

# INTERNATIONAL JOURNAL OF CURRENT RESEARCH IN CHEMISTRY AND PHARMACEUTICAL SCIENCES

(p-ISSN: 2348-5213; e-ISSN: 2348-5221)  
www.ijrcps.com



## Research Article

### THE EFFECT OF GALLIC ACID, NARINGIN, CHRISIN AND QUERCETIN AS FLAVONOIDS, ON THE THERMODYNAMIC STABILITY OF TYROSINASE

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## Abstract

**Background:** Tyrosinase is a copper-containing monooxygenase responsible for the biosynthesis of melanins and other polyphenolic compounds. Since tyrosinase function lead to the different biological process through animals and its deficiency may cause problems, thus the study of its activation or stabilizations is as important as its inhibition. **Method:** The thermodynamical stability and inhibition of mushroom tyrosinase (MT) from *Agaricus Bisporus* were investigated in the presence of gallic acid, naringin, chrysin, quercetin. Therefore, the protein denaturation and thermal scanning was studied. Sigmoid denaturation curves were analysed according to the two models of Pace theory to obtain the Gibbs free energy change of denaturation process. **Results and conclusion:** In the presence of gallic acid, kinetic assessment of the enzyme activity showed a non-competitive inhibition and the chrysin, naringin and quercetin induced a competitive inhibition.. Although these flavonoids induced MT thermal and chemical stability, the  $G^{\circ}H_2O$  magnitudes for sole enzyme, in presence of gallic acid, naringin, chrysin and quercetin was obtained 6.39, 7.21, 6.77, 8.56, and 8.04 respectively. Also, the melting points ( $T_m$ ) of enzyme in above conditions from thermal denaturation were calculated 56.2, 62.62, 59.09, 57.53 and 57.22oC, respectively. So, these flavonoids, induced physico-chemical stability of tyrosinase.

**Keywords:** Flavonoids; Tyrosinase; Enzyme stability.

## Introduction

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase responsible for the biosynthesis of melanins and other polyphenolic compounds (Uchida et al., 2014). It catalyses both the orthohydroxylation of monophenols and the oxidation of o-diphenols to o-quinones. Tyrosinase is widely distributed in mammals, plants and micro-organisms and plays a crucial role in

melanogenesis as the key enzyme (Kim et al., 2012). Mushroom tyrosinase has a molecular mass of 120 kDa that catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), and L-DOPA to DOPA quinone (Ha et al., 2011). Quinones chemically evolve to give rise to melanins or react with amino acids and proteins to enhance the colour products, which are brown, black, or red heterogeneous polymers (Huang et al., 2006).

The tyrosinase enzyme is responsible for skin, eye, inner ear and hair melanisation, and browning in fruits and vegetables. Its active site consists of two copper atoms and three states: met deoxy and oxy (Sharma et al., 2004). In a fresh preparation, %85 of the total enzyme is in the met-tyrosinase form, and its active site contains two copper ions that unable to bind to oxygen.

Only a small proportion of the enzyme is present as the oxy-form, which is the form that acts on monophenols (Gandía et al., 2003). The hydroxylation of L-tyrosine, the initial step in melanin synthesis, is of considerable importance since it is also the initial step in catecholamine synthesis.

The oxidation of monohydric phenols by tyrosinase exhibits an induction (or lag) period during which the rate of oxidation is accelerated (Cooksey et al., 1998). Chemical and spectroscopic studies on hemocyanin and tyrosinase have demonstrated that both proteins have similar active sites. Secondary structure predictions and homology to copper proteins structures have indicated that the CuA and CuB regions contain a  $\alpha$ -helical bundle creating a hydrophobic pocket near the protein surface (Park et al., 2003).

Since tyrosinase function lead to the different biological process through animals, plants, fungi, insects and other organisms, and its deficiency may cause problems as well as its over activity, thus the study of its activation and/or stabilizations is as important as its inhibition. Mushrooms also produce a variety of secondary metabolites, including phenolic

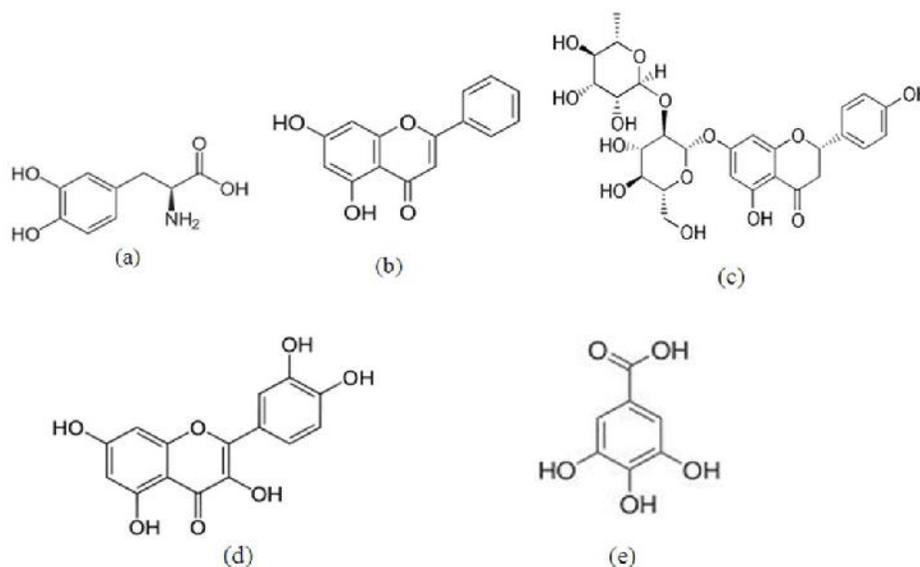
compounds, polyketides, terpenes and steroids which reduce oxidative damage in human tissues (Teissedre et al., 2000).

Mushrooms were considered as proper sources of proteins and phenolic-antioxidants, such as variegatic acid and gallic acid (Cheung et al., 2003). In this study, the importance of mushrooms have emphasized as a major natural food source containing the different antioxidants and tyrosinase. Tyrosinase inhibition has been considered due to its potential in the cosmetic, medicinal and agricultural industries. Therefore, this project was designed considering previous studies on inhibition, stability, structure and function of mushroom tyrosinase (MT) (Gheibi et al., 2005; Gheibi et al., 2006; Gheibi et al., 2009; Haghbeen et al., 2010), in order to investigate the effects of gallic acid, naringin, chrysin and quercetin on the inhibition and stability of mushroom tyrosinase..

## Materials and methods

### Materials and MT preparation

Mushroom tyrosinase (MT; EC 1.14.18.1) with specific activity 3400 units/mg,  $\text{Na}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$ , gallic acid, naringin, chrysin, quercetin and urea were purchased from Sigma chemical Co. (St. Louis, MO, USA). The chemical formula of all compounds used in this research is presented in figure 1. The buffer used in this research was 10 mM phosphate buffer solution (PBS), pH 6.8, and the salts were purchased from Merck Co. All experiments were carried out at 293 °K and all solutions were prepared in deionized distilled water.



**Figure 1.** Structures of L-Dopa (a), Chrysin(b), Naringin(c), Quercetin (d) and Gallic acid (e).

### Kinetic assessment of enzyme inhibition in the presence of flavonoids

MT activity was determined at 20 °C followed by increasing in absorbance at 475 nm accompanying the oxidation of the L-DOPA, as the enzyme substrate with the molar absorption coefficient of 3700 ( $M^{-1} cm^{-1}$ ). Spectrophotometric measurements were carried out with a UV- 2100 spectrophotometer. All of the catecholase reactions were carried out in 50mM phosphate buffer solutions at pH 6.8 and 20 °C.

30  $\mu$ l of mushroom tyrosinase solution (100 units), different concentrations of L-dopa (0.3, 0.6, 0.9& 1.2 mM), as a substrate, and different concentrations of each inhibitors were added to a cell and diluted to 1 ml by addition of phosphate buffer. The kinetics behavior of the enzyme follows by Michaelis–Menten kinetics. The  $K_m$  and maximal velocity ( $V_{max}$ ) of the tyrosinase activity were determined by the inverse reciprocal plots of Lineweaver-Burk. The inhibition type was determined by the Lineweaver-Burk plot, and the inhibition constant ( $K_i$ ) of the tyrosinase activity were determined by the secondary plots of the apparent  $K_m/V_{max}$  or  $1/V_{max}$  versus the concentration of the inhibitor.

$$1) \frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad 2) \text{ plots} = \frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i}\right)$$

### MT chemical stability measurements

To assess the effect of flavonoids on the chemical stability of the enzyme, chemical denaturation profiles of MT were recorded by titration of a protein solution 0.8mg/ml with aliquots from a 5 M stock solution of urea. These experiments were carried out in the absence and presence of 0.15mM gallic acid, 0.30mM naringin, 0.79mM chrysin and 0.74mM concentrations

of quercetin. Conformational change of the protein was obtained at wavelength of 280 nm by the spectrophotometric technique.

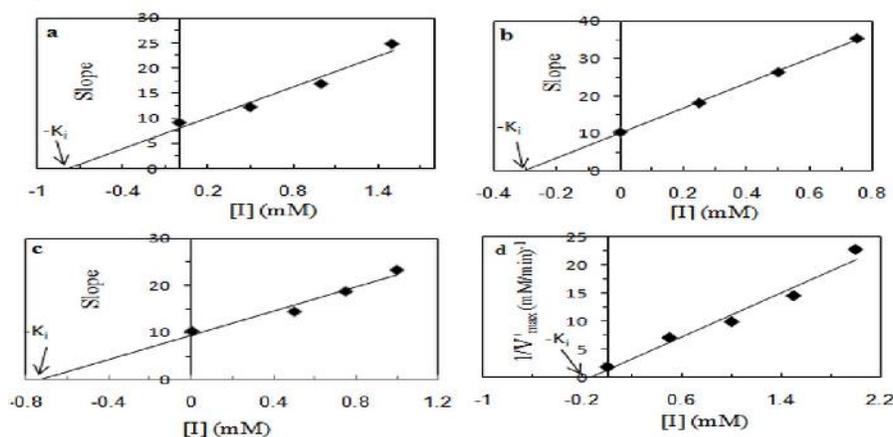
### Thermal analysis of MT

Fluorescent thermal intensity measurements were carried out on a spectrofluorimeter, Cary Eclipse model 100, equipped with a thermostatically controlled cuvette compartment. Different temperatures were from 20 to 95°C and emission spectra were recorded from 280 to 350 nm with excitation at 280 nm in increments of 1 nm and the intrinsic fluorescence thermal of MT, in the absence and presence of 0.15 mM gallic acid, 0.30 mM naringin, 0.79 mM chrysin and 0.74 mM of quercetin. MT complexes were measured by exciting the protein solution (0.8 mg/ml) in 10 mM PBS buffer at pH 6.8 and different temperature were from 20 to 95 °C, in 1 ml semi-micro quartz cuvettes with a 1 cm excitation light path.

## Results

### Inhibition of MT by falvonoids

The oxidation reaction of L-DOPA by mushroom tyrosinase follows Michaelis–Menten kinetics. As shown in figure 2, the inhibition constants ( $K_i$ ) of the enzyme in the presence of gallic acid, naringin, chrysin and quercetin were obtained by Lineweaver–Burk replots. For the inhibitor binding with free enzyme (E),  $K_i$  is obtained from the secondary plots of the slope versus concentration of gallic acid, naringin, chrysin and quercetin. The kinetic data of MT inhibition with gallic acid showed non-competitive inhibition, but the chrysin, naringin and quercetin induced a competitive manner of inhibition. The  $K_i$  obtained from fig 2 are 0.15, 0.30, 0.79 and 0.74 mM for gallic acid, naringin, chrysin and quercetin respectively.

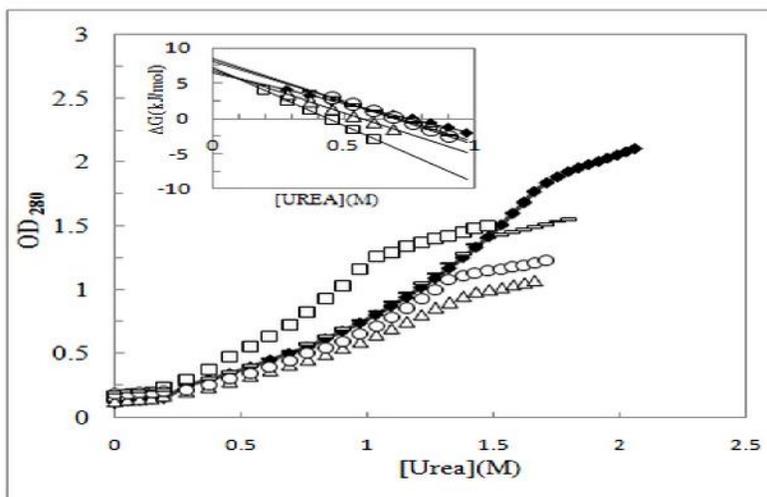


**Figure 2.** Secondary plots obtained from reciprocal plots of Lineweaver–Burk. The inhibition constant ( $K_i$ ) was determined by the X-intercept of the plots in the presence of different concentrations of chrysin (a), naringin (b), quercetin (c) and gallic acid (d) as MT inhibitors.

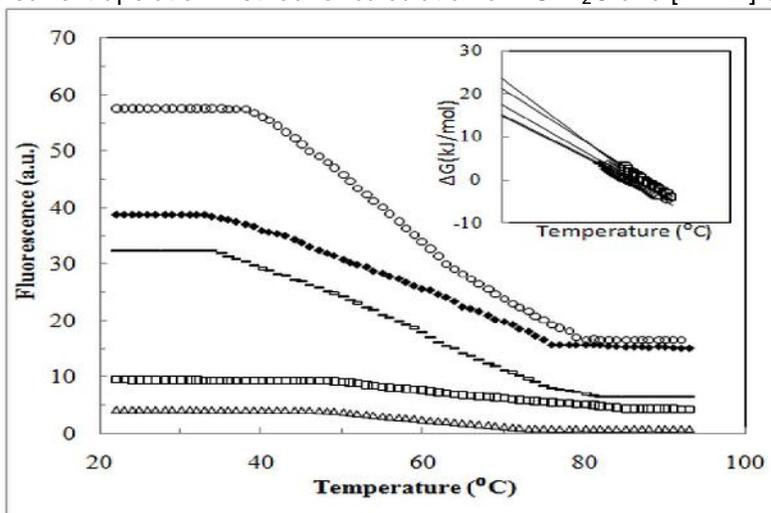
### Chemical and thermal denaturation

Chemical and thermal stability of MT was assessed in the absence and presence of flavonoids. Chemical and thermal denaturation profiles were obtained from

urea titration and thermal scanning, respectively, in the absence and presence of gallic acid, naringin, chrysin and quercetin in definite concentrations that has been depicted in figures 3 and 4.



**Figure 3.** Chemical denaturation curve of 0.8 mg/ml MT by urea solution at 280 nm in 10 mM PBS, 20°C and pH 6.8, in the absence ( ) and presence of 3 mM, naringin( ), quercetin ( ) and 1.5mM of gallic acid( ), chrysin( ). Inset: Linear extrapolation method for calculation of  $G^{\circ}_{H_2O}$  and  $[DTAB]_{1/2}$ .



**Figure 4.** a) Thermal denaturation curves obtained from molar .8mg/ml MT in PBS, and pH 6.8, in the absence ( ) and presence of 3 mM, naringin( ), quercetin ( ) and 1.5mM of gallic acid( ), chrysin( ). Inset: Linear extrapolation method for calculation of  $G^{\circ}_{25}$  and  $T_m$ .

Each profile is a sigmoidal curve, thus this process is described as a single denaturant-dependent step according to the two-step theory [15]. The determination of standard Gibbs free energy of denaturation ( $G^{\circ}$ ), as a criterion of conformational stability of a globular protein, is based on two state theory as follows:



By assuming two-state mechanism for protein denaturation, one can determine the process by monitoring changes in the absorbance or fluorescent thermal intensity, and hence calculate the denatured fraction of protein ( $F_d$ ) as well as determination of the equilibrium constant (K).

$$F_d = \frac{(Y_N - Y_{obs})}{(Y_N - Y_D)} \quad (2)$$

$$K = \frac{F_d}{(1 - F_d)} = \frac{(Y_N - Y_{obs})}{(Y_{obs} - Y_D)} \quad (3)$$

Where  $Y_{obs}$  is the observed variable parameter (e.g absorbance or thermal intensity) and  $Y_N$  and  $Y_D$  are the values of Y characteristics of a fully native and denatured conformation, respectively. The standard Gibbs free energy change ( $\Delta G^0$ ) for protein denaturation is given by the following equation:

$$\Delta G^0 = -RT \ln K \quad (4)$$

Where R is the universal gas constant and T is the absolute temperature.  $G^0$  varies linearly with denaturant concentration ([urea]) and temperature (T) over a limited region.

$$\Delta G^0 = \Delta G_{H_2O}^0 - m[\text{denaturant}] \quad (5)$$

**Table 1.** Inhibition constants ( $K_i$ ) of Chrysin, Gallic acid, Naringin and Quercetin.

	Chemical denaturation		Thermal denaturation	
	$[\text{UREA}]_{1/2} (\text{M})$	$\Delta G_{(H_2O)}^0 (\text{kJ/mol})$	$T_m (^\circ\text{C})$	$\Delta G_{(25^\circ\text{C})}^0 (\text{kJ/mol})$
Sole MT	0.75	6.39	56.2	14.22
Gallic acid 1.5mM	0.44	7.21	62.62	21.23
Naringin 3mM	0.56	6.77	59.09	23.46
Chrysin 1.5mM	0.69	6.77	57.53	17.49
Quercetin 3mM	0.71	8.04	57.22	15.05

The results of the present study showed that gallic acid, naringin, chrysin and quercetin induced the inhibition of MT activity and its physicochemical stability. So far, many flavonols have been isolated from plants, and some were identified as tyrosinase inhibitors, e.g. the inhibition of melanogenesis was addressed to gallic acid (GA, 3,4,5-trihydroxybenzoic acid) antioxidant activity in scavenging reactive oxygen species (Lien et al., 2014). Antioxidants such as ascorbic acid derivatives are used as melanogenesis inhibitory agents (Panich et al., 2011). The reduction of glutathione (GSH) is a well known biological antioxidant that acts as a quencher of oxidative insults, thereby playing a significant role in the inhibition of melanogenesis (Panich et al., 2013). Many phenolic compounds are known to show potent

$\Delta G_{H_2O}^0$  is the free energy of conformational stability in the absence of denaturant and m is a measure of the dependence of  $G^0$  on the denaturant concentration (Schein et al., 1990).  $\Delta G_{(25^\circ\text{C})}^0$  (the standard Gibbs free energy of protein denaturation at 25°C) can be obtained by equation 5 of Pace analysis (Saboury et al., 2004).  $\Delta G_{H_2O}^0$  and  $\Delta G_{(25^\circ\text{C})}^0$  are the most valuable criterions of protein conformational stability in the process of chemical and thermal denaturation, respectively.

In chemical denaturation,  $[\text{UREA}]_{1/2}$  is the denaturant concentration that need for protein receiving to half of its two-state transition. In thermal denaturation, protein melting point ( $T_m$ ) is a temperature that need for protein receiving to half of its two-state transition. Magnitudes of  $\Delta G_{H_2O}^0$  and  $\Delta G_{(25^\circ\text{C})}^0$ ,  $[\text{UREA}]_{1/2}$  and  $T_m$  determined from replots, are summarized in table 1.

antioxidant activity (Manach et al., 2004) and a number of naturally occurring melanogenic inhibitors contain a phenol structure (Chou et al., 2010; Kim et al., 2005; Nerya et al., 2003). GA is a naturally occurring polyphenol antioxidant that was recently shown to have potentially healthy effects (Nerya et al., 2003). However, the inhibitory action of GA on skin melanogenesis has not been fully explored. Flavonoids compounds act as inhibitors of mushroom tyrosinase due to their ability to chelate copper ion (Garcia et al., 2002; Conrad et al., 1994). Lineweaver-Burk plots showed different patterns of competitive inhibition for naringin, chrysin and quercetin and non-competitive inhibition for gallic acid as a phenolic compound. It has been reported that the inhibitory mode of flavonol inhibitors is usually competitive

inhibition for the oxidation of L-dopa by tyrosinase and the 3-hydroxy-4-keto moiety of the flavonol structure acts as the key role in copper chelation. Kinetic assessment of the enzyme activity in the presence of gallic acid, showed non-competitive and the chrysin, naringin and quercetin induced a competitive manner of inhibition, with the inhibitory constant ( $K_i$ ) of 0.15, 0.30, 0.79 and 0.74 mM, the order of gallic acid, naringin, chrysin and quercetin respectively. Considering the magnitudes of free energy of both chemical and thermal denaturation, experiments indicated the increase of the enzyme stability in the presence of different concentrations of the given flavonoids.

The inhibition of tyrosinase activity might depend on the hydroxyl groups on the phenolic compounds of the flavonoids, which may form hydrogen bonds with enzyme active site, inducing competitive manner of inhibition, leading to lower enzymatic activity. In line with this research, it has been reported that such enzyme inhibitions occurred through the hydroxyl group moiety of inhibitor that bind to the tyrosinase active site, resulting in steric hindrance or altered conformation of the enzyme (Baek et al., 2008; Kim et al., 2008). Gallic acid presented as an effective tyrosinase activity inhibitor (Kubo et al., 2003; Momtaz et al., 2008). Biochemically, the antioxidant activity of flavonoids may also be an important reason for tyrosinase inhibition activity (Kim et al., 2008).

Gallic acid, naringin, chrysin and quercetin can increase the stability of enzyme while protecting them from thermal and chemical denaturation mean while the enzymatic activity is reduced. Magnitudes of the  $G^{\circ}_{H_2O}$ ,  $G^{\circ}_{25}$ ,  $[DTAB]_{1/2}$  and determined from replots, are summarized in table 2. In chemical denaturation studies by urea,  $\Delta G^{\circ}_{H_2O}$  is estimated to be the highest for chrysin and quercetin, which may be due to their hydrophobic interactions which make the enzyme get a more stable structure.  $[UREA]_{1/2}$  is the

denaturant concentration that need for protein receiving to half of its two-state transition. Its magnitudes obtained 0.75 mM for sole enzyme and 0.44, 0.56, 0.69 and 0.71mM for gallic acid, naringin, chrysin and quercetin respectively. Comparison of these magnitudes reveals that the nature of enzyme medium in their presence gets a more stable structure.

In thermal denaturation, protein melting point,  $T_m$ , is a temperature that need for protein receiving to half of its two-state transition, that gallic acid have the highest value melting point  $T_m$  in comparison with another flavonoids. The increasing of  $T_m$  and  $G^{\circ}_{25}$  values

revealed as the best parameter that describing effect of flavonoids on stability of the enzyme.

$G^{\circ}_{H_2O}$  and  $G^{\circ}_{25}$  are the most valuable criterions of protein conformational stability in the process of chemical and thermal denaturation, respectively. So, like the amino acids (Sulaiman et al., 2014) and other osmolyts (Tripathi et al., 2010) these flavonoids induced thermodynamic stability, but in contrast, they showed inhibitory effects on the enzyme activity.

In overall, gallic acid, naringin, chrysin and quercetin can increase the stability of proteins through protecting them from thermal and chemical denaturations mean while they inhibit the enzymatic activity.

## Acknowledgement

We would like to thank the members of Cellular and Molecular Research Centre of Qazvin University of Medical Sciences and Imam Khomeini International University.

## Funding/support

This research was financially supported by Cellular and Molecular Research Centre of Qazvin University of Medical Sciences and Imam Khomeini International University.

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