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Evaluation of three methods for the extraction of antioxidants from leaf and aerial parts of *Lythrum salicaria* L. (Lythraceae)

Jamshidi, M., Shabani, E., Hashemi, Z. and *Ebrahimzadeh M. A.

Pharmaceutical Sciences Research Center, School of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran

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Abstract

Lythrum salicaria L. (Lythraceae) known as purple loosestrife used for its beneficial health effects against as many human diseases such as diarrhea, hemorrhoid, eczema internally and/or externally. The effects of the different extracting methods on the total phenolic (TP) and total flavonoid (TF) contents of *Lythrum salicaria* L. extracts were investigated. Dried leaf and flower of *L. salicaria* were extracted with three different methods (percolation method, ultrasonic assisted extraction and polyphenol fraction). The antioxidant activities of extracts were investigated by different methods, i.e. 1,1-diphenyl- 2-picryl hydrazyl radical (DPPH), nitric oxide and hydrogen peroxide scavenging activities as well as reducing power using standard methods. Phenol and flavonoid contents were also determined as gallic acid and quercetin equivalents, respectively. Extracts exhibited good antioxidant activity in all test models. In general, flower showed better activity than leaf extracts. Ultrasonic extract of flower showed the best activity in scavenging of hydrogen peroxide but polyphenol fraction was the more potent one in other tests. Its higher phenolic and flavonoid contents could be responsible for its antioxidant activity.

Introduction

The genus *Lythrum* belongs to *Lythraceae*, which spread throughout the world. It is well represented in the flora of Iran, at least with 30 species in the world and 8 species. *Lythrum salicaria* L. (*Lythraceae*) has a wide range of beneficial health effects (Humadi and Istudor, 2009). *L. salicaria* known as khon fam in Iran (Khanavi *et al*., 2011). It is known as a medicinal plant and it has been an important drug for centuries (Humadi and Istudor, 2009). The whole flowering plant and the flowering branch tips of this plant are used not only in folk medicine but also in pharmaceuticals. It is used internally for diarrhea, chronic intestinal catarrh, in the form of a decoction or a fluid extract. Externally, it is used to treat varicose veins, venous insufficiency, bleeding of the gums, hemorrhoid and eczema (Mantle *et al*., 2000; Rauha *et al*., 2000; Thomson, 2000).

Phytochemical investigation of *L. salicaria* has been demonstrated phenolic acids, flavonoids, tannins, anthocyanins, glycosides (salicairine), triterpenoides and organic acids (Rauha *et al.*, 2001; Becker *et al*., 2005). The phytochemical examination carried on this plant reported that tannins were the main compounds in *L. salicaria*. It contains a notable amount of flavons represented by flavon C-glycosides (vitexin, isovitexin, orientin and isoorientin) and anthocyanins, too. In addition, vescalagin, pedunculagin, vanoleic acid dilactone, 1,6-di-O-galloylglucose, 1-O-galloylglucose and 6-O-galloylglucose were identified. Sterols as β sitosterol were also detected in this plant (Bisset and Wichtl, 1994; Rauha *et al*., 2001; Becker *et al*., 2005; Bruneton, 2005).

Numerous reports have described the application of ultrasonically assisted extraction for deriving various components (Yang *et al*., 2008; Wang *et al.*, 2008). In these reports, many researches show that ultrasonic procedure could significantly improve extraction efficiency, reduce processing time, and decrease solvent consumption. This study aimed to investigate the *in vitro* antioxidant activities of extract from *Lythrum salicaria* leaf and flower using ultrasonically assisted extraction, comparing it with that of polyphenol rich extract and classic extraction method, i.e. percolation. Studies included 1,1-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, reducing power, hydrogen peroxide and nitric oxide radical scavenging activities. The total phenolic and total flavonoid 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

*Corresponding author.
Email: *zadeh20@yahoo.com*

involved in its antioxidant activity.

Materials and Methods

Plant material and preparation of freeze-dried extract

Leaves and flowers of *L. salicaria* were collected from Sari, Iran and identified by Dr. B. Eslami. A voucher specimen (No 891) has been deposited in Sari School of Pharmacy herbarium. Parts of plant were dried at r. t. and coarsely ground (2-3 mm) before extraction. Each part (100 g) was extracted by percolation using 70% ethanol for 24 h at r. t. The extract was then separated from the sample residue by filtration through Whatman No.1 filter paper. Extraction was repeated thrice. The resultant extracts were concentrated in a rotary evaporator until a crude solid extracts were obtained which was then freeze-dried for complete solvent removal and used as percolation (PER) extract.

Preparation of polyphenol fraction

Polyphenols were extracted according to our recently published paper (Rabiei *et al*., 2012). The extraction was performed twice at 20°C in a shaking incubator. Extracting time was 30 min, and extracting solvent was 100 ml of methanol/acetone/water (3.5/ 3.5/ 3) containing 1% formic acid. All extracts were collected and evaporated under vacuum at 35-40°C to remove methanol and acetone. Lipophilic pigments were then eliminated from the aqueous phase by extraction with petroleum ether. The aqueous phase was collected and subjected to further extraction for three times by ethyl acetate. Organic phases were collected and concentrated over a rotary vacuum until a crude solid extract was obtained, which was then freeze-dried for complete solvent removal and used as polyphenol (PP) fraction.

Ultrasonically assisted extraction

Parts of plant were extracted with 70% ethanol in an ultrasonic cleaning bath for one hour by indirect sonication at a frequency of 100 kHz and a temperature of 25 ± 3°C to yield ultrasonic extract. The extract was then separated from the sample residue by filtration. The resultant extract was concentrated in a rotary evaporator until a crude solid extract was obtained which was freeze-dried for complete solvent removal and used as ultrasonic (US) extract (Rabiei *et al.*, 2012).

Determination of total phenolic compounds and flavonoid content

Total phenolic compound contents were determined by the Folin-Ciocalteu method (Ebrahimzadeh *et al*., 2008). The extract samples (0.5 ml of different dilutions) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagent for 5 min and 2.0 ml of 75 g/l sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents (GAE). Flavonoid content of each extract was determined by following colorimetric method (Ebrahimzadeh *et al*., 2008). Briefly, 0.5 mL solution of each plant extracts in methanol were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water, and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam Perkin Elmer UV/ Visible spectrophotometer. Total flavonoid contents were calculated as quercetin equivalents (QE) from a calibration curve.

DPPH radical-scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts (Ebrahimzadeh *et al.*, 2008). Different concentrations of each extracts were added, at an equal volume, to methanolic solution of DPPH (100 μM). After 15 min at room temperature in the dark, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamin C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Reducing power determination

Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action (Ebrahimzadeh *et al.*, 2010a). The reducing power of extracts were determined according to the method of Yen and Chen (1995). Different amounts of each extracts (25-800 μg ml^{-1}) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, 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. Vitamin C was used as positive control.

Scavenging of hydrogen peroxide

The ability of the extracts to scavenge hydrogen peroxide was determined according to our recently published paper (Ebrahimzadeh *et al.*, 2009c). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extracts (0.1 - 1 mg ml^{-1}) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows: % scavenged $[H_2O_2] = [(A_o - A_1)/A_o] \times 100$. Where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract and standard.

Assay of nitric oxide-scavenging activity

The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of each extracts dissolved in water and incubated at room temperature for 150 min. After the incubation period, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (Ebrahimzadeh *et al.*, 2010a).

Statistical analysis

All the experimental results were centered using three parallel measurements of the mean ± SD. Analysis of variance (ANOVA) was performed. Duncan's new multiple-range test was used to determine the differences in means. All p values less than 0.05 were regarded as significant and p values less than 0.01 as very significant. The IC_{50} values were calculated from linear regression analysis.

Results and Discussion

Total phenol and flavonoid contents

Plants have evolved a wide range of mechanism to contend with health disorders, with a variety of antioxidant molecules and enzymes. Their phenolics are multifunctional and can act as reducing agents and quenchers of singlet oxygen. Studies have shown that consumption of foods and beverages rich in phenolic content is correlated with reduced the risk of atherosclerosis and cardiovascular disease (Manach *et al*., 2005). Antioxidant activity of plant origin components can be ascribed mainly to the presence of phenolic compounds. The Folin-Ciocalteu phenol reagent assay is used widely for a crude estimation of the amount of phenolic compounds in extracts. This method is based on the reducing power of the phenols, which react with this reagent to form chromogens that can be detected by a spectrophotometer at 760 nm (Ebrahimzadeh *et al*., 2009a).

Total phenol compounds, reported as gallic acid equivalents, were derived from a standard curve ($y = 0.0063x$, $r^2 = 0.987$). Total phenolic contents of L. salicaria leaf and flower were between 325.3 - 355.1 mg gallic acid equivalent (GAE) /g of extract. The total phenolic contents were in order of: polyphenol (PP) fraction > ultrasonic (US) extract > percolation (PER) extract. Flower had higher amounts of phenols than leaf in all extracts.

Flavonoids are widely distributed in the plant and are responsible for the colour of fruit and flowers (Cook and Samman, 1996). As the products of secondary metabolism in plants, they are of interest to the pharmaceutical and food industries because of their antioxidant activity (Ebrahimzadeh *et al*., 2009b). Using this method, flavonoids with some specific chemical structure can react with Al^{3+} and form a red complex, which gives a maximum absorption at 510 nm. Total flavonoid contents of extracts were between 39.59 - 66.34 mg quercetin equivalent (QE) /g of extract, as derived from a standard curve ($y = 0.0067x + 0.0132$, $r^2 = 0.999$). The total flavonoid contents were in order of: PP fraction > PER extract > US extract. Flavonoids may slow the pathogenesis of some disease by their scavenging effects, antioxidant recycling and metal chelating activity. Epidemiological evidence suggests an inverse relationship between intake of dietary flavonoids and risk of cardiovascular disease (Hertog *et al*., 1993). Phenolic compounds, such as flavonoids, phenolic acids, and tannins, are considered to be a major contributor to the antioxidant activity in medicinal plants. These antioxidants also possess various biological activities such as anti-inflammatory and anti-carcinogenic activities. These activities may be related to their antioxidant activity (Ebrahimzadeh *et al*., 2010a).

DPPH radical-scavenging activity

DPPH radical scavenging activity has been extensively used for screening antioxidants. DPPH

Table1. Total phenol and flavonoids contents and antioxidant activities of *L. salicaria* flower and leaf

Part used Extraction method	Total phenolic contents (GAE mg g^{-1})	Total flavonoid contents (QE mg g^{-1})	DPPH radical scavenging, IC_{50} (µg ml^{-1})[a]	NO scavenging activity, IC_{50} (mg ml^{-1})[b]	H_2O_2 scavenging activity, IC_{50} (µg ml^{-1})[c]
Flower					
Ultra	351.7 ± 13	18.69 ± 1.1	129.7 ± 2.5	0.85± 0.03	86.21 ± 2.75
Per	350 ± 22	39.59 ± 1.7	792.4 ± 17.6	1.79 ± 0.5	195.08 ± 3.91
PP	355.1 ± 15	66.34 ± 1.5	110.9 ± 3.8	0.69± 0.02	90.73 ± 1.85
Leaf					
Ultra	329.8 ± 10	20.09 ± 0.8	135.0 ± 4.1	0.76± 0.02	138,80 ± 4.96
Per	325.3 ± 19	29.62 ± 1.3	471.6 ± 13.3	1.41 ± 0.07	200.69± 13.38
PP	347 ± 15	60.87 ± 1.9	45.7 ± 1.4	0.73 ± 0.03	97.78 ± 2.11

Ultra: Ultrasonic; Per: Percolation; PP: Polyphenol Fraction.
[a] IC_{50} of BHA was 53.8 ± 3.17, vitamin C, 5.05 ± 0.12 and quercetin 5.28 ± 0.43 µg ml^{-1}, respectively.
[b] IC_{50} for quercetin was 0.16 ± 0.01 mg ml^{-1}.
[c] IC_{50} for vitamin C and quercetin were 21.4 ± 0.1 and 52.0 ± 3.1 µg ml^{-1}, respectively.

radical is scavenged by polyphenolic compounds through the donation of hydrogen and forming of reduced DPPH. Substances which are able to perform this reaction can be considered as antioxidants and radical scavengers. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm (Ebrahimzadeh *et al*., 2010a) Generally antioxidants will react with DPPH at a very rapid rate. Moreover, this method has been used widely to evaluate the radical scavenging ability of antioxidants from different plants due to its advantage of short time and sensibility (Ebrahimzadeh *et al.*, 2010a). Well-known antioxidant compounds, ascorbic acid, quercetin and BHA were used as positive control.

Extracts showed a concentration-dependent antiradical activity by inhibiting DPPH radical. IC_{50} for DPPH radical scavenging activity was between 45.7 – 792.4 µg ml^{-1}. The IC_{50} values for vitamin C, quercetin and BHA were 5.05 ± 0.12, 5.28 ± 0.43 and 53.8 ± 3.17 µg ml^{-1}, respectively. IC_{50} for DPPH radical-scavenging activity were in order of: PP fraction > US extract > PER extract. Polyphenol fraction with higher total phenol and flavonoid contents showed most potent activity than other fractions (Table 1).

Reducing power

Figure 1 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 400 µg ml^{-1} but were, however, less than that of vitamin C ($p < 0.001$).

Reducing power has been used as an antioxidant capability indicator of medicinal herbs. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+}. This is used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action (Ebrahimzadeh *et al*., 2010a). Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's

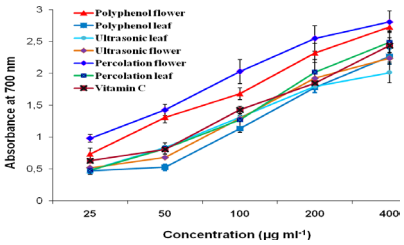

Figure 1. Reducing power of different extraction method of *L. salicaria* flower and leaf

Prussian blue at 700 nm. The greater the intensity of the color, the higher is the antioxidant activity of the sample. Figure 1 shows the dose- response curves for the reducing powers of *L. salicaria* flower and leaf. It was found that the reducing powers of extracts also increased with the increase of their concentrations. Flower showed significantly more potent reducing power than leaf extracts ($p < 0.01$). Percolation and polyphenol fractions of flower showed better activity than vitamin C which used as positive control ($p < 0.01$). Because the good reductive ability of percolation and polyphenol fractions, it was evident that these extracts did show reductive potential and could serve as strong electron donors, terminating the radical chain reaction.

Nitric oxide-scavenging activity

Table 1 shows the data for scavenging activities. The extracts showed weak nitric oxide scavenging activity of between 0.69 and 1.79 mg ml^{-1}. Inhibition increased with increasing concentration of the extracts. IC_{50} of quercetin was 0.16 ± 0.01 mg ml^{-1}. Activity was in order of PP fraction > US extract > PER extract. Again, Polyphenol fraction of flower showed the best activity (Table 1). In addition to ROS, nitric oxide is also implicated in inflammation, cancer and other pathological conditions. The plant/ plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health (Ebrahimzadeh *et al.*, 2010b).

Hydrogen peroxide scavenging

The differences in H_2O_2 scavenging capacities between the extracts may be attributed to the structural features of their active components, which determine their electron donating abilities. The extracts scavenged hydrogen peroxide in a concentration-dependent manner (Table 1). All extracts showed good scavenging activity. IC_{50} for H_2O_2 scavenging was 86.2 -200.6 µg ml^{-1} while IC_{50} values for vitamin

C and quercetin were 21.4 ± 0.1 and 52.0 ± 3.1 µg ml^{-1}, respectively (Table 1). Scavenging of H2O2 by extracts may be attributed to their phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water. The differences in H_2O_2 scavenging capacities between the extracts may be attributed to the structural features of their active components which determine their electron donating abilities. Polyphenol fraction with higher total phenol contents showed very good activity but here flower ultrasonic extract (with high total phenol contents) showed the most activity. Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems (Ebrahimzadeh *et al*., 2009c).

Conclusion

Lythrum salicaria leaf and flower extracts exhibited good antioxidant activity in all test models. The results showed that polyphenol fraction was rich in polyphenol and flavonoid compounds and that it had significant antioxidant and free radical scavenging activities in different test. Thus, this study gives strong support for expanding the investigations of the polyphenol fraction for use in the food industry. It also provides useful information on pharmacologic activities associated with the free radicals of a traditional folk remedy. Further investigation of individual compounds, their *in vivo* antioxidant activities is needed.

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