

EVALUATION OF THREE METHODS FOR THE EXTRACTION OF ANTIOXIDANTS FROM *VICIA FABA* L. BEAN AND HULLS

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Abstract— The efficiencies of three extraction methods (percolation, Soxhlet and ultrasonically assisted extraction) were evaluated for the extraction of antioxidants from *Vicia faba* L. bean and hulls. Antioxidant activities of extracts were evaluated using four different tests. Total phenolic and flavonoid contents of the extracts were determined using the Folin–Ciocalteu and aluminium chloride methods, respectively. Although the yield of extractions for ultrasonically assisted extraction was about half to one-fourth of that of other methods, the extraction ratio of total phenol was higher. Hull extracts had higher total phenolic and flavonoid contents and antioxidant activities than bean extracts. The hull ultrasonic extract showed the best DPPH ($IC_{50} = 56.9 \pm 2.5 \mu\text{g ml}^{-1}$) and NO radical scavenging ($11.3 \pm 0.5 \mu\text{g ml}^{-1}$). The hull percolation extract showed the best iron chelating ability ($171.8 \pm 6.8 \mu\text{g ml}^{-1}$) and reducing power. The results indicated that all extraction methods could effectively extract antioxidants from medicinal plants.

Keywords— Antioxidant activity, Extraction method, Soxhlet, Ultrasonic extraction, *Vicia faba*.

I. INTRODUCTION

Oxidation is an essential biological process for energy production in many living organisms. However, excessive reactive oxygen species (ROS), produced in vivo during some oxidative reactions, are not only strongly associated with lipid peroxidation but also involved in the development of various chronic diseases such as coronary heart diseases, atherosclerosis, cancer and aging (Valko *et al.*, 2007).

In recent years, the number of studies on the antioxidant activities of medicinal plants has increased dramatically due to increased interest in their potential for use as a rich and natural source of antioxidant compounds (Ghasemi *et al.*, 2009; Ebrahimzadeh *et al.*, 2009). Antioxidants are being used for the treatment and prevention of some diseases (Mahmoudi *et al.*, 2009). The *Faba bean* (*Vicia faba* L., Fabaceae) is a pulse crop commonly grown in many parts of the world. It has its origin in the East, and its consumption is popular in South America, Argentina and China (Haciseferogullari *et al.*, 2003). It is a source of energy, protein, folic acid, niacin, vitamin C, magnesium, potassium, iron and dietary fibre. Due to the high levels of lysine in their protein, the faba beans are an adequate complement to the protein of cereals (Azaza *et al.*, 2009). They have great potential in the snack food industry (Petitot *et al.*, 2010). The Iranian climate and favoured geographical location

have contributed to the diversity of medicinal plants. There are vast expanses of *V. faba* bean fields in northern Iran. Good HIV-1 reverse transcriptase activity has been reported (Fang *et al.*, 2011). Recently, good antioxidant activity has been reported for *V. faba* beans and hulls (Boudjou *et al.*, 2013).

The development of methods to extract bioactive compounds from plant materials, particularly by the pharmaceutical industry, has led to an increased need for ideal extraction methods that can obtain the maximum of the bioactive constituents in the shortest processing time at a low cost. In this study, the efficiencies of three methods used to extract antioxidants were evaluated. Ultrasonically assisted extraction, Soxhlet extraction and the percolation method were used for the extraction of the *V. faba* beans and hulls. The antioxidant capacities of the extracts were measured by 1,1-diphenyl picrylhydrazyl (DPPH) and nitric oxide radical scavenging activities, iron chelatory capacity and reducing power. The total phenolic and flavonoid contents of the extracts were determined using the Folin–Ciocalteu and aluminum chloride methods, respectively.

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

Vicia faba L. beans and hulls were collected, in May 2011 from Sari, Iran. The sample was authenticated by Dr. B. Eslami and the voucher specimen was deposited (No. 1137) in the Sari School of Pharmacy herbarium. Plant material was dried under dark conditions at room temperature for 2 weeks. The dry material was milled, obtaining 2-3 mm particles and then extracted by methanol for 24 h at room temperature. The extracts were then separated from the sample residues by filtration through the Whatman No.1 filter paper, with the process repeated three times. The resulting extracts were concentrated over a rotary vacuum at 35-40 °C until crude solid extracts were obtained, which then were freeze-dried (MPS-55 Freeze-drier, Cperon, Korea) for complete solvents removal.

C. Soxhlet assisted extraction

The powders of samples were extracted exhaustively in a Soxhlet extractor with methanol for 24 hours. The extracts were then concentrated in a rotary evaporator until the solvent was completely removed. The methanol extracts were kept in a well-closed container in the refrigerator until use.

D. Ultrasonically assisted extraction

Samples were extracted with methanol in an ultrasonic cleaning bath by indirect sonication at a frequency of 100 kHz and a temperature of $25 \pm 3^\circ\text{C}$ for 1 h to yield ultrasonic extracts. The extracts were then separated from the sample residue by filtration. The resultant extracts were concentrated in a rotary evaporator until crude solid extracts were obtained which were freeze-dried for complete solvent removal and used as ultrasonic extracts (Rabiei *et al.*, 2012).

E. Determination of the 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 a 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. The colorimetric aluminum chloride method was used for flavonoid determination (Chang *et al.*, 2002). Briefly, 0.5 ml of the extract in methanol was 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. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 mg ml^{-1} in methanol.

F. 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). One ml of every concentration of the extract (25-800 mg ml^{-1}) in methanol was added to one 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. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

G. 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 (1995). Different concentrations of the extract (2.5 ml) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium hexacyanoferrate (1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 2.5 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 FeCl_3 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.

H. Iron chelating activity

The chelating of ferrous ions by extracts was estimated by our recently published paper (Ebrahimzadeh *et al.*, 2008). To 1 ml of different concentrations of extracts were added 2.8 ml of distilled water, which were then mixed with 50 μl of 2 mM FeCl_2 and 150 μl of ferrozine (5 mM). 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- Fe^{2+} 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 the absorbance of the mixture containing the extract or standard. EDTA was used as a standard.

I. 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 the Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in H_3PO_4 2%) was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (Ebrahimzadeh *et al.*, 2010).

J. Statistical analysis

Experimental results are expressed as means $\pm$ SD. All measurements were replicated three times. The IC_{50} values were calculated using the 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 (Prior, 2003). Phenolic compounds, including phenolic acid

and flavonoids are antioxidant compounds widely prevalent in plants. These compounds also possess various biological activities and are able to reduce the incidence of cardiovascular diseases, of cancer and of degenerative diseases (Sun *et al.*, 2009). Polyphenols are important components in fruit tissues. Phenolic compounds are a class of antioxidant compounds which act as free radical terminators (Shahidi and Wanasundara, 1992). The total phenolic contents of *Vicia faba* L. bean and hulls were measured using the Folin–Ciocalteu reagent and were reported as gallic acid equivalents by reference to the standard curve (Table 1). The plant generally had high total phenolic contents. The total phenolic content of the extract ranged from 54.2 to 110.3 mg GAE/g. The Folin–Ciocalteu phenol reagent assay is used widely for a crude estimation of the amount of phenolic compounds present in an extract. This method is based on the reducing power of the phenolic hydroxyl groups, which react with the Folin–Ciocalteu phenol reagent to form chromogens that can be detected spectrophotometrically at 760 nm. In general, hulls had higher phenolic content than beans. This is consistent with the report of Boudjou *et al.*, (2013). In hulls, the total phenolic contents were highest in the Soxhlet extract followed by the ultrasonic and percolation extraction methods, respectively. The results obtained using the paired t-test indicated that the extraction efficiencies of the Soxhlet and ultrasonic methods were superior to that of percolation method ($P < 0.001$). This plant was a good source of phenol and contained very high amounts of total phenolics.

Flavonoids, which have the basic skeleton of diphenylpropanes ($C_6-C_3-C_6$) with different oxidations of the central pyran ring, are widely distributed in the plant kingdom and constitute about half of the 8,000 or so recognized phenols (Heim *et al.*, 2002). Flavonoids are the molecules responsible for the color of fruit and flowers. As the products of secondary metabolism in plants, they are of interest to the pharmaceutical and food industries because of their reported antioxidant activity (Paniwnyk *et al.*, 2001). Using this method, flavonoids with some specific chemical structures can react with Al^{3+} and form a red complex, which gives a maximum absorption at 510 nm. We therefore can only roughly estimate the amount of flavonoids present in an extract by using the spectrophotometric analysis method. 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 processes (Cook and Samman, 1996). Epidemiological evidence also suggests an inverse relationship between intake of dietary flavonoids and risk of cardio-

vascular disease (Hertog *et al.*, 1997). The total flavonoid content of the extract ranged from 22.6 to 62.8 mg QE/g. Furthermore, hulls had higher flavonoid contents than beans.

B. DPPH radical-scavenging activity

The hydrogen atoms, or the electron donation ability of the extracts, were measured from the bleaching of a purple-colored methanol solution of DPPH. As a very stable organic free radical with a deep violet color, DPPH gives maximum absorption at the range of 515 to 528 nm. Generally, antioxidants will react with DPPH, a nitrogen-centered radical converted to 1,1-diphenyl-2-picryl hydrazine, due to its hydrogen-donating ability, at a very rapid rate. The amount of DPPH reduced could be quantified by measuring a decrease in absorbance at 517 nm. Substances which are able to perform this reaction can be considered as antioxidants and radical scavengers. 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 (Koleva *et al.*, 2002). The capacity of extract to scavenge DPPH was measured and the results are shown in Table 1. Extracts showed a concentration-dependent antiradical activity by inhibiting DPPH radicals. This method is based on the reduction of the DPPH solution in the presence of a hydrogen or electron donating antioxidant. Substances which are able to perform this reaction can be considered as antioxidants and radical scavengers. The ultrasonic hull extract showed the best activity ($IC_{50} = 56.9 \pm 2.5 \mu g ml^{-1}$) followed by the Soxhlet bean extract ($IC_{50} = 697.0 \pm 23.3 \mu g ml^{-1}$). These extracts had high amounts of phenolic contents (Table 1). IC_{50} of standard compound BHA, vitamin C, and quercetin was 29.3 ± 5.9 , 3.7 ± 0.1 , and $3.9 \pm 0.2 \mu g ml^{-1}$, respectively. So the DPPH scavenging ability of the extracts may be attributed to their hydrogen donating ability that may reflect the role of phenols and flavonoids existing in the extract.

C. Metal chelating activity

Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydro peroxide decomposition reactions via Fenton chemistry. Therefore, minimizing Fe^{2+} concentration affords protection against oxidative damage. 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 several adverse effects which highlights the need to identify new chelators with an acceptable degree of tolerability. Therefore, natural products have received much attention (Ebrahimzadeh *et al.*, 2008). Ferrozine (Fig. 1) can quantitatively form complexes with Fe^{2+} . In the presence of other chelating agents, the complex formation is disrupted, resulting in a decrease in the redness of the complexes.

Table 1. Phenol and flavonoids contents and antioxidant activities of *Vicia faba*.

Part (Extraction yield)	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 (μg ml ⁻¹) ^b	Fe ²⁺ chelating ability, IC ₅₀ (μg ml ⁻¹) ^c
Bean					
Ultra (6.6)	54.2 ± 2.5	27.6 ± 1.1	918.7 ± 34.5	21.7 ± 1.3	450.2 ± 18
Per (14.6)	69.2 ± 2.8	22.6 ± 0.9	1143.7 ± 40.2	508.7 ± 26	657.4 ± 27
Sox (12.4)	56.6 ± 1.4	28.5 ± 1.1	697.0 ± 23.3	29.9 ± 2.1	826.9 ± 23
Hull					
Ultra (9.1)	103.4 ± 3.3	33.6 ± 1.3	56.9 ± 2.5	11.3 ± 0.5	560.0 ± 21
Per (32.3)	78.6 ± 3.6	62.8 ± 1.4	85.8 ± 3.8	34.7 ± 1.9	171.8 ± 6.8
Sox (39.5)	110.3 ± 6.1	60.5 ± 2.5	87.3 ± 3.4	23.6 ± 1.7	496.2 ± 26

Ultra: Ultrasonic; Per: Percolation; Sox: Soxhlet.

^a IC₅₀ of BHA was 29.3 ± 5.9, vitamin C, 3.7 ± 0.1 and quercetin 3.9 ± 0.2 μg ml⁻¹, respectively.

^b IC₅₀ for quercetin was 155.0 ± 6.4 μg ml⁻¹.

^c EDTA used as control (IC₅₀ = 4.2 ± 0.2 μg ml⁻¹).

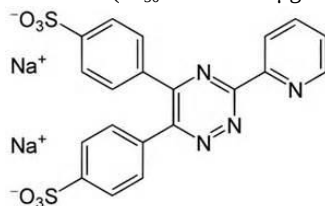


Figure 1. Chemical structure of ferrozine.

In this assay, both the extract and EDTA interfered with the formation of ferrous and ferrozine complexes, suggesting that they have chelating activity and capture ferrous ion before ferrozine. The absorbance of Fe²⁺-ferrozine complex was decreased dose-dependently, i.e. the activity increased with increasing concentrations. It has been reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion. Percolation extracts of hulls showed the best Fe²⁺ chelating ability. It was equipotent to quercetin which was used as a positive control ($p > 0.05$).

D. Nitric oxide-scavenging activity

Nitric oxide (NO) transmits signals from vascular endothelial cells to vascular smooth muscle cells and plays an important role in vital physiologic functions of many systems. It participates in pathways underlying a large group of disorders such as stroke, muscle diseases, primary headaches, inflammation, and neurodegenerative disorders such as Alzheimer's disease (Ebrahimzadeh *et al.*, 2010). In the nervous system, NO works as an atypical neural modulator that is involved in neurotransmitter release, learning and memory (Aliev *et al.*, 2009). The scavenging of NO is based on the principle that, sodium nitroprusside in aqueous solutions at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using the Griess reagent. Scavengers of NO compete with oxygen, leading to the reduced production of nitrite ions. In such situations, the use of herbal remediation, a NO scavenger, may prove useful. In our study, the extracts exhibited very potent nitric oxide-scavenging activity. The hull ultrasonic extract showed the best NO radical scavenging (IC₅₀=11.3 ± 0.5 μg ml⁻¹) at least 10 times more potent than quercetin which was used as standard (IC₅₀=155.0 ± 6.4 μg ml⁻¹)

($p < 0.001$). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation and other pathological conditions (Moncada *et al.*, 1991). Antinociceptive and CNS activities in some plants such as the *Hypericum* genus have been explained by scavenging of NO (Eslami *et al.*, 2011; Ozturk and Ozturk, 2001). There is some evidence that strongly suggests the involvement of NO signalling pathways in CNS disorders (Wegener and Volke, 2010; Aggarwal *et al.*, 2010).

E. Reducing power

Reducing power has been used as an antioxidant capability indicator of medicinal Herbs. Fe³⁺ reduction is an important mechanism of phenolic antioxidant action and often used as an indicator of electron donating activity. In this assay, the presence of reductants (antioxidants) in the samples would result in the reducing of Fe³⁺ to Fe²⁺ by donating an electron. The amount of Fe²⁺ complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Ebrahimzadeh *et al.*, 2009). Increasing absorbance at 700 nm indicates an increase in the reductive ability. The greater the intensity of the color, the higher is the antioxidant activity of the sample. Figure 2 shows the dose-response curves for the reducing powers of *V. faba* L. bean and hulls. It was found that the reducing powers of extracts also increased with the increases in their concentrations. Hull extracts had shown very potent reducing power which was much stronger than that of the bean extracts ($p < 0.01$). These fractions showed activity comparable to that of vitamin C ($p > 0.05$). Again, higher phenolic content in hulls leads to higher reducing power.

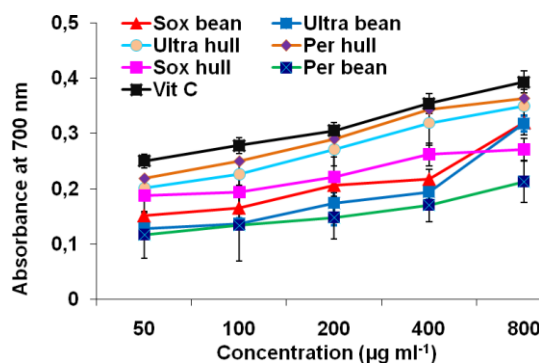


Figure 2. Reducing power of different fractions of *V. faba*. Vitamin C used as positive control.

Numerous reports have described the application of ultrasonically assisted extraction for deriving various components (Ma *et al.*, 2008; He *et al.*, 2010). In these reports, research showed that the ultrasonic procedure could significantly improve the extraction efficiency, reduce processing time, and decrease solvent consumption. The results obtained indicated that this extraction method can effectively extract antioxidants from *V. faba*. Although the yield of extractions were about half to one fourth of those of other methods (6.6 vs. 14.6 and 12.4 for bean and 9.12 vs. 32.3 and 39.5% for hulls), the ratio of extraction of the total phenol was higher. The hull ultrasonic extract showed the best DPPH ($IC_{50} = 56.9 \pm 2.5 \mu\text{g ml}^{-1}$) and NO radical scavenging ($11.3 \pm 0.5 \mu\text{g ml}^{-1}$). The Hull percolation extract showed the best iron chelating ability ($171.8 \pm 6.8 \mu\text{g ml}^{-1}$) and reducing power.

IV. CONCLUSIONS

The extraction efficiencies of three methods for the extraction of antioxidants from medicinal plants were evaluated. The results obtained indicated that these extraction methods could be used to effectively extract antioxidants from medicinal plants. These extraction methods can be applied to the analysis and purification of antioxidants in plants. This study also successfully identified *V. faba* with very high antioxidant capacities, which are potentially rich sources of natural antioxidants. Further investigation of individual compounds, with their *in vivo* antioxidant activities is needed.

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