Pharmaceutical Sciences, June 2016, 22, 81-86
doi: 10.15171/PS.2016.14
http://journals.tbzmed.ac.ir/PHARM

Research Article

Inhibitory Effects of *Urginea maritima* (L.) Baker, *Zhumeria majdae* Rech. F. and Wendelbo and *Physalis divaricata* D. Don Ethanolic Extracts on Mushroom Tyrosinase

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Article Info

Article History:
Received: 30 June 2015
Accepted: 16 March 2016
ePublished: 30 June 2016

Keywords:
-*Urginea maritima*
-*Zhumeria majdae*
-*Physalis divaricata*
-Tyrosinase inhibition

ABSTRACT

Background: Tyrosinase is a key enzyme in melanin synthesis from tyrosine. To prevent or treat pigmentation disorders, tyrosinase inhibitors have been used increasingly for medicinal and cosmetic products.
The aim of this study is to evaluate inhibitory effects of *Urginea maritima* (L.) Baker, *Zhumeria majdae* Rech.f. & Wendelbo and *Physalis divaricata* D.Don on mushroom tyrosinase.
Methods: The inhibitory activities of the hydroalcoholic extracts of plants against oxidation of L-DOPA (as a substrate) by mushroom tyrosinase were investigated. The amount of formed DOPAchrome was determined at 475 nm as optical density.
Results: The extracts showed anti-tyrosinase activity weaker than positive control (Kojic acid). The inhibitory activity of tested plants: *U.maritima*, *Z.majdae* and *P.divaricata* against mushroom tyrosinase were 38.61, 29.70 and 25.74 % at 1.67 mg/mL, respectively.
Conclusion: The most tyrosinase inhibitory activity was seen for *U.maritima*. However more investigations on human tyrosinase, toxicological and clinical studies are needed to confirm its activity.

Introduction

Tyrosinase (EC 1.14.18.1) is a multi-functional copper-containing enzyme which is present in a wide phylogenic spectrum from bacteria to mammals.[1] In the pathway of melanin synthesis, tyrosinase is a key enzyme which, catalyses ortho hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine or DOPA (monophenolas activity) and the oxidation of DOPA to DOPAquinone (diphenolase activity).[2]
Melanin plays a crucial role in protecting human skin from the harmful effects of ultraviolet (UV) radiations.[3] The over production of melanin results in hyperpigmentation conditions such as: melasma, freckles, senile lentigines, etc.[4]
Furthermore, tyrosinase is responsible for undesirable enzymatic browning in the plants during post-harvest handling and processing. The browning leads to a less attractive appearance and loss in nutritional quality of food and becomes a major problem in the food industries.[5,6]
Hence, the tyrosinase inhibitors could be used in medicine, cosmetics and food industries to prevention or treatment of pigmentation disorders and food browning. Thus, identification of novel tyrosinase inhibitors extremely is important.[7] Several tyrosinase inhibitors such as arbutin, kojic acid, hydroquinone and its derivatives are used in the clinics, but due to their low activities and cell toxicities, it is necessary to develop novel effective and feasible anti-tyrosinase agents.[8] Potent tyrosinase inhibitors such as steppogenin, norartocarpetin were isolated from plants, there are increasing efforts to find herbal tyrosinase inhibitors.[9,10]
Some plants of the Lamiaceae and liliaceae families had inhibitory effects on tyrosinase activity, such as *Hyptis suaveolens*, *Marrubium velutinum* and *Ocimum sanctum* from Lamiaceae family[11-13] and *Aloe vera*, *Paris polyphylla* from Liliaceae family.[11] Moreover, *Physalis edulis* from Solanaceae family could inhibit mushroom tyrosinase.[12] Hence, due to same effects of plants of a family, we selected *Zhumeria majdae* Rech.f. & Wendlbo (Lamiaceae), *Urginea maritima* (L) Baker (Liliaceae), *Physalis divaricata* D.Don (Solanaceae) for this experiment.
U.maritima (white squill or sea onion) is distributed in the Mediterranean area, North Africa and India.[14] This plant traditionally is used for treatment of asthma, cancer and cardiac failure.[15]
Some studies have been exhibited cardiovascular[16] and anti-insect[17] activities of *U.maritima* bulbs. In addition,

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U.maritima showed a strong cytotoxic effect against human cervical cell lines.[18]

Z.majdae is found in southeastern Iran and known mohrkhosh. It showed anti-inflammatory, antinociceptive,[19] antibacterial,[20] herpes simplex type 1 inhibitory,[21] antileishmanial, antiplasmodial, cytotoxic,[22] acetylcholinesterase inhibitory[23] and α-glucosidase inhibitory activities.[24]

P.divaricata is a summer weed in crop fields and distributed widely in west of Iran.[25] The antioxidant,[26] anti-inflammatory, antipyretic, analgesic,[27] antimicrobial[28] effects have been reported for other *physalis* species, while few studies have been done on *P.divaricata*.

This study was done to evaluate the inhibitory effects of *U. maritima*, *Z. majdae* and *p. divaricata* total extracts on mushroom tyrosinase.

Materials and Methods

Chemicals

Mushroom tyrosinase (3130 unit/mg) and L-DOPA (powder, ≥98.0 %) from Sigma Chemical Co, kojic acid (powder, ≥98.0 %) from Fluka Chemical Co, DMSO (Dimethylsulfoxide), ethanol and other chemicals used were of analytical grades.

Plant materials

The bulbs of *U.maritima*, the leaf of *Z.majdae* and the aerial parts of *P.divaricata* were collected from Khuzestan, Geno Mountains in Bandar Abbas and near Perspolis in 45 km from Shiraz, respectively. They were identified by the pharmacognosy department of Ahvaz Jundishapur University of Medical Sciences.

Preparation of plant extracts

The plant materials were grounded up and 72 h macerated with ethanol 80 % at the room temperature. The extracts were filtered and evaporated using a rotary evaporator (Heidolf, Germany) and lyophilized with freeze-dryer (Operon, Korea). The 100 mg of extracts was dissolved in three mL of DMSO subsequently diluted with 25 mM potassium phosphate buffer (pH 6.8).

Tyrosinase inhibitory assay

The inhibitory effects of the plant extracts on diphenolase activity of the mushroom tyrosinase were determined according to Karioti *et al*. with some modification.[13] In brief, 100 µL of the mushroom tyrosinase (9.63 unit/mL) in 25 mM potassium phosphate buffer (pH 6.8) was added to 50 µL of the different concentrations of the extracts (1.67, 0.83, 0.42, 0.21, 0.10 and 0.05 mg/mL). After incubation at the room temperature for five min, 100 µL of L-DOPA (2.5 mM) in 25 mM potassium phosphate buffer (pH 6.8) was added to a 96-well plate in a total volume of 250 µL. The amount of DOPAchrome formed in the reaction mixture was determined against the blank (extract and L-DOPA) at 475 nm as optical density in a microplate reader (Tecan sunrise, Switzerland) at 70-second intervals for 35 min.

Negative (DMSO) and positive (Kojic acid) controls were used. The concentrations 0.04, 0.032, 0.024, 0.016 and 0.008 mg/mL of kojic acid was used. The IC_{50} values of extracts were calculated. The IC_{50} value is the concentration of extract that inhibits 50 % of enzyme activity. Percent inhibition of tyrosinase activity was calculated as:

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

A: optical density at 475 nm of the mixture enzyme, L-DOPA and DMSO.

B: optical density at 475 nm of the mixture L-DOPA and DMSO.

C: optical density at 475 nm of the mixture enzyme, L-DOPA and extract.

D: optical density at 475 nm of the mixture L-DOPA and extract.

Kinetic analysis of tyrosinase inhibition

The inhibition type, Vm and Km of enzyme

The reaction mixture containing 100 µL of the mushroom tyrosinase solution, 25 mM potassium phosphate buffer (pH 6.8) and 50 µL of 0.21 mg/mL extracts were incubated at the room temperature for five min. Then 10, 20, 40, 60, 80 and 100 µL of L-DOPA solution (2.5 mM) were added to each well and optical density of reactions at 475 nm were read. The absorbance was recorded at 70-second intervals. The velocity (V) was calculated from the slope of the absorbance plot versus time. The Michaelis constant (K_m) and the maximal velocity (V_m) kinetic parameters of the tyrosinase activity were determined using a Linewear-Burk plot which is 1/V versus 1/[L-DOPA] and the equation was:

$$1/V = Km/Vm\,(1/S) + 1/Vm \quad \text{Eq.(2)}$$

The inhibition type was determined by changes type in V_m and K_m of the enzyme.

The inhibition constant (Ki) of enzyme

The mixture containing 50 µL of 0.42, 0.21, 0.10 and 0.05 mg/mL extracts and 100 µL of the enzyme incubated for 5 minutes at the room temperature. Then 10, 20, 40, 60, 80 and 100 µL of L-DOPA (2.5 mM) were added to each concentration of the extracts and the absorbance of the reactions at 475 nm in 70 seconds intervals were read.

The Linewear-Burk plot of each concentration of the extracts was drawn. The inhibition constant (K_i) was determined by the second plots of the apparent K_m/V_m (the slope of Linewear-Burk plot) versus the concentration of the extract. The K_i is the point which the graph intercepts the x-axis.

Results

We evaluated inhibitory effects of the total extracts of *U.maritima*, *Z.majdae* and *P.divaricata* on mushroom

tyrosinase with L-DOPA as the substrate. The extracts of *U.maritima*, *Z.majdae* and *P.divaricata* inhibited oxidation of L-DOPA by tyrosinase with 38.61, 29.70 and 25.74 % at 1.67 mg/mL, respectively. *U.maritima* was stronger than that of other plants in the inhibition of tyrosinase. All plants showed weaker anti-tyrosinase activity than that of the kojic acid.

Table 1. The Inhibitory effects of U.maritima, Z.majdae and P.divaricata extracts on the mushroom tyrosinase activity in different concentrations.

Conc (mg/mL)	*P.divaricata* (%)	*Z.majdae* (%)	*U.maritima* (%)
1.67	25.74	29.7	38.61
0.83	19.8	22.97	30.69
0.42	10.89	15.94	25.74
0.21	9.9	8.99	22.77
0.10	5.94	4.99	15.84
0.05	2.97	1.94	12.73

The extracts inhibited tyrosinase concentration-dependently as shown in Table 1. As the concentration of all plants increased, tyrosinase activity decreased.

We evaluated the inhibitory activity of the mentioned plants on tyrosinase in different concentration of L-DOPA to examine their inhibitory mechanism. All plants decreased K_m and V_m values of the mushroom tyrosinase, while Kojic acid decreased V_m value and increased K_m value (Table 2).

The inhibition constant (K_i) of *U.maritima* due to more inhibitory activity than other plants was determined. The Linewear-Burk plots of different concentrations of *U.maritima* and kojic acid in the presence of different concentrations of L-DOPA were drawn to determine the inhibition constant (K_i). Figure 1 shows linewear-Burk plots of 0.83 and 0.42 mg/mL of *U.maritima* (a) and 0.04, 0.032 mg/mL of kojic acid (b).

The K_i of *U.maritima* and kojic acid were estimated to be 5.98 and 1.2×10^{-2} mg/mL, respectively.

Discussion

Many studies have been done to screen new natural tyrosinase inhibitors for using in medicine, cosmetic and food industries to treat the hyperpigmentation disorders and prevent the food browning.

This study showed anti-tyrosinase activities of *U.maritima*, *Z.majdae* and *P.divaricata* which, inhibited oxidation of L-DOPA by mushroom tyrosinase weaker than kojic acid.

The inhibition type of all plants was determined to be uncompetitive due to decrease in K_m and V_m values of the enzyme. In other words, they only bind to enzyme-substrate complex.[29]

Kojic acid decreased the apparent value of V_m and increased value of K_m. Hence, the inhibition mode of kojic acid was determined the mixed type which, means it can bind to either free enzyme or enzyme-substrate complex.[29]

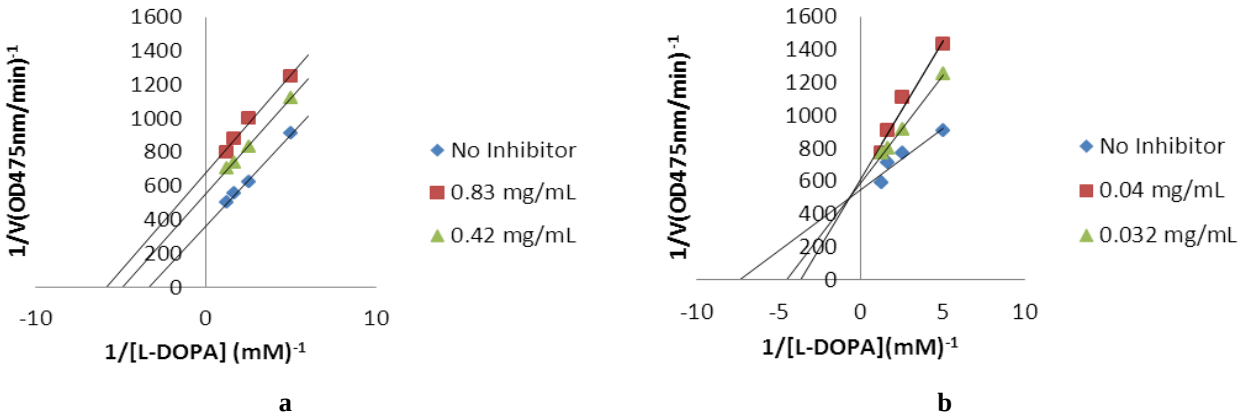
On base of our best knowledge, previous studies on tyrosinase inhibitory effects of all plants haven't been done.

U.maritima inhibited tyrosinase stronger than that of other the plants. Quercetin, taxifolin and kaempferol glycosides are found in white squill,[30] which exhibited tyrosinase inhibitory effects.[31-33] The anti-tyrosinase activity of white squill may probably due to the presence of quercetin, taxifolin and kaempferol glycosides.

Table 2. Kinetic parameters of tyrosinase in the presence and without of test samples.

	No inhibitor	*U.maritima*	*Z.majdae*	*P.divaricata*	Kojic acid
K_m (mM)	1.6×10^{-1}	0.9×10^{-1}	1.2×10^{-1}	0.9×10^{-1}	2.7×10^{-1}
Vm (U)[a]	1.7×10^{-3}	1.3×10^{-3}	1.5×10^{-3}	1.3×10^{-3}	1.6×10^{-3}

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



a **b**

Figure 1. Lineweaver–Burk plots in the absence (control) and in the presence of 0.83, 0.42 mg/mL of *U.maritima*(a) and 0.04, 0.032 mg/mL of kojic acid(b) with l-DOPA as the substrate are shown.

Squill showed antioxidant and free radical scavenging activities[34] and strong cytotoxic effect against cultured melanoma cell line.[35] Redox agents can inhibit melanogenesis through interference with copper atom in active site of tyrosinase, interacting with O-quinones, thus reduction of oxidative polymerization of melanin intermediates and scavenging ROS radicals generated in skin following UV which are able to stimulate melanogenesis.[36]

So, *U.maritima* can inhibit melanogenesis through anti-tyrosinase, antioxidant and cytotoxic activities.

Z.majdae inhibited oxidation of L-DOPA 29.7% at 1.67 mg/mL. The anti-tyrosinase effects of *Z.majdae* may probably contribute to its flavonoids content.

Also, *Z.majdae* exhibited antioxidant and free radical scavenging activities[20] which could relate to its anti-tyrosinase effect.

Few studies were done on *P.divaricata*. It had cytotoxic effect[37] and in this study, inhibited oxidation of L-DOPA by mushroom tyrosinase 25.74 % at 1.67 mg/mL. Other *Physalis* species also exhibited anti-tyrosinase activity. *P.edulis* roots was weaker than *P.divaricata* and showed 32% inhibition at 10 mg/mL on diphenolase activity of tyrosinase.[12] In another study: *P.alkekengi* inhibited tyrosinase more potent than *Alcea rosea*, *Bunium persicum B.Fedtsch* and *Marrubium vulgare L*. The IC_{50} value of *P.alkekengi* was 0.09 mg/mL and was stronger than *P.divaricata* in tyrosinase inhibition.[38]

The glycosides of quercetin and luteolin were isolated from *P.alkekengi*.[39] Quercetin and luteolin could inhibit tyrosinase.[31,40] Due to same chemical profiles of species of a genus, glycosides of quercetin and luteolin may probably contribute to the tyrosinase inhibitory activity of *P.divaricata*.

Conclusion

In this study the tyrosinase inhibitory activity of *U.maritima*, *Z.majdae* and *P.divaricata* on mushroom tyrosinase using L-DOPA as substrate for the first time were evaluated. All plants have the capability to inhibit mushroom tyrosinase, but their inhibitory activities of active compounds were not investigated. Moreover inhibitory activities of samples on tyrosine hydroxylation were not studied. So, further investigations of total extracts and their active compounds on human tyrosinase using L-DOPA and tyrosine as substrates, toxicological and clinical studies are needed to confirm these activities and safety of them.

Acknowledgements

The authors are grateful to the pharmacognosy department of Ahvaz Jundishapur University of Medical Sciences and also Ms.M.Feli for editing of article. This research is a part of a project granted (HC-009) by Ahvaz Jundishapur University of Medical Sciences.

Conflict of interests

The authors claim that there is no conflict of interest.

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