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Original Article

Inhibitory Activity on Tyrosinase and Antioxidant Activity of Methanol Extract of Various Aerial Parts of *Astragalus Siliquosus* Bioss. and *Verbascum Phoeniceum*

Mohammad Ali Zarei*, Masoumeh Babaei

Department of Biological Sciences, Faculty of Science, University of Kurdistan, Sanandaj, Iran

*Corresponding Author E-mail: mazarei@uok.ac.ir

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ABSTRACT

Background: Melanogenesis begins with the oxidation of *L*-tyrosine to *L*-Dopa by tyrosinase. Inhibition of tyrosinase could be effective in the treatment of complications such as skin lesions, eczema and melasma. Inhibition of tyrosinase could be effective in the treatment of those complications. The aim of this study was to determine inhibitory activity on tyrosinase and antioxidant activity of *Astragalus siliquosus* Bioss. and *Verbascum phoeniceum* L.

Methods: Methanol extracts of organs of the plants were prepared by maceration. Inhibitory effects of the extracts were evaluated in 96 wells at 492 nm wavelength. The antioxidant activity of the extracts was evaluated using DPPH free radical scavenging index and iron reduction test.

Results: For *A. siliquosus*, maximum inhibitory activity was of its flower extract (97% inhibition and IC_{50}=1.58 mg/mL) and of its stem extract (100% and IC_{50}=2.1 mg/mL). For *V. phoeniceum*, the highest inhibitory activity was of its leaf extract (87% inhibition and IC_{50}=3.2 mg/mL). Flower extract of *A. siliquosus* showed mixed Uncompetitive-non-competitive pattern of inhibitory activity, while the extract of the *V. phoeniceum* leaf showed mixed Competitive-non-competitive inhibitory pattern. All aerial parts of the *A. siliquosus* had 100% antioxidant activity with EC_{50}s of 0.089, 1.78, 1.25 mg/mL for its flower, leaf and stem, respectively. The leaves of the *V. phoeniceum* had a 100% antioxidant activity, with EC_{50} of 0.013 mg/mL.

Conclusions: Methanol extracts of flower and leaf organs of *A. siliquosus*, and leaf organ of *V. phoeniceum* had a reasonable inhibitory effect on tyrosinase activity. Future studies could be focused on those organs to separate potential agents with pharmaceutical and cosmetic applications.

Key words: *A. siliquosus*, Inhibitor, Methanol extract, Tyrosinase, *V. phoeniceum*

Introduction

Tyrosinase (monophenol monooxygenase EC: 1.14.18.1) is a dual-core copper-containing enzyme that converts monophenols to O-diphenols (monophenolase activity), and Oxidation of *O*-diphenols catalyses *O*-quinone derivatives (diphenolase activity) (Sánchez-Ferrer *et al*., 1995; Solomon *et al*., 2013). Tyrosinase use molecular oxygen as a co-substrate in the first reaction (Rolff *et al*., 2011). One of the oxygen atoms combines with the phenolic ring, while the other molecule converts the molecular oxygen to water. The end product is catalysed by tyrosinase dopaquinone, which is a precursor for melanin synthesis (Ito and Wakamatsu, 2008). In mammals, including humans, melanin is more commonly found in the skin and works to protect against UV rays (Jablonski and Chaplin, 2010). The relative amount of melanin is a key factor for skin colour as well as the development of ethnic biodiversity in humans. Plants use *O*-quinone to modify and harden their outer protective layer, such as seed cover, which is a protective agent against invaders. These examples show that tyrosinase function is related to the response to environmental stresses (Lai *et al*., 2017). Tyrosinase inhibitors are widely used in the cosmetics and agriculture industries. There are various natural and synthetic tyrosinase inhibitors. Plants are a rich source of bioactive chemicals that often have no harmful side effects. So, continued efforts have been made to search for tyrosinase inhibitors among them (Fernandes and Kerkar, 2017; Lee *et al*., 2016). In a screening study, Hassasni and Zarei reported the inhibitory effects of 70 plant species extracts on fungal tyrosinase activity in Kurdistan province, and identified eleven plants with inhibitory activity above 60% (Hassani, 2014). Morakinyo investigated the inhibitory effect of *Datura metel* leaf extracts on fungal tyrosinase activity, and methanol extract of plant and showed the most inhibitory effect on Tyrosinase (Morakinyo Sanni and Omotoyinbo, 2016). Hyo Hee Yanga demonstrated the inhibitory effect of tyrosinase on the aerial parts of *Humulus japonicas* (Yanga *et al*., 2017). In a study, Aromagam (2019) showed the inhibitory and antioxidant effect of methanolic extract of different parts of *Astragalus ponticus* Pall on Tyrosinase. The present study was designed to determine the organs of two plants (*Astragalus siliquosus* Bioss.) and (*Verbascum phoeniceum* L.) responsible for tyrosinase inhibitory activity. The second aim of this study was to investigate the antioxidant capacity of the methanol extracts of the above-mentioned plant organs.

Experimental

Plant materials

In late spring, aerial parts of *Verbascum phoeniceum* and *Astragalus siliquosus* were collected by a systematic senior plant expert from Kurdistan province rangelands and systematically recorded in the herbarium of the Agricultural Research and Natural Resources Research Centre. They were transferred to the Department of Biological Sciences, University of Kurdistan. The aerial parts of the plants, including flower, leaf and stem, were then separated. These isolated organs were completely dried in shade conditions for several days. The dried plants were first examined for contamination with soil or other plants and then cut into smaller pieces by horticultural scissors. They were then milled into a soft powder.

Preparation of crude extract

To prepare the methanol extract of each organ, 20 g of their powder was soaked in 200 mL of pure methanol for 72 h with sporadic shaking in room temperature. Then filtered with Whatman No. 42, filter paper, the solid residue was discarded and the filtered portion used to

obtain pure extract. Filter fluid for each organ was concentrated using a rotary evaporator at 65 ºC, and 50 RPM for 40 minutes. The residues were poured into a flat glass container for complete drying. Extracts were kept at -20 ºC until subsequent steps.

Inhibition assay for yeast Tyrosinase activity

In this study, the modified method of (Khatib *et al.*, 2005) was used to measure the inhibitory effect of tyrosinase activity on the extract. All extracts were tested in 9 concentrations of 0.001, 0.01, 0.1, 1, 10, 100, 500, 1000 and 1500 mg/mL. Kojic acid was used as positive control and 2 mM catechol solution as a substrate. 50 mM phosphate buffer with pH = 6.5 was used to maintain the pH of the reaction medium. All assays were performed in 96 well microplates using a microplate reader and in final volume of 200 µl. Each well contained 110 µl of phosphate, 10 µl of extract and 30 µl of tyrosinase enzyme solution, incubated at room temperature for 5 min. Then 50 µl of substrate was added to each. The microplate was then transferred to a microplate reader after 20 minutes of incubation at room temperature. The absorption was read 30 times at one-minute intervals at 492 nm. The assays were performed in 3 replicates each and the blank well contained all test wells except the enzyme (30 µl buffer was added instead). Kojic acid was used as positive control and buffer was used as negative control. Using the inhibitory formula, the percentage of inhibition of tyrosinase was obtained.

$$\% \text{ Inhibition} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Extract)}}{\text{Absorbance (Control)}} \times 100$$

The IC_{50} value, defined as the concentration of the sample, which has been defined to inhibit 50% tyrosinase, is estimated using nonlinear regression analysis using Sigma Plot software. All values are expressed as Mean ± Standard Deviation.

Kinetic analysis of tyrosinase inhibition

In order to determine the type of inhibition applied by the extracts with the highest inhibition percentage, a double reversed graph of Lineweaver-Burk was drawn based on the enzymatic reaction in the presence of different concentrations of inhibitors. The V_{max} and K_m for each control and the apparent V_{max} and K_m for each inhibitor concentrations were determined, and the K_i inhibitory constant was calculated using secondary plots at different inhibitor concentrations (Palmer and Bonner, 2007).

Determination of antioxidant

To evaluate the antioxidant properties of the extracts, two conventional methods including DPPH free radical scavenging ability and iron resuscitation strength test were used. The first approach is to trap the DPPH radicals by the ability to hydrogen donating by extract (Chung *et al.*, 2006). 1,1-diphenyl-2-picrylhydrazyl (DPPH) is a violet stable radical which turns into yellow non-radical diphenylpicrylhydrazine by reducing by an electron-donor agent (Molyneux, 2004). In the second approach, the reducing power of the extracts was evaluated by its potency to reduce Ferric iron to its ferrous state. Iron reducing is often used as an electron donating potential measurement, which is a suitable method for evaluating the antioxidant activity of phenolic compound (Hinneburg *et al.*, 2006).

DPPH free radical-scavenging capacity

Fu method was used to investigate the antioxidant activity and inhibition of DPPH free radicals by extracts (Fu *et al*., 2014). The assays were performed in 96 well microplates in total volume of 200 µl using microplate reader. First, 100 µl of 0.1 mM DPPH solution (dissolved in methanol) was added to the wells and then 100 µl of different concentrations of the extract was added. The microplate was then incubated for 30 min at 25 °C. After 30 min, the absorbance was measured at 492 nm. Ascorbic acid was used as positive control and DPPH solution in methanol was used as negative control. At the end of the assay, the absorbance of the blank plate was subtracted from the absorbance of the corresponding wells, and then the final absorbance was calculated by subtracting the Blank well from the test well. Antioxidant activity was calculated for 3 replicates of each extract and then averaged. Finally, the amount of DPPH radical trapping was calculated by following equation:

$$\%\ RSA = \frac{Absorbance\ (Control) - Absorbance\ (Extract)}{Absorbance\ (Control)} \times 100$$

The DPPH solution was also used as a negative control. Ascorbic acid was used as a positive control with concentrations of 0.031, 0.25, 0.5, 1, 2, 5, 8 and 12 µg/mL. EC_{50} values indicate the concentration of the sample needed to scavenge 50% of the DPPH free radicals.

Determination of iron reducing power

The iron reduction power of extracts was also measured according to the method of Yen and Chen with some modifications (Yen and Chen, 1995). The extract was prepared with different concentrations of 50, 100, 200, 400, 600 and 800 µg/mL. Phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%) were then added. The mixture was incubated at 45 °C for 30 minutes. At the end of incubation, 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture, centrifuged for 10 minutes at 2383 g. After the end of the centrifugation, 2.5 mL of top solution was mixed with 2.5 mL distilled water and $FeCl_3$ (0.5 mL, 0.1%) and absorbance were measured at 700 nm. The higher the absorbance of the reaction mixture, the greater the reduction power of the sample.

Statistical analysis

The percentage of inhibition was calculated for three replicates of each extract and the mean and standard deviation were calculated. All these steps were performed using Excel software.

Results

Inhibition of tyrosinase activity

In this study, it was observed that the highest inhibitory activity of *A. siliquosus* was related to 1500 mg/mL stem methanol extract and 1000 mg/mL flower extract (Figure 1). Also, the highest inhibitory activity of *V. phoeniceum* was related to 1500 mg/mL methanol extract of leaf (Figure 2).

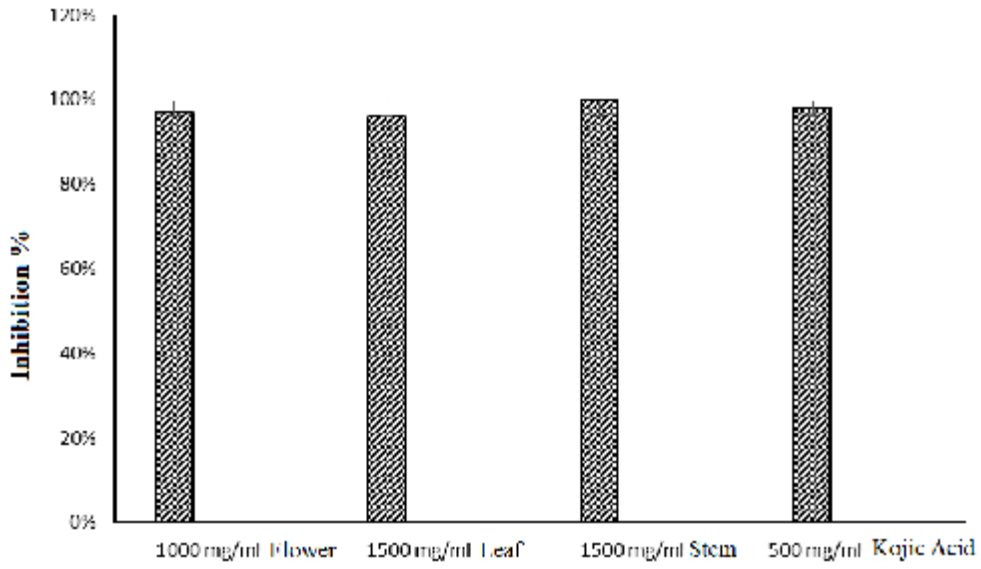

Figure 1. Tyrosinase inhibition percent by methanol extract from different parts of *A. siliqusus* and Kojic acid

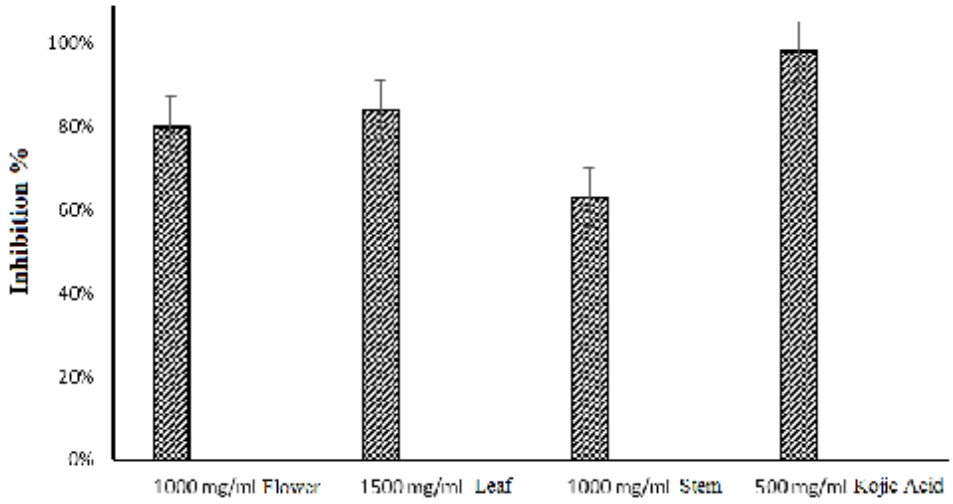

Figure 2. Tyrosinase inhibition percent by methanol extract from different parts of *V. phoeniceum* and Kojic acid

According to the results of the enzyme kinetic study, methanol extract of *A. siliquosus* showed an Uncompetitive-non-competitive mixed inhibition pattern (Figure 3), and the extract of *V. phoeniceum* leaf organ, showed a pattern of Competitive-Noncompetitive mixed inhibition (Figure 4). Inhibitory constant (Ki) values for the active plant extracts were calculated by plotting slops of primary plots against the inhibitor concentrations (Figures 5 and 6).

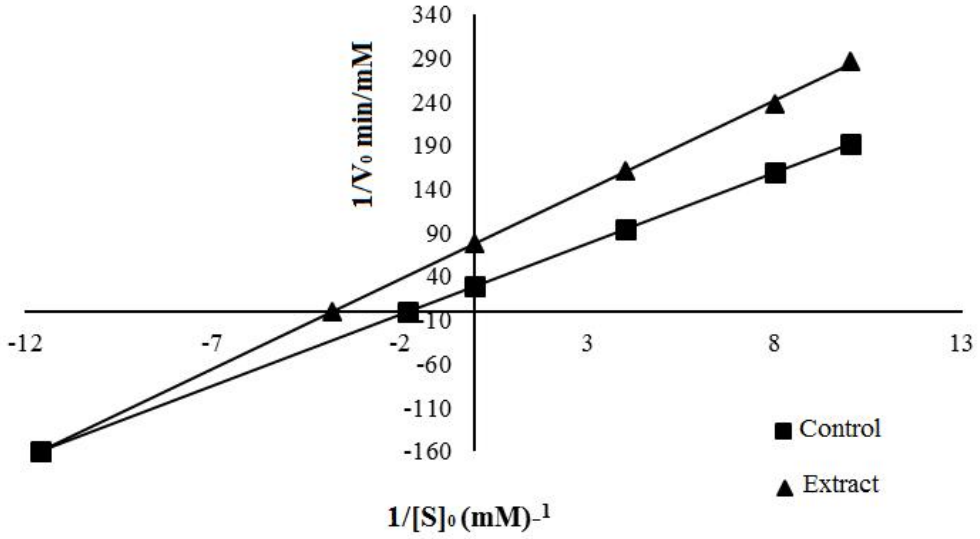

Figure 3. Lineweaver-Burke plot shows an Uncompetitive-non-competitive inhibition of tyrosinase by the flower extract of *A. siliquosus*

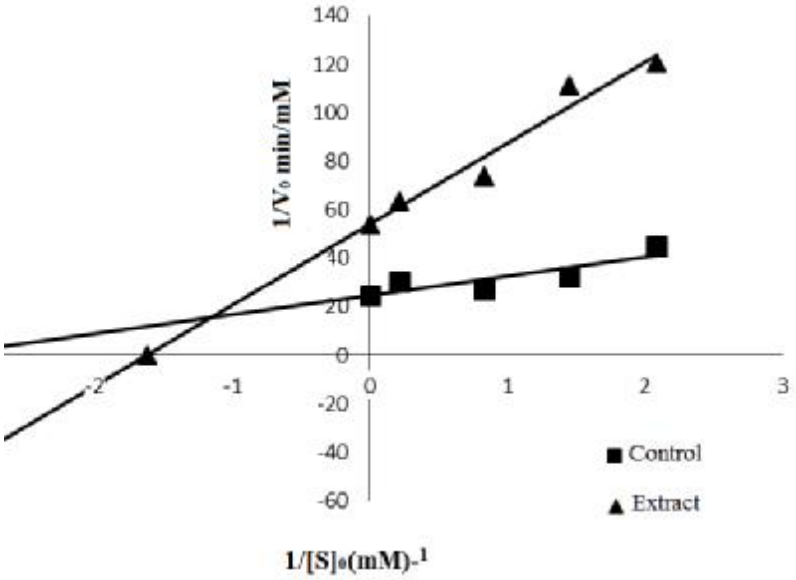

Figure 4. Lineweaver-Burke plot shows a Competitive-Noncompetitive inhibition of tyrosinase by the leaf extract of *V. phoeniceum*

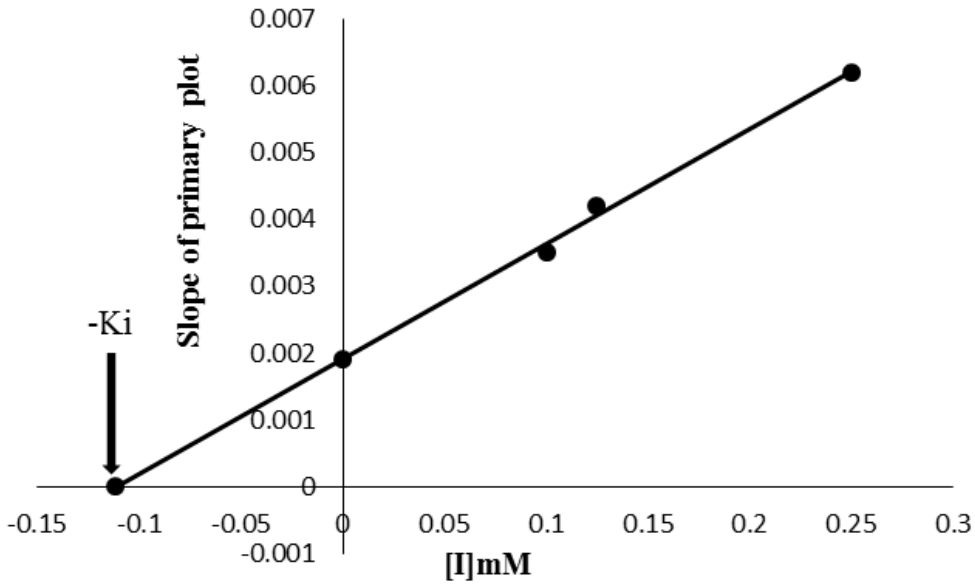

Figure 5. Secondary plots for the methanol extract of *A. siliquosus* flower to determine the inhibitor constant

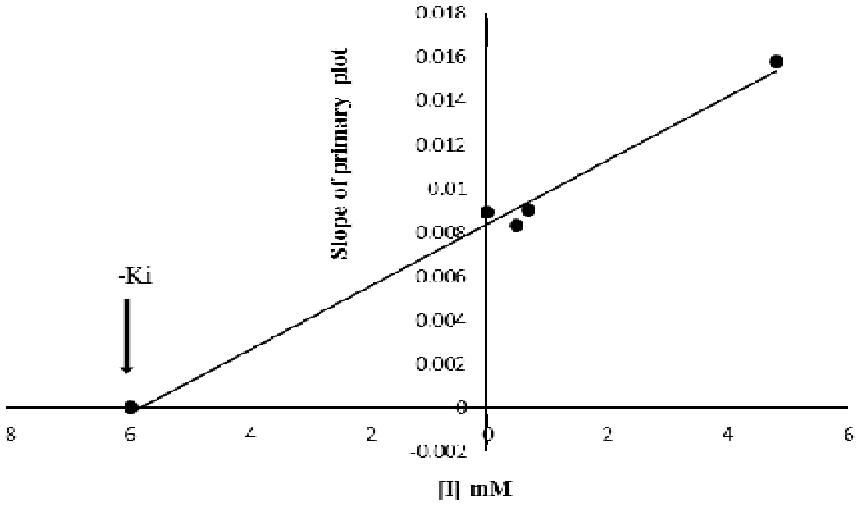

Figure 6. Secondary plots for the methanol extract of *V. phoeniceum* leaf, to determine the inhibitor constant

Antioxidant activity

The percentage of DPPH free radical reduction of and EC_{50} of methanol extract of each organ of *A. siliquosus* and *V. phoeniceum* is given in Table 1. Percentage of DPPH reduction and EC_{50} showed 100 mg/mL methanol extract of each aerial organ. Antioxidant activity also was determined by the ability of iron reduction for aerial extracts of *A. siliquosus* and *V. phoeniceum* at different concentrations of the extract. Vitamin C was used as positive control. The results are shown in Figures 7 and 8.

Table 1. DPPH radical scavenging activity and EC_{50} values was measured for aerial part *A. siliquosus* and *V. phoeniceum*

Systematic name	Organ	DPPH %Red	EC_{50} (mg/mL)
Astragalus siliqusus Bioss	Flower	100	0.089
	Leaf	100	1.25
	Stem	100	1.78
Verbascum phoeniceum L.	Flower	94	0.079
	Leaf	100	0.013
	Stem	95	1.12

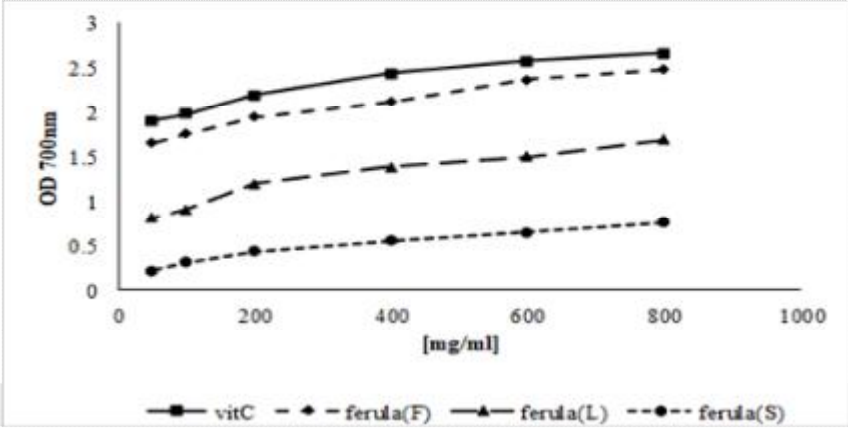

Figure 7. Iron reduction potential for hexane extract of *Ferula haussknechtii* (ascorbic acid as standard)

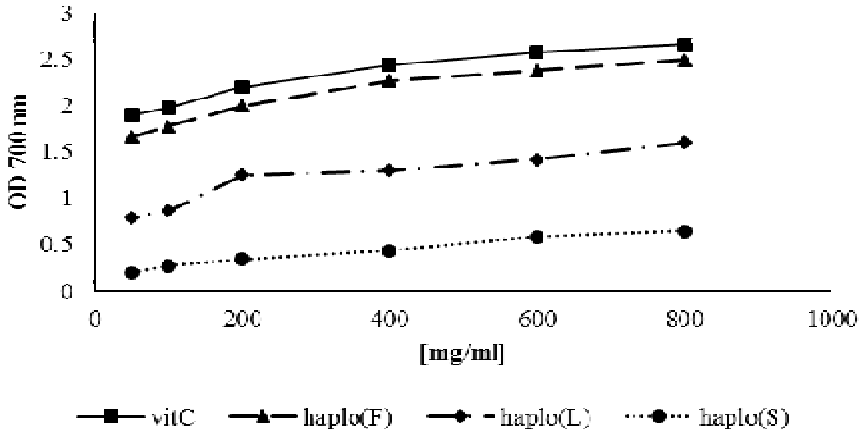

Figure 8. Iron reduction potential for hexane extract of *Haplophyllum acutifolium* (ascorbic acid as standard)

Discussion

Medicinal plants play an important role in the health care industry in both developed and developing countries. The general public believes that medicinal plants have minimal or no side effects. Medicinal herbs are often used in medicine, industry, agriculture, and the food field. They are also used to reduce the side effects of drugs and toxic agents. In the medical field,

many researchers have high hopes for medicinal plants for the treatment of various diseases such as cancer (Jamshidi-Kia *et al*., 2018). For example, during the screening of natural products for the development of the cosmetic industry, it was found that the aerial extract of *Humulus japanicus* has tyrosinase inhibitory activity. This plant has been reported to be an antitumor and antioxidant too (Yang *et al*., 2017). During a screening study, the inhibitory effect of 70 plant species extracts on fungal tyrosinase activity was investigated and several plants with high inhibitory activity were introduced, two of which were *Verbascum phoeniceum* L. and *Astragalus siliqusus* Bioss (Hassani and Zarei, 2019). The *V. phoeniceum* belongs to the Scrophulariaceae family and is the largest genus of this family. This family has a high distribution in the world, especially in cold and temperate regions. This genus has approximately 360 species, 43 species of which are endemic in Iran and its distribution is limited to some highland areas of Kurdistan province (GBIF Secretariat 2019). Verbuscum is used as an anti-cough in some lung diseases such as bronchitis. It has strong antioxidant, antibacterial, antifungal and antimicrobial properties that can be used in pharmaceuticals, food industries, cosmetics and sanitation (Jamshidi-Kia *et al*., 2018). Astragalus plants from the family Fabaceae have more than 900 annual and perennial herbaceous plants in Iran, most of which are exclusive to western parts of Iran (Sanandaji and Mozaffarian, 2010). Plants of this genus have numerous health benefits such as: immune system enhancer, anti-cancer, anti-diabetic, anti-influenza effects (Siahpoosh and Amraee, 2011). The highest distribution of this genus is in cold-arid regions such as the Northern Hemisphere and South America, and especially in Southeast Asia (Dong, 2003). Anti Tyrosinase activity of hexane extract from another species of this genus (*Astragalus vegetus* Bunge) has been reported earlier (Hashemi and Zarei, 2014). According to the results of this study, the highest inhibitory activity of *A. siliquosus* was related to 1500 mg/mL stem methanol extract (100%) and 1000 mg/mL of its flower (97%). The highest inhibitory activity of *V. phoeniceum* was related to 1500 mg/mL methanol extract (87%). Calculation IC_{50} for flower and stem extract of *A. siliqusus* was 1.58 mg/mL and 2.13 mg/mL, respectively, and the leaf organ of *V. phoeniceum* IC_{50} was 3.155 mg/mL. This indicates that there is a stronger inhibitor in the organs mentioned. The results of Enzyme kinetic study that the flower extract of *A. siliqusus* showed mixed Uncompetitive-non-competitive inhibition pattern, and leaf methanol extract of *V. phoeniceum*, showed mixed Competitive-non-competitive inhibition pattern.

In the non-competitive inhibition, the inhibitor binds to the enzyme-substrate complex. Previous binding of the substrate to the enzyme is probably required to form the site of inhibitor binding on the enzyme. In competitive inhibition, due to the existence of common binding sites or spatial interference of these sites, there was competition between the inhibitor and the substrate for binding to the enzyme, and only one of them could bind to the enzyme. In non-competitive inhibition it can be inferred that the inhibitor in the extract binds to the enzyme and to the enzyme-substrate complex (Palmer and Bonner, 2007). The results of DPPH free radical scavenging assay by aerial parts of *A. siliqusus* and *V. phoeniceum* plants against positive control (ascorbic acid) showed that the antioxidant activity in aerial organs was concentration dependent just like ascorbic acid. Phenolic compounds have been found in *A. siliqusus* and *V. phoeniceum* plants (Jamshidi-Kia *et al*., 2018; Siahpoosh and Amraee, 2011). Since phenolic and flavonoid compounds have antioxidant and inhibitory effects on tyrosinase (Shukla *et al*., 2016), it can be said that such compounds cause antioxidant and inhibitory activity on tyrosinase in the above-mentioned plants.

Conclusions

The results showed that flower and stem methanolic extract of *A. siliqusus* and leaf of *V. phoeniceum* significantly inhibited tyrosinase activity. Therefore, these studies may be used in future research with the aim of identifying potential sources of new drugs for the treatment of diseases caused by hyperactivity of tyrosinase. Due to the use of tyrosinase inhibitors in the food, pharmaceutical, cosmetic and agricultural industries, these plants may be used also as sources for the extraction of tyrosinase inhibitors. Since the two plants have high antioxidant properties, they can be used as natural antioxidant sources.

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