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Inhibitory Effects of *Physalis alkekengi* L., *Alcea rosea* L., *Bunium persicum* B. Fedtsch. and *Marrubium vulgare* L. on Mushroom Tyrosinase

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Background: The key enzyme in the process of melanin biosynthesis is tyrosinase. Skin hyperpigmentation and browning of foods are undesirable phenomena which tyrosinase represents. Therefore, tyrosinase inhibitors have been used increasingly for medicinal and cosmetic products.

Objectives: In this study, inhibitory effects of four plants including: *physalis alkekengi* L., *Alcea rosea* L., *Bunium persicum* B. Fedtsch. and *Marrubium vulgare* L. on diphenolase activity of mushroom tyrosinase were evaluated.

Materials and Methods: The inhibitory activities of hydroalcoholic extracts of plants against oxidation of L-Dopa (as a substrate) by mushroom tyrosinase were investigated.

Results: The hydroalcoholic extract of *P. alkekengi* showed the most tyrosinase inhibitory effect with IC50 of 0.09 mg/mL vs. 0.38, 0.38 and 2.82 mg/mL of *B. persicum*, *A. rosea* and *M. vulgare*, respectively. *M. vulgare* exhibited uncompetitive inhibition and other plants showed mixed type inhibition on mushroom tyrosinase.

Conclusions: All plants could inhibit mushroom tyrosinase, but more investigations on human tyrosinase and clinical studies are needed.

Keywords: Hyperpigmentation; Tyrosinase; Inhibition; Plants

1. Background

Melanin is a pigment with a polymeric complicated structure, which plays an important role in determination of mammalian skin and hair color. It is widely distributed in organisms, including fungi, plants and animals. Another role of melanin is absorption of Ultraviolet (UV) rays to protect the skin (1). Melanin synthesis occurs in a special kind of skin cells named melanocytes, which is stimulated by UV radiations (2). Keratinocytes are another kind of cells that surround melanocytes. Melanin is transported to keratinocytes after its synthesis (3).

Melanogenesis is a process that results in melanin production. This process is a combination of two kinds of enzymatic and chemical reactions. The enzymatic reactions are catalyzed by tyrosinase, which oxides L-tyrosine to dopaquinone. This step is a rate-limiting step in melanogenesis (4). Tyrosinase is a key enzyme that catalyses hydroxylation of monophenols to O-diphenols (monophenolase activity) and oxidation of O-diphenols to quinones (diphenolase activity) (5). Next reactions occur spontaneously at physiological pH value (4).

Abnormal accumulation of melanin leads to hyperpigmentation disorders. The enzymatic browning of vegetables and fruits is another issue that tyrosinase represents (4). Tyrosinase could contribute to neurodegenerative diseases such as Parkinson through formation of the neurotoxin 5-S-cysteinyl-dopamine during the oxidation of dopamine to an O-quinone and reaction with cellular thiols (6).

These undesirable phenomena encouraged researchers to find new tyrosinase inhibitors for use in cosmetic, medicine and food industries to prevent or treat hyperpigmentation disorders and food browning (4). The common tyrosinase inhibitors such as hydroquinone regardless of potent tyrosinase inhibitory effect, have limitations in use, because of its mutagenicity and cytotoxicity effects (7). Also very few tyrosinase inhibitors are used in food industry due to off-flavors, off-odors, food safety and economic values (1). Furthermore, these adverse effects spurred us to find natural tyrosinase inhibitors.

It has been shown that *Physalis edulis* L., *Marrubium velutinum* L., *Marrubium cylleneum* L., *Althea officinalis* L. and

Cuminum cyminum L. could inhibit tyrosinase (8-10). Since close plants and other species of a genus may have the same effects, we selected *physalis alkekengi*, *Marrubium vulgare* L., *Alcea rosea* L. and *Bunium persicum* B. Fedtsch for this study.

P. alkekengi (*Solanaceae*), known as winter cherry, is an indigenous plant to central and southern Europe, China and Indochina and naturalized in the U.S (11). It has antimicrobial (12), antioxidant (13), hypoglycemic (14), antineoplastic (15), anti-inflammatory (16), antispasmodic (17) and anti-fertility (18) effects. Winter cherry in folk medicine is used as a diuretic in kidney and bladder ailments and in the treatment of gout and rheumatism (11).

B. persicum (*Apiaceae*) is distributed in central Asia, Iran, Pakistan, Afghanistan, Keshmir and Pamir (19). The *B. persicum* exhibits antimicrobial (20), antifungal (21), antioxidant (22), α-amylase inhibitory (23), anticonvulsant (24), antinociceptive, anti-inflammatory (25) and antihistaminic (26) effects.

A. rosea (*Malvaceae*), known as Hollyhock is widely grown in gardens and parks in the Southern Europe and Asia. Some pharmacological effects including antibacterial, analgesic, anti-inflammatory and cytotoxic activities (27-29) have been reported.

M. vulgare (*Lamiaceae*) is a medicinal plant used in folk medicine for many diseases including acute and chronic bronchitis, whooping cough, asthma, tuberculosis, pulmonary catarrh, respiratory infections, diarrhea, jaundice, debility and painful menstruation (11). It has antibacterial, antifungal (30), antioxidant (31), analgesic (32), anti-diabetic (33), antihepatotoxic (34), hypotensive (35), antispasmodic (36) and antinociceptive (37) activities.

2. Objectives

P. alkekengi, *M. vulgare*, *A. rosea* and *B. persicum* are some plants available in Iran, which have not been subjected for tyrosinase inhibitory effects before, so the aim of this study was to evaluate inhibitory effects of these plants on mushroom tyrosinase activity.

3. Materials and Methods

3.1. Chemicals

Mushroom tyrosinase (lyophilized powder, 3130 unit/mg solid) and L-dopa (powder, ≥ 98 %) from Sigma Chemical Co, kojic acid (powder, ≥ 98.0 %) from Fluka Chemical Co, DMSO (Dimethyl sulfoxide), ethanol and n-hexane from Sigma Chemical Co were used.

3.2. Plant Materials

P. alkekengi (from sites near Tehran), *A. rosea* (from Isfahan), *B. persicum* (from Chubar Mountains in Kerman) and *M. vulgare* (from 1000 m above the sea level near Tehran) were collected. They were identified by the pharmacognosy department of Ahvaz Jundishapur University of Medical Sciences. Then the plants were shade dried at room temperature and milled.

3.3. Preparation of Extracts

The aerial parts of *P. alkekengi*, *A. rosea* and *M. vulgare* and seeds of *B. persicum* were extracted with ethanol 80% for 72 hours. After filtering, the *B. persicum* extract was defatted with n-hexane. The extracts were concentrated by rotary evaporator (Heidolph, Germany). Then the concentrated extracts were freeze-dried by vacuum freeze dryer (Operon, Korea) and the yields were 7.76, 12.35, 10.56, 6.69 and 8.62% for *P. alkekengi*, *A. rosea*, *B. persicum* (total), *B. persicum* (defatted) and *M. vulgare*, respectively. The obtained extracts powder (0.1 g) was dissolved in three mL of DMSO. Then the extracts were diluted with 25 mM phosphate Buffer (pH 6.8).

3.4. Tyrosinase Inhibition Assay

The tyrosinase activity was measured according to Karioti et al. (8) with some modifications. First, 50 μL of each extracts in six different concentrations (8.3, 4.2, 2.1, 1.0, 0.52 and 0.26 mg/mL) was mixed with 100 μL of mushroom tyrosinase (9.63 U/mL) and incubated at 25°C for five minutes. Then 100 μL of 5 mM of L-Dopa solution (substrate for tyrosinase) was added to the mixture reaction. The amount of dopachrome as a product was immediately measured at 475 nm of optical density.

The increased absorbance at 475 nm was recorded during 35 minutes. DMSO and kojic acid were used as negative and positive controls, respectively. The concentrations 0.04, 0.08, 0.12 and 0.16 mg/mL of kojic acid were used. The inhibitory effects of test samples on the enzyme activity were expressed as percent inhibition and IC50 values were calculated. IC50 value is the concentration of the inhibitor that inhibits 50% of tyrosinase activity under experimental conditions and obtained through plot of inhibition percent versus Log conc (mg/mL).

$$\%\text{Inhibition} = \{[(A-B)-(C-D)]/(A-B)\} \times 100 \quad (1)$$

A: optical density of L-Dopa, DMSO and enzyme.
B: optical density of L-Dopa and DMSO.
C: optical density of test sample (kojic acid and the extracts), enzyme and L-Dopa.
D: optical density of test sample (kojic acid and the extracts) and L-Dopa.

3.5. Determination of Enzyme Kinetic Parameters

Fifty microliters of different concentrations (0.52, 1.04, 2.08 and 4.16 mg/mL) of extracts were incubated with 100 μL of mushroom tyrosinase at 25°C for five minutes. Then different volumes (100, 80, 60, 40, 20 and 10 μL) of 5 mM L-Dopa in phosphate buffer (pH 6.8) were added to the reaction mixture. Kinetic parameters, K_m and V_m of tyrosinase activity were calculated by linear regression from

Lineweaver–Burk plots. The inhibition constant (K_i) of an inhibitor was obtained from the secondary plot of Lineweaver–Burk plots. The secondary plot consists of vertical axis that is the slops of Lineweaver–Burk plots and horizontal axis that is inhibitor concentrations. The intercept on the horizontal axis is K_i (38).

3.6. Statistical Analysis

The measurements were performed in triplicate and mean was used in calculations.

4. Results

4.1. Inhibitory Effects of Plants on Mushroom Tyrosinase Activity

In this study, tyrosinase inhibitory effects of *P. alkekengi*, *A. rosea*, *B. persicum* and *M. vulgare* on diphenolase activity of mushroom tyrosinase were evaluated. As with increasing the concentration of all plants, the activity of tyrosinase decreased. Therefore, plants had a dose-dependent inhibitory effect on tyrosinase (Figure 1).

All total extracts of plants inhibited tyrosinase weaker than kojic acid (IC_{50} = 0.014 mg/mL). IC_{50} values of *P. alkekengi*, *A. rosea*, total and defatted extracts of *B. persicum* and *M. vulgare* were calculated 0.09, 0.38, 0.37, 0.38 and 2.82 mg/mL, respectively.

4.2. Kinetic Study of Mushroom Tyrosinase Activity

The kinetic behavior of the samples for the inhibition of tyrosinase was also analyzed by lineweaver-Burk plots. kojic acid, *P. alkekengi*, *A. rosea* and *B. persicum* increased the K_m, but the K_m was decreased when *M. vulgare* was used. All the extracts and kojic acid decreased V_m value (Table 1). Lineweaver–Burk plots of *P. alkekengi* different concentrations were shown in Figure 2. As *P. alkekengi* exhibited the most tyrosinase inhibitory activity, its K_i was calculated as 0.41 mg/mL (Figure 3).

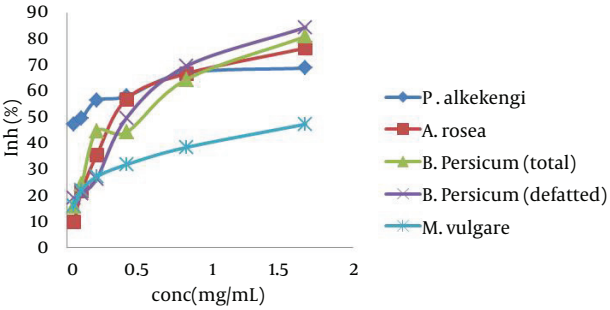

Figure 1. The Effect of Different Concentrations of *P. alkekengi*, *A. rosea*, *B. persicum* and *M. vulgare* on Mushroom Tyrosinase Activity for the Catalysis of L-Dopa

Table 1. Kinetic Parameters of Enzyme With and Without Extracts

Variable	No Inhibitor	Kojic Acid	*P. alkekengi*	*A. rosea*	*B. persicum* (Total)	*B. persicum* (Defatted)	*M. Vulgare*
Km (mM)	0.16	0.45	0.22	0.3	0.17	0.19	0.11
Vm (U) [a]	4.3	2	1.5	2.2	1.6	1.6	2.1

[a] Unite: One unit (U) of enzymatic activity was defined as the amount of enzyme needed for increasing 0.001 unit of absorbance per min at 475 nm under the experimental conditions.

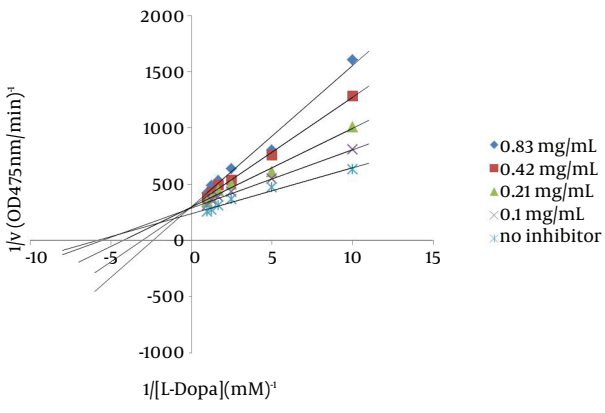

Figure 2. Lineweaver–Burk Plots of Mushroom Tyrosinase and L-Dopa With and Without *P. alkekengi* Different Concentrations

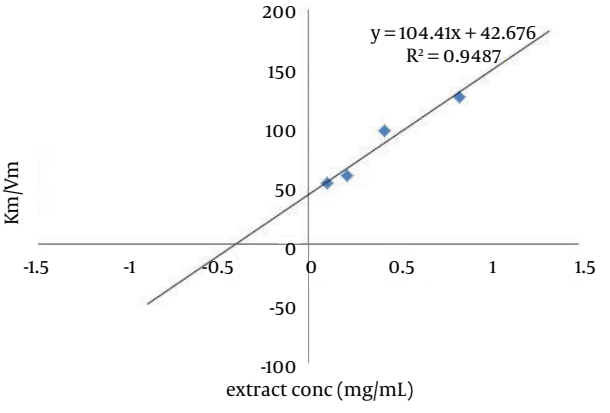

Figure 3. The Slope of Lineweaver–Burk Plots Versus the Concentration of *P. alkekengi* for Determination of Inhibitory Constant (K_i) of Tyrosinase

5. Discussion

The tyrosinase inhibitors could be used in medicine, cosmetic and food industries to treat or prevent hyperpigmentation disorders and food browning. Based on our best knowledge, studies on anti-tyrosinase effect of *A. rosea*, *B. persicum* and *M. vulgare* have not been performed before.

Among test plants, *P. alkekengi* showed the most tyrosinase inhibitory effects, indicating its potent tyrosinase inhibitors or high content of active compounds with tyrosinase inhibitory effects.

Other species of this genus, *P. divaricata* and *P. edulis* exhibited tyrosinase inhibitory activities. *P. divaricata* showed tyrosinase inhibitory effect with IC_{50} value of 3.34 mg/mL (39) and *P. edulis* inhibited tyrosinase 32% at 10 mg/mL (9), while *P. alkekengi* showed tyrosinase inhibitory activity of 32% at 0.03 mg/mL. Therefore, *P. alkekengi* showed more potent tyrosinase inhibitory activity than *P. edulis* and *P. divaricata*.

The fruits of *P. alkekengi* have a lot of ascorbic acid (40). Ascorbic acid can prevent enzymatic reactions of tyrosinase through trapping the O-dopaquinone intermediate (1). Steroids can inhibit tyrosinase and so far three steroids from the aerial parts of *Trifolium balansae* were isolated which showed potent tyrosinase inhibitory effects (41). Some steroidal compounds such as physalin were isolated from *P. alkekengi* (42), which may contribute to its tyrosinase inhibitory activity.

Total and defatted extracts of *B. persicum* exhibited similar inhibitory effects on mushroom tyrosinase, which shows that non-polar compounds of *B. persicum* do not contribute to its tyrosinase inhibitory effect. In another study, *B. persicum* showed 42% inhibition at 1.14 mg/mL for diphenolase activity of mushroom tyrosinase (43). This difference could be due to various experimental conditions.

Cuminaldehyde, kaempferol, caffeic acid and p-coumaric acid are found in *B. persicum* (44) and can inhibit mushroom tyrosinase (1, 45-47). The tyrosinase inhibitory effect of *B. persicum* may be related to these compounds.

C. cyminum another kind of cumin could inhibit tyrosinase more potent than *B. persicum*, 45.8% at 50 μg/mL (10). It may contribute to more content of Cuminaldehyde in *C. cyminum* than *B. persicum* (44, 48). Gholamhoseinian et al. reported that *C. cyminum* had more anti-tyrosinase effect than *B. persicum* (43).

A. officinalis, another species of *Althea*, inhibited tyrosinase activity 48% (9) at 10 mg/mL, when *A. rosea* showed the same inhibition at 1.75 mg/mL. Therefore, *A. rosea* was more potent than *A. officinalis* in tyrosinase inhibition.

The flowers of *A. rosea* are the source of polyphenolic compounds such as anthocyanins (49) shown to have tyrosinase inhibitory activity (4). Other species of *Marrubium*, *M. cylleneum* and *M. velutinum* inhibited tyrosinase by 35.44% and 30.56% at 0.033 mg/mL, respectively and *M. vulgare* showed 16% inhibition at 0.05 mg/mL (8). Therefore, *M. vulgare* was weaker than *M. velutinum* and *M. cylleneum* in tyrosinase inhibition, which may be related to their high content of tyrosinase inhibitors. Amongst 45 isolated compounds with tyrosinase inhibitory activity from *M. velutinum* and *M. cylleneum*, quercetin showed the most activity (49.67% at 0.051 mM) (8). Some of these compounds such as several flavonoids (ladanein, glycoside derivatives of luteolin, chrysoeriol and apigenin) and cinnamic acid derivatives were isolated from *M. vulgare* (8, 33) that may contribute to its inhibitory activity.

Kojic acid, *P. alkekengi*, *A. rosea* and *B. persicum* exhibited mixed inhibition. They can bind to free enzyme and enzyme-substrate complex and reduce the affinity of substrate for the mushroom tyrosinase (50). However, *M. vulgare* showed uncompetitive inhibition. It can bind to enzyme-substrate complex and produce deactivated ESI complex and cannot bind to free enzyme (50). Although *B. persicum* inhibited tyrosinase through mixed mechanism, cuminaldehyde showed uncompetitive inhibition in previous studies (1).

In this study, we used crude extracts. Active compounds have a key role in inhibitory effect on mushroom tyrosinase. We did not isolate, identify and study pure active compounds. It can be performed on most potent herbs in future. According to this study, the extract of *P. alkekengi* had the most inhibitory effect on mushroom tyrosinase, but in vivo and clinical studies are needed to confirm the inhibitory effect and safety of *P. alkekengi*. Study active compounds of *P. alkekengi* could lead to development of new and effective tyrosinase inhibitors.

Antibacterial, antioxidant and anti-tyrosinase effects have been shown for *P. alkekengi*, *A. rosea*, *B. persicum* and *M. vulgare*; therefore, these plants could be considered as good food additives to prevent food browning and growth of microbes, but more studies on anti-browning effects and safety of these plants should be performed.

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Authors' Contributions

Study concept and design: Foroogh Namjoyan, Alireza Jahangiri and Mohammad Ebrahim Azemi, Acquisition of data: Elaheh Arkian, Analysis and interpretation of data: Alireza Jahangiri and Elaheh Arkian, Drafting of the manuscript: Elaheh Arkian and Hamideh Mousavi, Critical revision of the manuscript for important intellectual content: Foroogh Namjoyan, Mohammad Ebrahim Azemi and Alireza Jahangiri, Statistical analysis: Elaheh Arkian.

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