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Research Article

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Comparison of Classical and Ultrasound-Assisted Extraction Methods on Antioxidant Activities of Pharmaceutically Active Compounds from *Valeriana Officinalis*

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Abstract

Medicinal plants are good sources and rich of substances which having nutraceutical and health benefits. Valeriana officinalis L. (Valerianaceae) is a known medicinal plant used in traditional medicine worldwide. The aim of present study was to evaluate the efficiencies of three methods for evaluated for antioxidant from Valeriana officinalis. Besides, the impact of extraction methods on total phenollic and flavonoids contents and antioxidant activities of Valeriana root was investigated. The dried-root was extracted by three different methods including maceration, ultrasonic assisted, and Soxhlet assisted extraction. Antioxidant capacity was assessed using four different methods: DPPH and nitric oxide (NO) free radicals scavenging, reducing power and iron chelating activity. The total phenolic and flavonoid contents were also identified. The ultrasonic extract showed highest amount of total phenolics and flavonoids contents. In DPPH radical scavenging activity, the ultrasonic assisted extract, (IC50=0.546 µg/ml) had a higher activity from other extracts. In reducing power assay, maceration extract showed the highest activity. In DPPH radical scavenging activity, IC50 for ultrasonic extract, soxhlet assisted extraction and maceration extract were 0.546, 0.816 and 0.678 µg/ml, respectively. Here, the maceratin extract showed the highest activity, too. The results clearly indicated the extraction methods used in this study significantly affected antioxidant capacities and total phenolic and flavonoids contents. Ultrasonic assisted extraction and Soxhlet methods were found to be more efficient in extraction of antioxidant components of valeriana.

Keywords: Valeriana, Extraction Method, Antioxidant, Total Phenolic, Total Flavonoids

Introduction

The roles of oxidants have been known in many acute and chronic diseases including cancer, cardio vascular troubles and neurodegenerative diseases. Medicinal plants contain a wide variety of natural antioxidant, such as phenolic acids, flavonoids and tannins, which posses more antioxidant property than dietary plants [1]. Recently, attention has been focused on medicinal plants for their phenolic compounds and related potent antioxidant activity. The investigations have been demonstrated that natural antioxidants are of great value in prevention or treatment of pathological disorders [2]. The genus *Valeriana officinalis* belongs to Valerianaceae, which spread throughout the world. Valerian species (Valerianaceae) have numerous medicinal uses as stone inhibitor, expectorant, anti-inflammatory and sedative properties. Antioxidant activities of root of this plant have been reported recently [3]. In this study, the efficiencies of three methods (ultrasonically assisted extraction, Soxhlet extraction and maceration method) were evaluated for extract antioxidants from V.officinalis. The antioxidant capacity was assessed using four different methods: 1,1-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) free radical scavengering, reducing power and iron chelating activity. The total phenolic and total flovonoids contents also were investigated. This study is an attempt to establish a scientific basis for the use of this plant in traditional medicine, and find the possible mechanism involved in its antioxidant activity.

Materials and Methods

Plant material and Preparation of Extracts

Roots of *V. officinalis* were collected from Lorestan, Iran. The roots of plant were dried in shade at room temperature and then were grounded in a course powder with the help of a suitable grinder. The powder has to store in an airtight container under cool and dry

place until further analysis required. 50 g of dried powdered sample were macerated for 24h with 300 ml of methanol. Extraction was repeated thrice. The extracts were filtered through Whatman filter paper No.1, and the resultant extracts were evaporated under rotavapour until a solid sample was obtained (maceration extracts). The sample was extracted exhaustively in a Soxhlet extractor with methanol for 24h. The crude solid extracts were freeze-dried for complete solvent removal and used as Soxhlet extracts. Sample was extracted with methanol in an ultrasonic bath at a frequency of 100 kHz for one hour (3×20 min) to yield ultrasonic extracts [4].

Ultrasonically Assisted Extraction

The roots of plant were extracted with 300 ml of methanol in an ultrasonic cleaning bath for one hour by indirect sonication at a frequency of 100 kHz and a temperature of 25°C to yield ultrasonic extract. The extract was filtered through Whatman filter paper No.1, and the filtrate was concentrated under reduced pressure at 40°C. The extract was dried, weighed (2.4 g), stored at 4°C in storage vials and used as ultrasonic extract [5].

Total Phenolic Content

The total phenolic contents of the extract were determined by Folin–Ciocalteu method [6]. The extract samples (0.5 ml of different dilutions) were mixed with 2.5 ml of 0.2N Folin-Ciocalteu reagents for 5 min followed by the addition of 2 ml of 20% (w/v) sodium carbonate. The mixture was allowed to stand for a further 2 hours in the dark, and absorbance of reaction was measured at 760 nm. The standard curve was drawn by 20-100 µg ml^{-1} solution of Gallic acid. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.

Total Flavonoid Content

The total flavonoid contents were measured by the aluminum chloride colorimetric method. Briefly, 0.5 ml solution of extracts in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum Chloride, 0.1 ml of 1M potassium acetate, and 2.8 ml of distilled water, and left at room temperature for 30 minutes. The mixture was allowed to stand for 15 min, and absorbance of the reaction mixture was measured at 415 nm. The total flavonoid contents were determined as quercetin equivalent from a standard curve which was prepared by quercetin at concentrations 10-100 µg ml^{-1}. The total flavonoid contents were calculated in terms of quercetin equivalents (QE) from a calibration curve, and the result was expressed as mg quercetin equivalents (QE) per g dry weight.

Antioxidant Properties

DPPH Radical-Scavenging Activity

Two ml of different concentrations of each extracts were added to 2 ml methanolic solution of DPPH (100 µM). After 15 minutes at room temperature in the dark, while continuously recording the absorbance decreased at 517 nm. BHT was used as standard controls. IC50 values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals [7].

Iron Chelating Capacity

To examine the strong iron chelating properties of the extracts, different concentrations of extracts were added a solution of 0.05 ml of ferrous Chloride (2mM). 0.2 ml of ferrozine solution was added. After 10 minutes, the absorbance of the solution was recorded at 562 nm. EDTA was used as a standard [8].

Ferric Reducing Power Assay

The reducing powers of extract were determined according the method of Yen and Chen (1995). Fe (II) reduction is often used as an indicator of electron donating potential, which is a significant mechanism of phenolic antioxidant activity [9]. Briefly, different amounts of extracts (25-800 µg ml^(-1)in water were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as positive control.

Nitric Oxide-Scavenging Activity

Nitric oxide was generated from sodium nitroprusside and measured by the Greiss reaction. Sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) spontaneously producing nitric oxide (NO), which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. For the experiment, sodium nitroprusside (10 mM) in phosphate-buffered saline was mixed with different concentrations (5–200 µg/ml) of each methanol extracts were dissolved in methanol and incubated at 30°C for 2 hours. The same reaction mixture without the extract but the equivalent amount of ethanol served as the control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H3PO4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 546 nm. Quercetin was used as positive control [10].

Statistical Analysis

All experiments were performed thrice and the results averaged data were expressed as mean ± SD. Linear regression analysis was used to calculate IC_{50} for each plant extract.

Discussion and Results

Total Phenolic Compounds and Flavonoid Contents

Natural phenolic compounds can act as reducing agents and quenchers of singlet oxygen and some of which can play an important role in cancer prevention and treatment [11]. Investigations have shown that consumption of foods and beverages rich in phenolic content is related to reduce the risk of atherosclerosis and cardiovascular disease [12]. Phenolic compounds from medicinal herbs and dietary plants include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others. Various bioactivities of phenolic compounds are responsible for their chemopreventive properties (e.g., antioxidant, anticarcinogenic, or antimutagenic and anti-inflammatory effects) and also contribute to their inducing apoptosis by arresting cell cycle, regulating carcinogen metabolism and ontogenesis expression, inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation, and blocking signaling pathways [13].

The total phenolic contents three methods (ultrasonically assisted extraction, Soxhlet extraction and maceration method) were estimated by Folin Ciocalteu's method using Gallic acid as standard. The reagent is formed from a mixture of phosphotungstic acid and phosphomolybdic acid which after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten and molybdenum. The blue coloration produced has a maximum absorption in the region of 760 nm and proportional to the total quantity of phenolic compounds originally present [14]. The gallic acid solution of concentration (20-100 ppm) conformed to Beer's Law at 760 nm with a regression co-efficient (R2) = 0.9941. The plot has a slope (m) = 0.0011 and intercept = 0.0205. The equation of standard curve is y = 0.0011x + 0.0205 (Figure 1). Total phenolic contents of *V. officinalis* root between 312.3-342.7 mg Gallic acid equivalent (GAE) /g of extract (Table 1). The total phenolic contents were in order of: Ultrasonic extract > Soxhlet extract > Maceration extract, respectively.

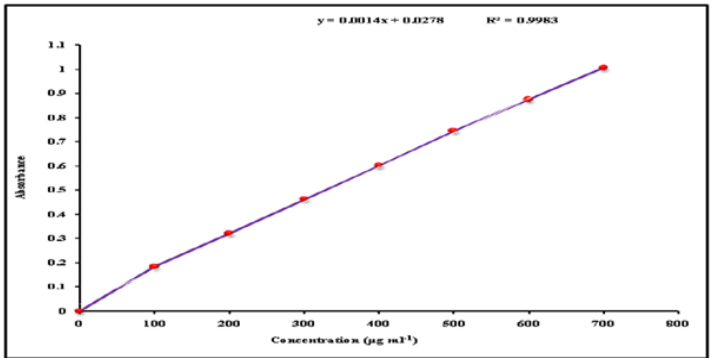

Figure 1: Total phenolic content for standard Gallic acid

Flavonoids have been linked to reducing the risk of major chronic diseases including cancer because they have powerful antioxidant activities in vitro, being able to scavenge a wide range of reactive species (e.g., hydroxyl radicals, peroxyl radicals, hypochlorous acid, and superoxide radicals) [15]. Many flavonoids chelate transition metal ions such as iron and copper, decreasing their ability to promote reactive species formation. These activities of flavonoids are related to their structures. Flavones containing more hydroxyl groups exhibit very high radical scavenging activity, for example, myricetin, quercetin, rutin, and quercetin are well-known potent antioxidants. Flavones with additional catechol structure (3-galloyl group) have significantly enhanced antiradical activity [16]. Moreover, glycosylation of hydroxyl groups and substitution of other substituents (e.g., methoxy groups) also affects the antioxidant activity of flavonoids. The total flavonoid content three methods (ultrasonically assisted extraction, Soxhlet extraction and maceration method) were measured with the aluminum chloride colorimetric assay using quercetin as standard. Aluminum chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavones, which gives a maximum absorption at 510 nm [17]. The quercetin solution of concentration (10-100 ppm) conformed to Beer's Law at 415 nm with a regression co-efficient (R^2) = 0.9996. The plot has a slope (m) = 0.0125 and intercept = 0.0145. The equation of standard curve is y = 0.0125x + 0.0145 (Figure 2). Total flavonoid contents of extracts were between 79.6-127.3 mg quercetin equivalent (QE) /g of extract, as derived from a standard curve (y = 0.0125x + 0.0145, R^2= 0.9996) (Table 1). The total flavonoid contents were in order of: Ultrasonic extract > Soxhlet extract > Maceration extract, respectively. Ultrasonic extract had higher flavonoids contents than other extracts, too. Data obtained from the total phenolic content and total flavonoid content and methods support the key role of phenolic compounds in free radical scavenging and/or reducing systems.

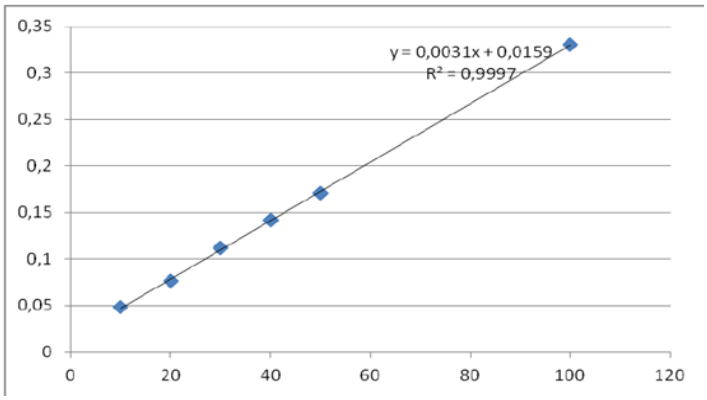

Figure 2: Total flavonoid content for standard quercetin

DPPH Radical-Scavenging Activity

Plants rich in secondary metabolites, including phenolic, flavonoids and carotenoids, have antioxidant activity due to their redox properties and chemical structures. The methanolic root extract of *V. officinalis* had strong antioxidant activity against all the free radicals investigated. DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances [18]. In the DPPH assay, the antioxidants are able to donate hydrogen to reduce the stable radical DPPH to the yellow-colored non-radical diphenyl-picrylhydrazine (DPPH-H). DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants based on the absorption change of DPPH at 517 nm measured spectrophotometrically [19]. The capacities of extracts to scavenge DPPH are shown in Table 1. Ultrasonic extract showed the highest activity (IC50 = 0.546 ± 5.3 µg ml-1) followed by Soxhlet extract with IC50 = 0.678 ± 2.6 µg ml-1. BHA was used as positive control and IC50 of BHA was 0.529 ± 3.2 µg ml-1. Extracts showed a concentration-dependent antiradical activity by inhibiting DPPH radical. Ultrasonic extract with higher phenol and flavonoids contents showed the best activity and maceration extract with lowest phenol and flavonoid contents were the least active.

Reducing Power

Antioxidant compounds reduce Fe^{3+}-ferricyanide complexes to the ferrous (Fe^{2+}) form. The Prussian blue colored complex is formed by adding FeCl3 and then ferric form (Fe^{2+}) converted to the ferrous form (Fe^{2+}). Therefore, the amount of reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm. In this assay, the yellow color of the test solution changes to green or blue depending on the reducing power of the antioxidant. A higher absorbance indicates higher ferric reducing power [20]. Figure 3 shows the dose-response curves for the reducing powers of the extracts. The reducing power of the extracts increased with increase in their concentrations. The extracts exhibited fairly good reducing power at 25 and 800 µg ml-1 but they were less than that of vitamin C as positive control ($p < 0.001$) and merely the maceration extract more than vitamin C. Because the good reduc-

tive ability of Maceration extract, it was evident that these extracts showed reductive potential and could serve as strong electron donors, terminating the radical chain reaction.

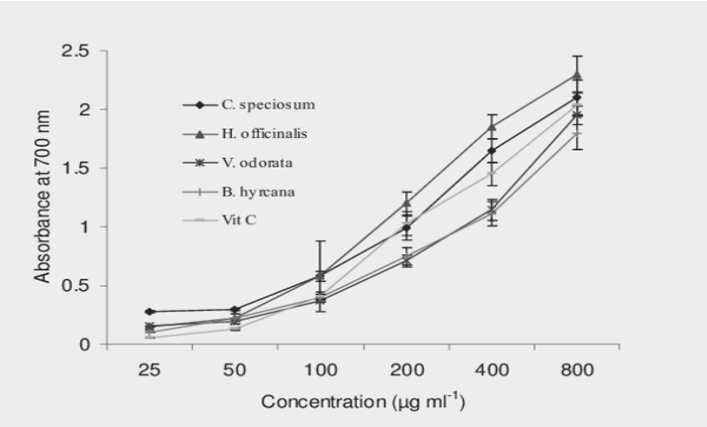

Figure 3: Reducing power of different extraction methods of *V. officinalis* root

Nitric Oxide-Scavenging Activity

Nitric oxide (NO) is a free radical produced in mammalian cells, involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases. Nitric oxide is a very unstable species under aerobic conditions. It reacts with O_2 to produce stable product nitrate and nitrite through intermediates NO_2, N_2O_4, and N_3O_4. NO scavenging capacity is determined by the decrease in the absorbance at 550 nm, induced by antioxidants. It is estimated by using Griess reagent and in presence of test compound which is a scavenger that the amount of nitrous acid will decrease [21]. The association of nitric oxide (NO) with cardiovascular disease has long been recognized and the extensive research on this topic has revealed both pro- and anti-atherosclerotic effects [22]. The results of NO scavenging activity of extracts are shown in Table 1. Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25°C for 2 h resulted in linear time-dependent nitrite production, which is reduced by the tested methanolic extracts of roots of *Valeriana officinalis*. This may be due to the antioxidant principles in the extract, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. It is to be noted that ultrasonic extract a greater inhibition comparative to others but less than quercetin which has shown 0.19 inhibition of NO. Nitric oxide scavenging activity of extracts between 0.59 and 1.69 mg ml-1. Inhibition increased with increasing concentration of the extracts. Activity was in order of Ultrasonic extract > Soxhlet extract > Maceration extract. Phenolic compounds and flavonoids have been reported to be associated with ant oxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals [23]. The nitric oxide scavenging activity of flavonoids and phenolic compounds are known, we can speculate that these constituents might be responsible for the observed nitric oxide scavenging activity [24].

Table 1: Phenol and flavonoid contents and antioxidant activities of *V. officinalis* root.

Table 1. Phenol and flavonoid contents and antioxidant activities of Officinalis root. a IC50 of BHA was 0.529±3.2 g/ml. b EDTA used as control (IC50 =16.3±0.3 g/ml).c IC50 for quercetin was 0.19±0.02 g/ml.

Extraction Method	Total phenolic contents (GAE mg/g)	Total flavonoid contents (QE mg/g)	DPPH radical scavenging IC_{50} (µg/ml)[a]	Fe^{2+} chelating ability IC_{50} (µg/ml)[b]	NO scavenging activity IC_{50} (µg/ml)[c]
0.59±0.03	202±8.3	0.546±5.3	127.3±2.4	390.6±1.3	Ultrasonic
0.76±0.04	186±5.2	0.678±2.6	102.4±1.7	342.7±2.7	Soxhlet
1.69±0.6	421±14.2	0.816±4.2	79.6±1.5	312.3±3.2	Maceration

Iron Chelating Capacity

Ferrozine can quantitatively form complexes with Fe2+. The absorbance of Fe2+-ferrozine complex was decreased dose-dependently. Soxhlet extract showed the best activity followed by ultrasonic extract. Soxhlet extract had the highest amount of phenol and flavonoid contents (Table 1). It is known that flavonoids with a certain structure and particularly hydroxyl position in the molecule can act as proton donating and show radical scavenging activity [25].

Conclusion

The extraction efficiencies of the three methods for the extraction of antioxidants from *V. officinalis* root were evaluated. The results clearly indicated the extraction methods used in this study significantly affected antioxidant capacities and total phenolic and flavonoids contents. Ultrasonic assisted extraction and Soxhlet methods were found to be more efficient in extraction of antioxidant components of valeriana [26].

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