

## ANTIOXIDANT ACTIVITY OF VICIA CANESCENCE

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### Summary

In present study, antioxidant and antihemolytic activities of aerial parts of *Vicia canescence* were investigated. Antioxidant activity was investigated in three different models. In addition, antihemolytic effect of extract was determined in H<sub>2</sub>O<sub>2</sub>-induced hemolysis. The extract showed different level of antioxidant activity in studied models. Extract showed moderate Fe<sup>2+</sup> chelating ability. IC<sub>50</sub> was 846 ± 33.8 µg ml<sup>-1</sup>. Extract also exhibited low antioxidant activity in hemoglobin-induced linoleic acid system but were capable of scavenging hydrogen peroxide in a concentration dependent manner. Extract show moderate antihemolytic activity against H<sub>2</sub>O<sub>2</sub>-induced hemolysis. The total amount of phenolic compounds in extract was determined as gallic acid equivalents (55.5 ± 2.49) and total flavonoid contents were calculated as quercetin equivalents (16.12 ± 0.61) from a calibration curve.

**Key words:** Antioxidant, chelating, phenol, *Vicia canescence*

### Introduction

*Vicia* genus member of *Papilionaceae* family has 45 species in Iran (1). *V. sativum* seeds coat and embryo cotyledons are the source of cathepsin D inhibitor. Cathepsin D is a lysosomal protease which plays an important role in cancer invasion and metastasis. The extract from *V. sativum* showed insecticidal activity. Its flavonol glycosides have exhibited hepatoprotective effect against CCl<sub>4</sub> induced hepatotoxicity (2). Previously condensed tannins, rutin, isoquercitrin, astragaln, diosmine, galactosylpinitols, vicinin, prunasin, beta-cyanolanin, and mucilage have been isolated from *V. sativum* (2). Antioxidant activity of *V. faba* (3), *V. charca* and *V. sativa* (4) and *V. sativum* (2) have been reported previously. Also anti-inflammatory and antinociceptive activity of *V. sativa* (5) and antimicrobial and cytotoxic activity of *V. faba* (3) have been reported.

To best of author knowledge there is no scientific report on *canescence* biological activity. The aim of this study was to determine the antioxidant and antihemolytic activities of *V. canescence* aerial parts in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

### Materials and methods

**Plant materials:** *V. canescence* aerial parts were collected from bashm neck, south of kiasar, sari Iran, in July 2009. After identification of the plant by Dr. Bahman Eslami (Assistance professor of plant systematic) a voucher (No. 545) has been deposited in the faculty of pharmacy herbarium.

**Chemicals:** Ferrozine, Linoleic acid, Trichloroacetic acid (TCA), Potassium ferricyanide were purchased from Sigma Chemicals Co. (USA). Vitamin C, Hydrogen peroxide, Ethylenediaminetetraacetic acid (EDTA) and Ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

**Freeze-dried extract:** Aerial parts of plant were dried at room temperature and coarsely ground before extraction. A known amount of aerial parts of plant was extracted at room temperature by percolation method using ethanol/water (70/30). The resulting extract was concentrated over a rotary vacuum until a crude solid extract was obtained, which was then freeze-dried for complete solvent removal.

**Determination of total phenolic compounds and flavonoid content:** Total phenolic compound contents were determined by the Folin-Ciocalteu method (6). 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<sup>-1</sup> 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 our recently published papers (7, 8). Briefly, 0.5 ml solution of plant extract 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 spectrophotometer (Perkin Elmer). Total flavonoid contents were calculated as quercetin from a calibration curve.

### Antioxidant activity:

**Iron ion chelating activity:** The chelating of ferrous ions by *V. canescence* was estimated by the method of Dinis *et al.*, (11, 12). Briefly, the extract (25 to 1600 µg ml<sup>-1</sup>) was added to a solution of 2 mM FeCl<sub>2</sub> (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe<sup>2+</sup> complex formation was calculated as  $[(A_0 - A_s)/A_s] \times 100$ , where A<sub>0</sub> was the absorbance of the control, and A<sub>s</sub> was the absorbance of the extract/ standard. Na<sub>2</sub>EDTA was used as positive control.

**Scavenging of hydrogen peroxide:** The ability of the extract to scavenge hydrogen peroxide was determined according to our recently published papers (13). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extract (0.2-2 mg ml<sup>-1</sup>) 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<sub>2</sub>O<sub>2</sub>] = [(A<sub>0</sub> - A<sub>1</sub>) / A<sub>0</sub>] × 100 where A<sub>0</sub> was the absorbance of the control and A<sub>1</sub> was the absorbance in the presence of the sample of extract and standard.

**Antioxidant activity in a hemoglobin-induced linoleic acid system:** The antioxidant activity of extracts was determined by a modified photometry assay (14). Reaction mixtures (200 ml) containing 10 ml of each extract (10–400 mg), 1 mmol/l of linoleic acid emulsion, 40 mmol/l of phosphate buffer (pH 6.5), and 0.0016% hemoglobin, were incubated at 37°C for 45 min. After the incubation, 2.5 ml of 0.6% HCl in ethanol was added to stop the lipid peroxidation. The amount of peroxide value was measured in triplicate using the thiocyanate method by reading the absorbance at 480nm after colouring with 100 ml of 0.02 mol/l of FeCl<sub>2</sub> and 50 ml of ammonium thiocyanate (30 g/100 ml). Vitamin C was used as positive control.

#### **Antihemolytic activity:**

**Preparation of rat erythrocytes:** All the animal experiments were carried out with the approval of institutional animal ethical committee. Male Wistar rats were sacrificed under anesthesia and blood was collected by heart puncture in heparinized tubes. Erythrocytes were isolated and stored according to the method described by Yuan *et al.* (14). Briefly blood samples collected were centrifuged (1500×g, 10 min) at 4 °C, erythrocytes were separated from the plasma and buffy coat and were washed three times by centrifugation (1500×g, 5 min) in 10 volumes of 10 mM phosphate buffered saline (pH 7.4; PBS). The supernatant and buffy coats of white cells were carefully removed with each wash. Washed erythrocytes stored at 4 °C and used within 6 h for further studies.

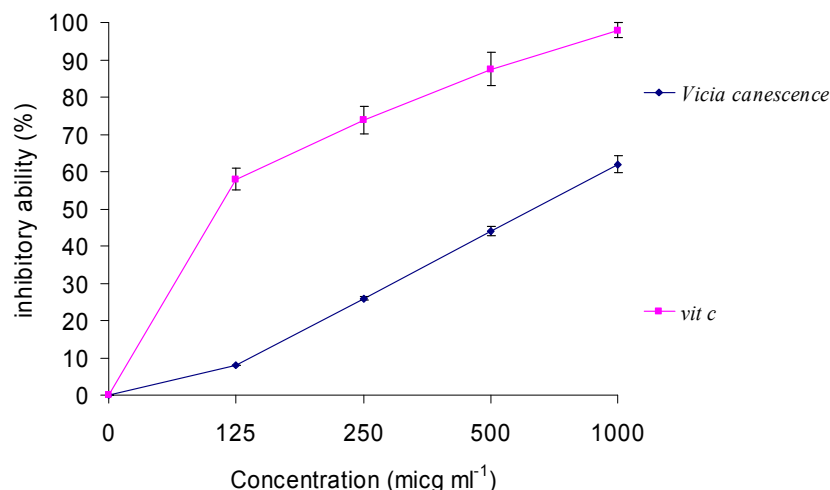
**Antihemolytic activity of extract against H<sub>2</sub>O<sub>2</sub> induced hemolysis:** Antihemolytic activity of the extract was assessed as described by Ebrahimzadeh *et al.* (15). The erythrocytes from male Wistar rat blood were separated by centrifugation and washed with PBS. The erythrocytes were then diluted with PBS to give 4% suspension. 1 g of extract/mL of saline buffer were added to 2 mL of the erythrocyte suspension and the volume was made up to 5 mL with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 mL of H<sub>2</sub>O<sub>2</sub> solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H<sub>2</sub>O<sub>2</sub> in the reaction mixture was adjusted to bring about 90% hemolysis of blood cells after 240 min. After incubation the reaction mixture was centrifuged at 1500 rpm for 10 min and the extend of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation.

**Statistical analysis:** Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance (p < 0.05) and the means separated by Duncan's multiple range test. The IC<sub>50</sub> values were calculated from linear regression analysis.

### Results and discussion

The total phenolic content was  $55.5 \pm 2.49$  mg gallic acid equivalent  $\text{g}^{-1}$  of extract powder by reference to standard curve ( $y = 0.0054x + 0.0628$ ,  $r^2 = 0.987$ ). The total flavonoid content was  $16.12 \pm 0.61$  mg quercetin equivalent  $\text{g}^{-1}$  of extract powder, by reference to standard curve ( $y = 0.0063x$ ,  $r^2 = 0.999$ ). This plant showed high total phenol and flavonoid contents. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (16,17,18). Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases (19, 20, 21). 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 human and thereby improves quality of life and overall survival in some diseases such as Thalassemia major (22). In addition, brain iron dysregulation and its association with amyloid precursor protein plaque formation are implicated in Alzheimer's disease (AD) pathology and so iron chelation could be considered a rational therapeutic strategy for AD (12). Foods are often contaminated with transition metal ions which may be introduced by processing methods. Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry (23). These processes can be delayed by iron chelation and deactivation. The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (24). Because  $\text{Fe}^{2+}$  also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing  $\text{Fe}^{2+}$  concentration in Fenton reactions affords protection against oxidative damage. The chelating of ferrous ions by the extract was estimated by the method of Dinis *et al.*, (25). Ferrozine can quantitatively form complexes with  $\text{Fe}^{2+}$ . In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The absorbance of  $\text{Fe}^{2+}$ -ferrozine complex was decreased dose-dependently, i.e. the activity was increased on increasing concentration from 25 to 1600  $\mu\text{g ml}^{-1}$ . *V. Canescence* extract showed moderate  $\text{Fe}^{2+}$  chelating ability.  $\text{IC}_{50}$  was  $846 \pm 33.8$   $\mu\text{g ml}^{-1}$ . EDTA showed very strong activity ( $\text{IC}_{50} = 18$   $\mu\text{g ml}^{-1}$ ). 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 (19). Scavenging of  $\text{H}_2\text{O}_2$  by extract may be attributed to its phenolics, and other active components which can donate electrons to  $\text{H}_2\text{O}_2$ , thus neutralizing it to water (18). *V. caescence* extract was capable of scavenging hydrogen peroxide in a concentration dependent manner.  $\text{IC}_{50}$  for  $\text{H}_2\text{O}_2$  scavenging activity was  $897 \pm 36.7$   $\mu\text{g ml}^{-1}$ . The  $\text{IC}_{50}$  values for vitamin C was 21.4  $\mu\text{g ml}^{-1}$ , 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  $\text{H}_2\text{O}_2$  is very important throughout food systems (18). Erythrocytes are considered as prime targets for free radical attack owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the  $\text{O}_2$  transport associated with redox active hemoglobin molecules, which are potent promoters of reactive  $\text{O}_2$  species. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation (26).

The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical (27). Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Tested extracts show good activity in hemoglobin-induced linoleic acid system but There were significant differences between extracts and vit C ( $p < 0.001$ ) (Figure 1).



Hemoglobin-induced lipid peroxidation of ethanol/water (70: 30) extract of *V. canescence*. Vitamin C used as control.

Hemolysis has a long history of use in measuring free radical damage and its inhibition by antioxidants but only few studies have been performed with erythrocytes in whole blood. In this study, we used a biological test based on free radical-induced erythrocytes lysis in rat blood. This assay is useful either for screening studies on various molecules and their metabolites, especially on the one hand molecules having an oxidizing or antioxidizing activity and on the other hand molecule having a long-term action (15). Lipid oxidation of rat blood erythrocyte membrane mediated by H<sub>2</sub>O<sub>2</sub> induces membrane damage and subsequently hemolysis. The extract showed moderate inhibitory activity (IC<sub>50</sub> = 1111 ± 25 µg ml<sup>-1</sup>). Vitamin C exhibited better activity with IC<sub>50</sub> = 235 ± 9 µg mL<sup>-1</sup>.

### Conclusion

Our study indicated the of *V. canescence* aerial parts extract exhibited good but different levels of antioxidant activity in some of models studied. Further investigation of individual compounds and the mechanisms of activities is needed.

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