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ANTIOXIDANT PROPERTIES OF *GALIUM VERUM*

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ABSTRACT

The genus *Galium* (Rubiaceae), comprised of approximately 1300 species and used for the treatment of a variety of pathological conditions in folk medicine of many cultures. In this study, antioxidant activities of aerial parts of *G. verum* were investigated employing various in vitro assay systems, i.e. DPPH and nitric oxide radical scavenging, reducing power and H_2O_2 scavenging. IC_{50} for DPPH radical-scavenging activity was 59.6 ± 0.04 µg ml^{-1}. The extract exhibited potent reducing power at 50 - 800 µg ml^{-1} that were comparable with Vitamin C. Also, extract showed very strong nitric oxide-scavenging. IC50 was 1.7 ± 0.01 µg ml^{-1}. Extract was capable of scavenging hydrogen peroxide in a concentration dependent manner. It showed very strong activity. At 50 µg ml^{-1}, percentage of inhibition was 92.5 %. The extract exhibited very potent antioxidant activities in all tested models. The total amount of phenolic compounds in extract was determined as gallic acid equivalents (753 ± 21 mg g^{-1} of extract) and total flavonoid contents were calculated as quercetin equivalents (151.25 ± 8.2 mg g^{-1} of extract) from a calibration curve. This plant was a good source of phenols and contains very high amount of total flavonoids and phenolic compounds. The very potent antioxidant activity may be attributed to the presence of phenols and flavonoids in the extract.

Keywords: Antioxidant activity, *Galium verum*, Radical scavenging, Reducing power.

INTRODUCTION

Free radicals and reactive oxygen species (ROS) are well known inducers of cellular and tissue pathogenesis leading to more than 100 diseased including inflammatory disorders, malaria, AIDS, heart disease, stroke, arteriosclerosis, diabetes and cancer, Parkinson´s and Alzheimer`s diseases, and aging processes (Di Matteo V. 2003; Nabavi SF et al. 2016). Minimizing oxidative damage may well be one of the most important approaches to the primary prevention of these diseases and health problems. Antioxidants provide protection to living organisms from this damage caused by uncontrolled production of ROS and the concomitant lipid peroxidation, protein damage and DNA strand breaking (Ghosal S. 1996). Antioxidants have been detected in a large number of foods and plant extracts. Among the various medicinal plants, some endemic and edible species are of particular interest because they may be used for producing raw materials or preparations containing phytochemicals with significant antioxidant capacities and high content of mineral with health benefits (Ebrahimzadeh MA et al. 2010a). Although there are some synthetic antioxidant compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are commonly used in processed foods, it has been reported that these compounds may have side effects (Nabavi SF et al. 2016). So the use of traditional medicine is widespread, and plants still present a large source of

natural antioxidants that might serve as leads for the development of novel drugs.The genus Galium (Rubiaceae), comprised of approximately 1300 species, is used for the treatment of a variety of pathological conditions such as hepatitis and skin infections, as a sedative, diuretic and antidiarrheal and in the treatment of some complaints, gout and epilepsy in folk medicine of many cultures (Bolivar P et al. 2011). Compounds isolated from the genus Galium include iridoid glucosides, triterpene saponins, anthraquinones and flavonoids (Banthorpe DV et al. 1995; De Rosa S et al. 2000). High content of chlorogenic, caffeic and coumaric acids have been reported from *G. verum* (Danila AO et al. 2011). The genus Galium is represented in Iran by about 50 species and *Galium verum* (Lady´s Bedstraw or Yellow Bedstraw, called Shir Panir in Persian) is one of these group plants (Mozaffarian V. 2007). Gallium species are traditionally used to coagulate milk because of an enzyme in their chemical composition. For this reason, this plant is known as "yogurt herb" (Shafaghat A et al. 2010). Antioxidant properties of *G. verum* from Serbia ant Turkey have been reported (Lakic NS et al. 2010; Demirezer LO et al. 2006). In this study, the antioxidant activity of aerial parts of *G. verum* L. were examined employing four various *in vitro* assay systems, i.e. DPPH and nitric oxide radical scavenging, reducing power and scavenging of hydrogen peroxide, in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

MATERIALS AND METHODS

Plant material and preparation of freeze-dried extract

Aerial parts of *Galium verum* L. were collected from Gadook in north of Iran, in summer 2014 and identified by Dr. B. Eslami. A voucher (No. 870) has been deposited in Sari School of Pharmacy Herbarium. The materials were dried at room temperature and coarsely ground (2-3 mm) before extraction. 100 g of powder was extracted at room temperature by percolation using methanol for 24 h at room temperature. The extract was then separated from the sample residue by filtration through Whatman No.1 filter paper, and repeated three times. 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 (yield, 21.5%).

Determination of total phenolic compounds and flavonoid content

Total phenolic compound contents were determined by the Folin-Ciocalteau method (Ebrahimzadeh MA et al. 2008). The extract sample (0.5 ml) was mixed with 2.5 ml of 0.2 N Folin-Ciocalteau 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. Total flavonoids were estimated using $AlCl_3$ colorimetric method (Ebrahimzadeh MA et al. 2008). To 0.5 ml of sample, 0.5 ml of 2% AlCl3 ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid contents were calculated as quercetin 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 extract (Ebrahimzadeh MA et al. 2010b). Different concentrations of extract were added, at an equal volume, to methanolic solution of DPPH (100 M) at dark. After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamin C and BHA were used as standard controls. IC50 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. The reducing power of *G. verum* was determined according to the method of Yen and Chen. 2.5 ml of extract (25-800 g ml^{-1}) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 ml, 1%). The mixture was incubated at 50 ^{o}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 FeCl3 (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 (Ebrahimzadeh MA et al. 2010b).

Assay of nitric oxide-scavenging activity

In this experiment, 1 ml of sodium nitroprusside (10 mM) in phosphate-buffered saline was mixed with 1 ml of extract (at different concentrations) dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extract but with an equivalent volume of water, served as control. Following the incubation period, 0.5 ml of Griess reagent was added. The absorbance was measured at 546 nm. Quercetin was served as positive control (Ebrahimzadeh MA et al. 2010c).

Scavenging of hydrogen peroxide

The ability of the extract to scavenge hydrogen peroxide was determined according to our recently published paper (Ebrahimzadeh MA et al. 2010b). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). 1.4 ml of extract (at different concentrations) in distilled water was added to a H2O2 solution (0.6 ml, 40 mM). The absorbance of H2O2 at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without H2O2. The percentage of hydrogen peroxide scavenging by the extract and standard compounds was calculated as follows: % Scavenged [H2O2] = [(Ao − A1)/Ao] × 100 where Ao was the absorbance of the control and A1 was the absorbance in the presence of the sample of extract and standard.

RESULTS AND DISCUSSIONS

Total phenol and flavonoid contents

Plants have been used traditionally for the treatment and prophylaxis of different disorders. The protection has been attributed to plant antioxidants such as polyphenols and vitamins C, E and β-carotene (Prior R. 2003). The total phenol content was measured by Folin Ciocalteu reagent in terms of gallic acid equivalent by reference to standard curve ($y = 0.005x + 0.062$, $r^2 = 1$) (Fig. 1). The total phenolic content of aerial parts of *G. verum* was 753 ± 21 mg gallic acid equivalent/g of extract. This plant is a good source of phenols and contains very high amount of total phenolics. The total flavonoid contents were 151.25 ± 8.2 mg quercetin equivalent/g of extract, by reference to standard curve ($y = 0.006x + 0.014$, $r^2 = 0.998$) (Fig. 2). It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of the actions of flavonoids are through scavenging or chelating processes (Kessler M et al. 2003). The compounds, such as flavonoids, which contain hydroxyl groups, are responsible for the radical scavenging effect in the plants (Ebrahimzadeh MA et al. 2009). Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases (Zhao CC et al. 2006). According to our study, the contents of these phytochemicals in *G. verum* can explain its very strong antioxidant activity.

DPPH radical-scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical DPPH method is an easy, rapid, and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (Ebrahimzadeh MA et al. 2010b). The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (Danila AO et al. 2011). DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron-donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Ebrahimzadeh MA et al. 2010b). The amount of reduced DPPH could be quantified by measuring the decrease in absorbance at 517 nm. The results of previous studies with different antioxidant assay on *G. verum* suggest that polyphenol content should be considered as an important feature of this plant, as some of its effects, such as antioxidant activity, could be attributed to the presence of these constituents (Ebrahimzadeh MA et al. 2008a). The capacity of extract to scavenge DPPH was measured and the results are shown in Figure 3. It was found that the radical- scavenging activities of extract increased with increasing concentration. IC_{50} for DPPH

radical-scavenging activity was 59.6 ± 0.04 µg ml^{-1}. The IC_{50} values for Ascorbic acid and BHA were 8.78 ± 0.21 and 92.9 ± 4.5 µg ml^{-1}, respectively.

Reducing power of G. verum extract

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 4 shows the dose-response curves for the reducing powers of the extracts from *G. verum*. It was found that the reducing power of extract increased with the increase of their concentration. The extract exhibited strong reducing power at 50 - 800 µg ml^{-1} that were comparable with Vitamin C ($P > 0.05$, at 200-800 µg ml^{-1}). Because the reductive ability of the extract was significantly comparable to Vitamin C, it was evident that the extract showed reductive potential and could serve as electron donor, terminating the radical chain reaction.

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 (Ebrahimzadeh MA et al. 2010c). The extracts showed very strong nitric oxide-scavenging. IC_{50} was 1.7 ± 0.01 µg ml^{-1}.The percentage of inhibition was increased with increasing concentration of the extract. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Shafaghat A et al. 2010). Natural 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.

Scavenging of hydrogen peroxide

Scavenging of H_2O_2 by *G. verum* extract may be attributed to its phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water (Demirezer L et al. 2006). Extract was capable of scavenging hydrogen peroxide in a concentration dependent manner. It showed very strong activity. At 50 µg ml^{-1}, percentage of inhibition was 92.5 %. The IC50 values for ascorbic acid and BHA were 21.4 and 52.0 µg ml^{-1}, respectively. 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 MA et al. 2010b).

Figure 1
Standard curve of gallic acid.

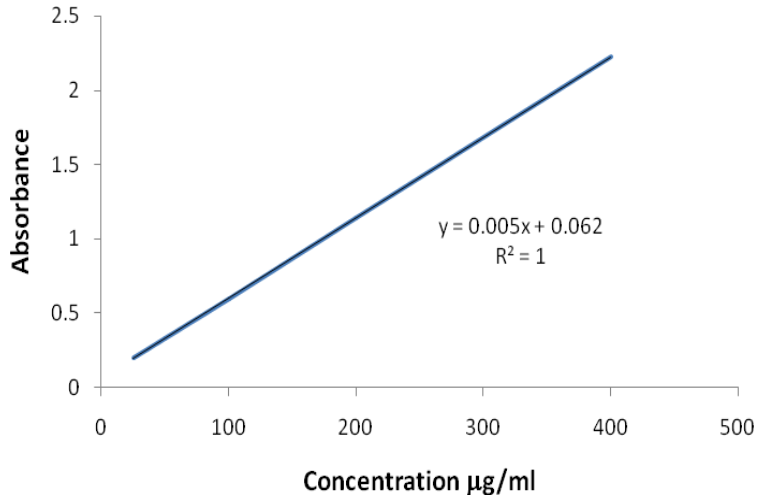


Figure 2
Standard curve of quercetin.

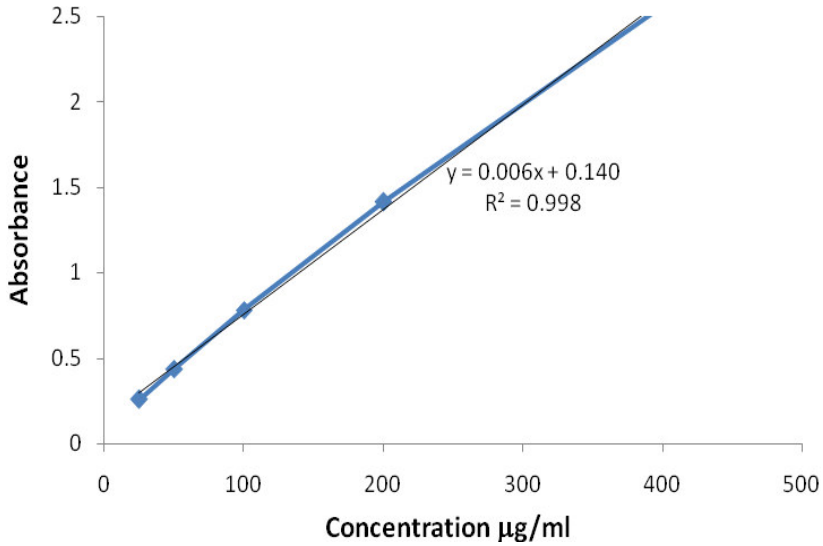


Figure 3
DPPH radical-scavenging activity of Galium verum. BHA used as standard.

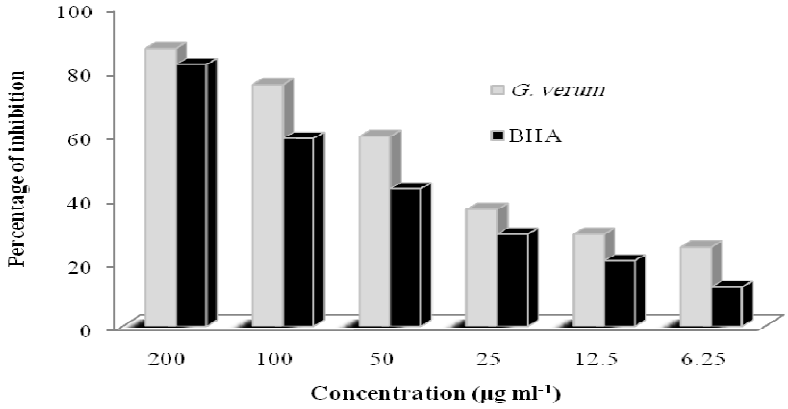
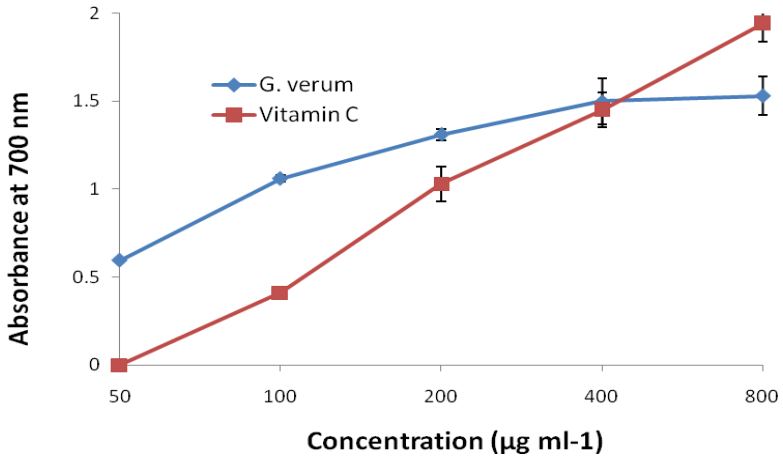


Figure 4
Reducing power of Galium verum. Vitamin C used as standard



CONCLUSION

G. verum aerial parts exhibited good but different levels of antioxidant activities in nearly all the models studied. This plant was a good source of phenols and contains very high amount of total flavonoids and phenolic compounds. Its potent antioxidant activity may be attributed to the presence of phenols and flavonoids in the extract. Further investigations of individual compounds as cost effective food/feed additives for human health and their in vivo antioxidant activities or other effects are needed.

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CONFLICT OF INTEREST

Conflict of interest declared none

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