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Research Paper

Antioxidant and free radical scavenging activities of *Hypericum perforatum* L. (st. John's wort)

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Abstract: *Hypericum perforatum* is used as a folk remedy for the treatment of various neurological disorders. In this study, antioxidant activity of flower-bearing branches of *H. perforatum* was investigated employing various in vitro assay systems. IC₅₀ for DPPH radical-scavenging activity was $96.0 \pm 3.7 \mu\text{g ml}^{-1}$. Extract showed potent nitric oxide scavenging activity between 6.25 and $100 \mu\text{g ml}^{-1}$. Percentage of inhibition was increased with increasing concentrations of the extract. IC₅₀ was $21.1 \pm 1.8 \mu\text{g ml}^{-1}$. Extract showed good reducing power that was comparable with vitamin C ($p > 0.05$). Extract showed very weak Fe²⁺ chelating ability. Percentage of inhibition was 41% at $200 \mu\text{g ml}^{-1}$ in scavenging of H₂O₂. This plant was a good source of phenols and contains very high amount of total phenolics. Total phenol compounds, as determined by the Folin Ciocalteu method, was $505.7 \pm 18 \text{ mg GAE}$ and the total flavonoid content, by AlCl₃ method was $23.8 \pm 1.6 \text{ mg QE}$. Antioxidant activity may be attributed, at least in part, to the presence of high amount of phenols and flavonoids in the extract. Potent nitric oxide scavenging activity may explain reported anti-inflammatory and CNS activities of this plant. These activities may be partially mediated by NO pathway.

Keywords: Antioxidant activity, *Hypericum perforatum*, DPPH, Nitric oxide

Introduction

Reactive oxygen species (ROS) are generated by many redox processes that normally occur in the metabolism of aerobic cells which are highly reactive and harmful to the cells. If not eliminated, they can damage important molecules, such as proteins, DNA, and lipids (Fang and Yang, 2002). Oxidative stress is an important contributor to the pathophysiology of a variety of pathological conditions such as cardiovascular dysfunctions, atherosclerosis, inflammation, carcinogenesis, drug toxicity and neurodegenerative diseases (Aruoma, 1998). The study of antioxidant status during a free radical challenge can be used as an index of protection against the development of these degenerative processes in experimental condition for therapeutic measures. Cells express several defense mechanisms that help prevent the damaging effects of ROS. However, these endogenous systems are often insufficient for complete scavenging of ROS (Anderson, 1999). Hence, certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the ROS in human body. The harmful action of free radicals can be blocked by these antioxidant substances, which scavenge the free radicals and detoxify the organism (Dehpour et al., 2009). Thus, recent studies have investigated the potential of plant products as antioxidants against various diseases induced by free radicals (Zakizadeh et al., 2011). Recently, interest has considerably increased in finding naturally occurring antioxidant to replace synthetic antioxidants, which were restricted due to their side effects such as carcinogenesis (Wichi, 1988). The search for newer natural antioxidants, especially of plant origin, has been increasing ever since.

Hypericum perforatum L. (St. John's Wort, Hypericaceae) is a member of the genus *Hypericum*, of which there are 400 species worldwide (Kizil et al., 2004). It is native to Europe, Asia, Africa and North America. In recent years, the consumption of *H. perforatum* derived products has increased dramatically, and it is presently one of the most consumed medicinal plants in the world (Wills et al., 2000). The plant has a wide range of medicinal applications, including skin wounds, eczema, burns, diseases of the alimentary tract and psychological disorders (Butterweck, 2003). Most recent interest in *H. perforatum* has focused on its antidepressant effects (Butterweck, 2003); however, the herb has shown other activities including wound-healing (Pesin Suntar et al., 2010), antifungal (Milosevic et al., 2007), antimycobacterial (Fitzpatrick, 1954), antibacterial (Saddiqe et al., 2010) antiviral (Serkedjieva et al., 1990) and anti-HIV/anti-AIDS activities (Wood et al., 1990). The extract of the flowering aerial parts of *H. perforatum* has been used as a remedy against various ailments including urogenital inflammations, diabetes mellitus, neuralgia, heart diseases, the common cold, gastrointestinal disorders, jaundice, hepatic and biliary disorders, hemorrhoids and peptic ulcers in Turkish folk medicine (Yesilada et al., 1995). Its anticonvulsant (Hosseinzadeh et al., 2005), anti-inflammatory (Mascolo et al., 1987) and analgesic (Ozturk and Ozturk, 2001) activities have also been reported. Animal experiments have revealed that *H. perforatum* will also be valuable in the clinical management of abuse and dependence of various substances such as alcohol, nicotine and caffeine (Coskun et al., 2006). Aerial parts of the plant contain a spectrum of six major natural product groups: naphthodianthrones (e.g. hypericin), phloroglucinols (e.g. hyperforin), flavonoids (such as kaempferol and quercetin), biflavones, phenylpropanes and proanthocyanidins. Additionally, lesser amounts of tannins, xanthenes, essential oils, and amino acids are present (Nahrstedt et al., 1997). In spite of many reports, little is known about antioxidant activity of this plant. A flavonoid-rich extract of *H. perforatum* L. by adsorption on macroporous resin (Zou et al., 2004), aqueous ethanolic extract at 80 °C (Giotti et al., 2009) and its effectiveness on brain oxidative status of rats treated with amnesic dose of scopolamine (El-Sherbiny et al., 2003) have been reported. In the recent years, in our laboratories, some of the widely used Iranian medicinal plants used in folk medicine have been selected for the investigation of their chemical constituents, pharmacological and biological activities in an attempt to establish a scientific basis for their ethno medical uses (Ebrahimzadeh et al., 2010; Mahmoudi et al., 2009; Nabavi et al., 2010). Recently we have published some biological activities of *H. scabrum* aerial part (Eslami et al., 2011). In continuation of our research program, in order to scientifically evaluation of ethnomedical uses of *Hypericum spp.*, in vitro antioxidant activity of *H. perforatum* was evaluated in different model included 1,1-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide and nitric

oxide radical scavenging activities, iron chelatory capacity and reducing power. A possible relationship between total phenol and flavonoids and pharmacological activities was considered.

Martial mad Methods

Plant materials and preparation of freeze-dried extract

Flower-bearing branches of *Hypericum perforatum* (*Hypericaceae*) was collected from sari, Iran, in spring 2011. Sample identified by Dr. Bahman Eslami. A voucher specimen (No 861) has been deposited in Sari School of Pharmacy herbarium. Sample was dried at room temperature and coarsely ground before extraction. A known amount of sample was extracted at room temperature by percolation method using methanol. The resulting extract was concentrated over a rotary vacuum until a crude extract was obtained, which was then freeze-dried for complete solvent removal.

DPPH radical-scavenging activity

Spectrophotometric analyses were recorded on double beam Perkins Elmer UV/Visible-spectrophotometer to determine the DPPH free radical scavenging ability (Ebrahimzadeh et al., 2010). Different concentrations of extract were added, at an equal volume to methanolic solution of DPPH (100 μ M). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. BHA was used as standard controls. IC₅₀ values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Iron chelating activity

The ability of extract to chelate ferrous ions was estimated by our recently published paper (Ebrahimzadeh et al., 2008). Briefly, different concentrations of extract were added to a solution of 2 mM FeCl₂ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml), and the mixture was then shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. The percentage inhibition of ferrozine-Fe²⁺ complex formation was calculated as $[(A_0 - A_1)/A_0] \times 100$, where A₀ was the absorbance of the control, and A₁ was absorbance of mixture containing extract or standard. EDTA was used as a standard.

Assay of nitric oxide-scavenging activity

For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of *H. perforatum* extract dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extracts but with an equivalent amount of water, served as control. 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 (Dehpour et al., 2009).

Scavenging of hydrogen peroxide

The ability of the extract to scavenge hydrogen peroxide was determined according to our recently published paper (Nabavi et al., 2010). 1.4 ml of extract (0.1-3.2 mg ml⁻¹) in distilled water was added to a hydrogen peroxide solution (0.6 ml, 40 mM in phosphate buffer, pH 7.4). The absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging was calculated as follows: % Scavenged [H₂O₂] = $[(A_0 - A_1)/A_0] \times 100$ where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of extract or standard.

Reducing power determination

The reducing power of extract was determined according to our recently published paper (Nabavi et al., 2010). 2.5 ml of extract (25-800 μ g/ml) in water were mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide [K₃Fe(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 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.

Determination of total phenolic compounds and flavonoid contents

Total phenolic compound contents were determined by the Folin-Ciocalteu method (Ebrahimzadeh et al., 2008). The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteu reagent for 5 min and 2.0 ml of 75 g l⁻¹ 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. The total flavonoid content was measured by a colorimetric assay (Ebrahimzadeh et al., 2008). Briefly, 0.5 ml solution of extract in methanol were 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. Total flavonoid contents were calculated as quercetin from a calibration curve.

Statistical analysis

Experimental results are expressed as means \pm SD. All measurements were replicated three times. Statistical analyses were performed by Student's t-test. The values of P lower than 0.05 were considered statistically significant. The IC₅₀ values were calculated from linear regression analysis.

Results and Discussion

Plants have been used traditionally for the treatment and prophylaxis of different disorders. This protection has been attributed to their antioxidant components such as polyphenols (Prior, 2003). Polyphenols are important components in fruit tissues. These

compounds are thought to be instrumental in combating oxidative stress. They can prevent some oxidation-related diseases such as atherosclerosis, cardiovascular and neurodegenerative diseases and cancer (Sun et al., 2009). Total phenol compounds, as determined by Folin Ciocalteu method, are reported as gallic acid equivalents by reference to standard curve ($y = 0.0054x + 0.0628$, $r^2 = 0.987$). The total phenolic content of extract was 505.7 ± 18 mg gallic acid equivalent/g of extract (GAE). A sample (from Greece) contained 257 ± 4 GAE (Gioti et al., 2009). This plant was a good source of phenols and contains very high amount of total phenolics. The total flavonoid content was 23.8 ± 1.6 mg quercetin equivalent/g of extract powder, respectively, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.999$). Flavonoids comprise the major group of biologically active compounds in *H. perforatum* (2–4%). The flavonol aglycones identified so far include kaempferol, luteolin, myricetin and quercetin. Hyperoside (hyperin) and rutin usually dominate among the glycosides of *H. perforatum* followed by quercitrin and isoquercitrin (Saddiqe et al., 2010; Zou et al., 2004). Martonfi et al. (2001) identified a rutin-free chemotype growing in Italy. Flavonoids form a ubiquitous group of polyphenolic substances typically produced by plants. Flavonoids are of great interest for their bioactivities, which are basically related to their anti-oxidative properties (Cote et al., 2010). Flavonoids may slow the pathogenesis of atherosclerosis and cardiovascular 60 diseases by their ROS scavenging effects. There is inverse relationship between intake of dietary flavonoids and risk of cardiovascular disease (Cook and Samman, 1996). Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases (Tripoli et al., 2007).

DPPH assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH. The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples. It 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 et al., 2010). It was found that the radical-scavenging activity of extract increased with increasing concentrations (Figure 1). IC_{50} for DPPH radical-scavenging activity was $96.0 \pm 3.7 \mu\text{g ml}^{-1}$. The IC_{50} values for BHA was $92.9 \pm 4.5 \mu\text{g ml}^{-1}$. The aerial parts of a sample from Greece were studied to assess their composition and their antioxidant activity. Best activity was presented by shoots with leaves and flower extracts (ethanol, 60%) which was comparable to BHT (Gioti et al., 2009). A flavonoid rich extract was an effective scavenger in quenching DPPH with IC_{50} of $10.63 \mu\text{g ml}^{-1}$ (Zou et al., 2004).

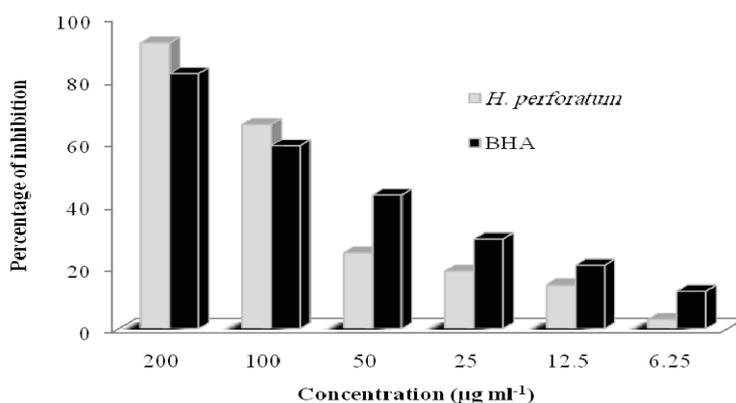


Figure 1. DPPH radical-scavenging activity of *H. perforatum*. BHA used as standard.

Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications in humans and thereby improves quality of life and overall survival in some diseases such as Thalassemia. Clinically useful iron chelators have some adverse effects which remains an urgent need to identify other chelators with an acceptable degree of tolerability. Therefore, much research has focused on natural product (Ebrahimzadeh et al., 2008). Ferrozine can quantitatively form complexes with iron. In this assay, both the extract and EDTA interfered with the complex formation of iron with ferrozine, although the iron chelating activity of the extract was weak. Activity in the metal chelating test indicated that some compounds in the extract are electron donors and can react with free radicals to convert them into more stable products and terminate radical chain reactions. The absorbance of Fe^{2+} -ferrozine complex was decreased dose dependently from 0.4 to 3.2 mg ml^{-1} . It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Ebrahimzadeh et al., 2008). Extract showed very weak Fe^{2+} chelating ability. IC_{50} was $2.1 \pm 0.3 \text{ mg ml}^{-1}$. EDTA showed very strong activity ($IC_{50} = 18 \mu\text{g ml}^{-1}$). It has been suggested that chelation of metal ion is main antioxidant action of flavonoid-rich extract of this plant (Zou et al., 2004). Our results do not confirm this report at least for iron chelation.

The scavenging of NO 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 NO compete with oxygen, leading to reduced production of nitrite ions. The extract showed potent nitric oxide-scavenging activity between 6.25–100 $\mu\text{g ml}^{-1}$. Percentage of inhibition was increased with increasing concentrations of the extract. IC_{50} was $21.1 \pm 1.8 \mu\text{g ml}^{-1}$. IC_{50} for quercetin was $17.01 \pm 0.30 \mu\text{g ml}^{-1}$. Scavenging activity may help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. In addition to ROS, NO is also implicated in inflammation and other pathological conditions (Eslami et al., 2011). This may explain good anti-inflammatory activity of this plant (Mascalo et al., 1987). NO has been associated with a variety of physiologic processes in the human body since it was identified as a novel signal molecule. It plays an important role in vital physiologic functions. In CNS, it works as an atypical neural modulator that is involved in neurotransmitter release, neuronal excitability and learning and memory (Aliev et al., 2009).

CNS activity of this plant (Butterweck, 2003; Hosseinzadeh et al., 2005) may be partially mediated by NO pathway. There are some evidences that strongly suggest involvement of NO signaling pathway in CNS disorders (Wegener and Volke, 2010; Aggarwal et al., 2010).

In assay of reducing power, 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. Figure 2 shows the dose-response curves for the reducing power of extract. Reducing power of extract increased with the increase of its concentrations. It showed strong reducing power which was comparable with vitamin C which used as standard. Such a linear correlation between concentration of flavonoid-rich extract of *H. perforatum* L. has been reported with a coefficient of $r^2 = 0.9991$ (Zou et al., 2004).

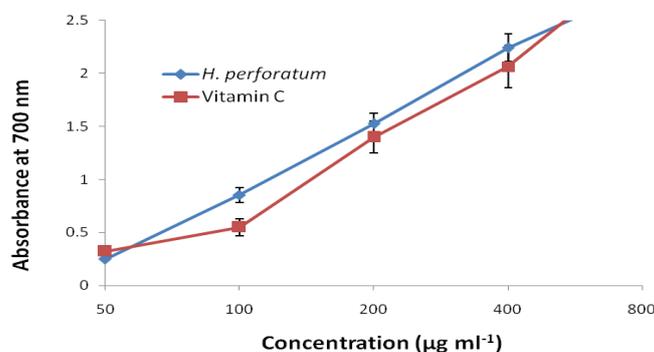


Figure 2. Reducing power of *H. perforatum* extract.

Scavenging of H_2O_2 , may be attributed to its phenolics, which can donate electrons to H_2O_2 thus neutralizing it to water. Extract was capable of scavenging H_2O_2 in a concentration dependent manner. Extract showed good activity. Inhibition was 41% at $200 \mu\text{g ml}^{-1}$ and 92.6% at $400 \mu\text{g ml}^{-1}$. The IC_{50} values for ascorbic acid and BHA were 21.4 and $52.0 \mu\text{g ml}^{-1}$, 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 et al., 2010).

Conclusion

Methanolic extract of *H. perforatum* exhibited high levels of antioxidant activity in nearly all models studied. Especially its potent nitric oxide scavenging activity may explain reported anti-inflammatory and CNS activities of this plant. These activities may be partially mediated by NO pathway. Further investigation of individual compounds, with their invivo antioxidant activities and different antioxidant mechanisms is needed.

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