. Based on the results, various extracts of the plant that are rich in phenolic compounds can be used in prevention and treatment of diseases associated with oxidative stress.

Benzoic acid, 3-hydroxy-4-methoxybenzoic acid, benzoyl paeoniflorin, paeoniflorin, and oleanolic acid have been isolated from several *Paeonia* species like *P. suffruticosa*, and *Paeonia lactiflora* [27-30]. Benzoic acid is used for the treatment of fungal skin diseases such as tinea in some ointments [31]. A study has shown that

veratric acid could prevent the cardiovascular dysfunction, cardiac and aortic fibrosis and lipid peroxidation, that may introduce veratric acid as a beneficial molecule in the treatment of hypertension [32,33]. The oleanolic acid, a pentacyclic triterpenoid compound, has been reported from many medicinal plant species including *P. lactiflora* [14]. Oleanolic acid and its derivatives are called multifunctional compounds with several biological activities such as anti-inflammatory, cardio protective, hepato-protective, gastro-protective, antitumor, antiviral, anti-diabetic, antimicrobial, anti-parasitic, wound healing and analgesic properties in addition to prompting apoptosis in preclinical models of cancer [34,35].

Based on our findings, the fractions and especially the ethyl acetate fraction of the plant that is rich in phenolic compounds can be suggested in prevention and treatment of diseases associated with oxidative stress, therefore further pharmacological and phytochemical studies are needed to prove useful effects of *Paeonia daurica* ssp. *macrophylla*.

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Author contributions

Seyde Nargess Sadati performed plant preparation, extraction, DPPH and FRAP tests, total phenolics content, and brine shrimp fatality of the extracts and also drafted the manuscript; Nilofar Taleb Kashefi as student, has done all tests and separation of compounds; Narguess Yassa conceived the study, and guided the isolation and identification of substances and edited the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

dry wt: dry weight