

ANTIOXIDANT ACTIVITIES OF *HYPERICUM FURSEI* L.B.S. ALIPOUR[†], M.A. EBRAHIMZADEH[‡], B. ESLAMI[†] and Z. RAHMANI[‡]

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Abstract— In this study, antioxidant activities of *H. fursei* were investigated. Different model were used for assay. IC₅₀ for DPPH radical-scavenging activity was 7.5±1.2 µg ml⁻¹. The IC₅₀ value for BHA was 92.9±4.5 µg ml⁻¹. The extract showed very good reducing power that was comparable with vitamin C (p> 0.05) but showed weak nitric oxide scavenging activity and Fe²⁺ chelating ability. Extract showed good activity in scavenging of H₂O₂. Inhibition was 99.1% at 800 µg ml⁻¹. The IC₅₀ values for extract and BHA were 333.1±14.7 and 52.0±4.5 µg ml⁻¹, respectively. Total phenol compounds, by the Folin Ciocalteu method, was 274±9.6 mg gallic acid equivalent/g of extract and the total flavonoid content, by AlCl₃ method was 23.9 ± 1.4 mg quercetin equivalent/g of extract. Antioxidant activity may be attributed to the presence of phenols and flavonoids in the extract.

Keywords— antioxidant activity, *Hypericum fursei*, reducing power, iron chelating activity.

I. INTRODUCTION

Despite the immense technological advancement in modern medicine, many people from all over the world still rely on traditional medicine and medicinal plants for their daily healthcare needs because they are safe (Eisenberg *et al.*, 1998). 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.*, 2006; Dehpour *et al.*, 2009; Ebrahimzadeh *et al.*, 2009a; Mahmoudi *et al.*, 2009; Hajizadeh *et al.*, 2010). Free radicals, together with secondarily formed radicals are known to play an important role in the pathogenesis of many chronic conditions like atherosclerosis, arthritis, diabetes, ischemia, reperfusion injuries, central nervous system injury and cancer (Halliwell, 1997). Hence, 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, including antioxidant enzymes and nonenzymatic compounds that help prevent the damaging effects of free radicals. However, these endogenous systems are often insufficient for complete scavenging of these species (Anderson, 1999). Hence, certain amounts of exog-

enous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the free radicals in human body. The harmful action of free radicals can be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism (Dehpour *et al.*, 2009). 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. Plants have been a constant source of drugs and recently, much emphasis has been placed on finding new therapeutic agents from medicinal plants.

Hypericum (*Hypericaceae*) genus which contains more than 400 species occurs throughout the world and is well represented in the Mediterranean and the Near East Areas (Kizil *et al.*, 2004) and grows naturally between altitudes 750-3200 m in turkey and Iran. Recently there has been increasing interest in the genus *Hypericum*, because it is a source of a variety of compounds (Kizil *et al.*, 2004). Modern studies have been focused on the activity of extracts of these plants against certain viruses and bacteria and on their possible applications as medicines for various diseases (Ceakir *et al.*, 1997). Antimicrobial, antifungal, antiviral, antinociceptive and cytotoxic, antioxidant, antihypoxic and antidepressant and anticonvulsant activity of some of *Hypericum* species have been reported (Decosterd *et al.*, 1991; Yesilada *et al.*, 1993; Erdogan *et al.*, 2004; Hosseinzadeh *et al.*, 2005; Kizil *et al.*, 2008; 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. fursei* 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 antioxidant activity was considered. To the best of our knowledge, the present study is the first report on antioxidant activity of *H. fursei*.

II. METHODS

A. Chemicals

Ferrozine, Trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH) and Potassium ferricyanide were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Sodium nitrite, Butylated hydroxyanisole (BHA), Ascorbic acid, Gallic acid, Sulfanilamide, Ferric

chloride and N-(1-naphthyl) ethylenediamine dihydrochloride, Ethylenediaminetetraacetic acid (EDTA) were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

B. Plant material and preparation of freeze-dried extract

H. fursei. (*Hypericaceae*) aerial part was obtained in summer of 2010 from Veresk, northern of Iran. The sample was authenticated by Dr. Bahman Eslami and the voucher specimen was deposited (No. HS139) has been deposited in the Sari School of Pharmacy herbarium. Plant material was dried under dark conditions at room temperature for 10 days. The dry material was milled, obtaining 2-5 mm particles and then extracted by 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 resulting extracts were concentrated over a rotary vacuum at 35°C until a crude solid extract was obtained which then was freeze-dried (MPS-55 Freeze-drier, Cperon, Korea) for complete solvent removal (23%).

C. 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 (Dehpour *et al.*, 2009). Two ml of different concentrations of extract (6.25-200 mg ml⁻¹) in methanol were added to two ml of 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 three times. The percentage of inhibition was calculated as follows: % inhibition = $[(A_0 - A_1)/A_0] \times 100$ where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of extract or standard. Vitamin C, BHA and quercetin were used as standard controls. IC₅₀ values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

D. 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 the extract was determined according to the method described by Yen and Chen (2005). Different concentrations of the extract (1 ml) were mixed with 1 ml phosphate buffer (0.2 M, pH 6.6) and 1 ml potassium hexacyanoferrate (1%), followed by incubation at 50 °C in a water bath for 20 min. After incubation, 1 ml of TCA (10%) was added to terminate the reaction. The mixture was then centrifuged at 3000 rpm for 10 min. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water and then 0.2 ml of FeCl₃ solution (0.1% in water) was added. The absorbance was measured at 700 nm against an appropriate blank. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

E. Iron chelating activity

The ability of extract to chelate ferrous ions was estimated by method of Dinis *et al.* (1994). 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. 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 A_0 was the absorbance of the control, and A_1 was absorbance of mixture containing extract or standard. EDTA was used as a standard.

F. Assay of nitric oxide-scavenging activity

The procedure was performed based on the method by Sreejayan and Rao (1997). 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 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 (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in H₃PO₄ 2%) was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (Ebrahimzadeh *et al.*, 2010).

G. Scavenging of hydrogen peroxide

The ability of the extract to scavenge hydrogen peroxide was determined according to the method of Ruch (Elmastaş *et al.*, 2006). Extract (0.1-3.2 mg ml⁻¹) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM in phosphate buffer, pH 7.4). The absorbance of H₂O₂ at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without H₂O₂. The percentage of H₂O₂ scavenging was calculated as follows: % Scavenged [H₂O₂] = $[(A_0 - A_1)/A_0] \times 100$ where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of extract or standard.

H. Determination of total phenolic compounds and flavonoid contents

Total phenol contents were determined by Folin-Ciocalteu reagent (McDonald *et al.*, 2001). The extract sample (0.5 ml) was 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 was then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 mg ml⁻¹ solutions of gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of gallic acid equivalent (mg g⁻¹ of dry mass), which is a common reference compound. Colorimetric aluminum chloride method was used for flavonoid determination (Chang *et al.*, 2002). Briefly, 0.5 ml solution of extract in methanol was mixed with 1.5 ml of methanol, 0.1 ml of 10% alumi-

num 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. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg ml⁻¹ in methanol.

I. Statistical analysis

Experimental results are expressed as means $\pm$ SD. All measurements were replicated three times. The IC₅₀ values were calculated from linear regression analysis.

III. RESULTS AND DISCUSSION

A. Total phenol and flavonoid contents

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 *et al.*, 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). Phenolic compounds are a class of antioxidant compounds which act as free radical terminators (Shahidi and Wanasundara, 1992). 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 274 ± 9.6 mg gallic acid equivalent/g of extract. This plant was a good source of phenols and contains very high amount of total phenolics. The total flavonoid content was 23.9 ± 1.4 mg quercetin equivalent/g of extract powder, respectively, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.999$). 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). It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. Flavonoids may slow the pathogenesis of atherosclerosis and cardiovascular diseases by their ROS scavenging effects. The mechanisms of action of flavonoids are through scavenging or chelating process (Cook and Samman, 1996).

B. DPPH radical-scavenging activity

DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts (Koleva *et al.*, 2002). The capacity of extract to scavenge DPPH was measured and the results are shown in Fig. 1. The antioxidants react with DPPH radical, a purple colored stable free radical and convert it into a yellow colored diphenyl-picryl hydrazine. The amount of DPPH reduced could be quantified by measuring a decrease in absorbance at 517 nm. Extract reduced DPPH radicals in a dose dependent manner. IC₅₀ of extract and stand-

ard compound, BHA were 7.5 ± 1.2 and 92.9 ± 4.5 $\mu\text{g ml}^{-1}$. The extract showed higher activity than control in this

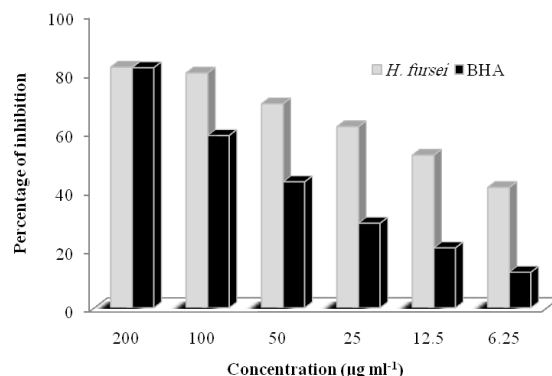


Figure 1. DPPH radical-scavenging activity of *H. fursei*. BHA used as standard.

study. So the DPPH scavenging ability of the extracts may be attributed to its hydrogen donating ability that probably shows the role of phenols and flavonoids existing in the extract.

C. Metal chelating activity

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 (Van Acker *et al.*, 1996). Clinically useful iron chelators have some adverse effects which remain 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 complexation 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, i.e. the activity was increased on increasing concentrations from 0.2 to 0.8 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). *H. fursei* extract showed very weak Fe^{2+} chelating ability. It showed only 18.5% inhibition at 0.2 mg ml⁻¹. EDTA showed very strong activity (IC₅₀ = 18 $\mu\text{g ml}^{-1}$).

D. Nitric oxide-scavenging activity

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 42% inhibition at 0.2 mg

ml⁻¹. The % inhibition was increased with increasing concentrations of the extract. IC₅₀ for quercetin, as standard, was 17.01 ± 0.03 mg 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 reactive oxygen species, nitric oxide is also implicated in inflammation and other pathological conditions (Moncada *et al.*, 1991). 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 many systems. In the CNS, NO works as an atypical neural modulator that is involved in neurotransmitter release, neuronal excitability, and learning and memory (Aliev *et al.*, 2009). Scavenging of NO explains good antinociceptive and CNS activities in *Hypericum* genus (Eslami *et al.*, 2011; Ozturk, 1997; Ozturk and Ozturk, 2001; Hosseinzadeh, *et al.*, 2005). There are some evidences that strongly suggest involvement of NO signaling pathway in CNS disorders (Wegener and Volke, 2010; Aggarwal *et al.*, 2010).

E. Scavenging of hydrogen peroxide

This effect may be attributed to its phenolics, which can donate electrons to H₂O₂ thus neutralizing it to water. Extract was capable of scavenging H₂O₂ in a concentration dependent manner. Extract showed good activity. Inhibition was 99.1% at 800 µg ml⁻¹. The IC₅₀ values for extract, ascorbic acid and BHA were 333.1 ± 14.7, 21.4 ± 1.9 and 52.0 ± 4.5 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 H₂O₂ is very important throughout food systems (Ebrahimzadeh *et al.*, 2009b).

F. Reducing power

In this assay, the presence of antioxidants in the samples would result in the reducing of Fe³⁺ to Fe²⁺ by donating an electron. Amount of Fe²⁺ complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Ebrahimzadeh *et al.*, 2009b). Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 2 shows the dose-response curves for the reducing power of the 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 (p>0.05).

III. CONCLUSIONS

The methanolic extract of *H. fursei* exhibited high levels of antioxidant activity in three models studied (DPPH and hydrogen peroxide radical scavenging activities and reducing power). Further investigation of individual compounds, with their *in vivo* antioxidant activities and different antioxidant mechanisms is needed.

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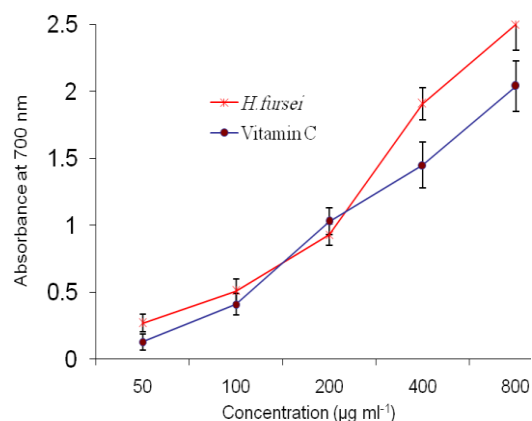


Figure 2. Reducing power of *H. fursei* extract.

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