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Biotech Health Sci. 2014 August; 1(2): e22242.
Published online 2014 August 28. **Research Article**

Inhibitory Effects of Quercetin and Kaempferol as two Propolis Derived Flavonoids on Tyrosinase Enzyme

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Received: February 12, 2014; **Revised:** March 17, 2014; **Accepted:** March 30, 2014

Background: Tyrosinase is a copper-containing enzyme, which is widely distributed in microorganisms, animals and plants. It is also a key enzyme in melanin biosynthesis, which plays a crucial role in determining the color of mammalian skin and hair. In addition, unfavorable enzymatic browning of plant-derived foods by tyrosinase causes a decrease in nutritional quality and economic loss of food products.
Objectives: In the present study the activity of this enzyme was examined against quersetin and kaempferol as two potentially flavonoid inhibitors.
Materials and Methods: In this work, the effects of quercetin and kaempferol as propolis-derived compounds on activity of mushroom tyrosinase (MT) were studied. These flavonoids showed inhibitory activity on catecholase and cresolase reactions in presence of caffeic acid and p-comaric acid, respectively. The inhibition mode of quercetin and kaempferol were competitive towards both catecholase and cresolase activities of the enzyme.
Results: The inhibition constants (Ki) were determined as 0.072 and 0.112 mM for catecholase activity, and 0.016 and 0.06 mM for cresolase activity, respectively.
Conclusions: In general, quercetin and kaempferol can be used as good candidates in melanogenesis inhibition. Moreover they should be considered as good blockers of enzyme activity in hyper pigmentation and clinical application.

*Keywords:*Motor Activity; Quercetin; Kaempferol

1. Background

Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme, which is widely distributed in microorganisms, animals and plants. It is also a key enzyme in melanin biosynthesis, which plays a crucial role in determining the color of mammalian skin and hair. In addition, unfavorable enzymatic browning of plant-derived foods by tyrosinase leads to a decrease in nutritional quality and economic loss of food products (1, 2). On the other hand, the activity of this enzyme in the skin causes excessive production of melanin, which results in the creation of dermatological disorders such as melanoma and other skin hyperpigmentations and depigmentation (3-5). In cosmetic applications, tyrosinase inhibitors can be considered as skin whitening agents (6). Therefore, tyrosinase inhibitors may be clinically helpful in dealing with skin cancers and cosmetics.

The formation of melanin in the human body is influenced or reduced by several mechanisms, including anti-oxidation, direct tyrosinase inhibition, melanin inhibition of migration from cell to cell and hormonal activities, etc. (7). In fact, the tyrosinase enzyme catalyzes the hydroxylation of tyrosine to form 3,4-dihydroxyphenylalanine (L-DOPA), and also catalyzes the reaction leading to formation of DOPA quinine from L-DOPA (8). Quinones, in turn, develop chemically to form melanins and other polyphenolic compounds (9). Quinones chemically evolve to give rise to melanins or react with amino acids and proteins to enhance the color products, which are brown, black, or red heterogeneous polymers (10).

Flavonoids and phenolics are major groups of non-essential dietary components that have been associated with the inhibition of atherosclerosis and cancer (11). Flavonoids and polyphenolic crude extracts have been reported to possess xanthine oxidase inhibitory activity (12). A potential source of such compounds is mushrooms (13). Mushrooms accumulate a wide variety of secondary metabolites including phenolic compounds. Mushrooms have long been widely appreciated for their good flavor and texture. They are recognized as a nutritious food as well as an important source of biologically active compounds of medicinal value (14-16). Flavonoids and phenols have been shown to possess important antioxidant activities toward highly active free radicals, which are principally based on the redox potentials of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure (17).

2. Objectives

Pursuing our previous studies on inhibition and stabil-

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ity of mushroom tyrosinase (MT), in the present study the activity of this enzyme was examined against quersetin and kaempferol as two potentially flavonoid inhibitors.

3. Materials and Methods

3.1. Materials and Mushroom Tyrosine Preparation

Mushroom tyrosine (EC 1.14.18.1) with a molecular weight of 120 KDa and p-hydroxycinnamic (p-coumaric acid λ max = 288 nm, ε = 19400/Mcm) and 3,4-dihydroxycinnamic acid (caffeic acid, λ max = 311 nm, ε = 12000/Mcm) were used in this research. The buffer used throughout this research was 10 mM phosphate buffer solution (PBS), pH = 6.8 and dihydroxy caffeic acid was purchased from Merck.

3.2. Measurement of Cresolase and Catecholase Reactions of Mushroom Tyrosine

Tyrosinase catalyzes two successive kinetic processes; the first is the ortho-hydroxylation of monophenols to diphenols (cresolase activity) and the second is oxidation of diphenols to quinone (catecholase activity). Kinetics assays were done in the presence of p-coumaric and caffeic acids as mono and di-phenol substrates, respectively. All enzymatic reactions were performed in the presence of different substrates in 10 mM phosphate buffer, PH of 6.8 and temperature of 293 K, and the absorbance of the final products were measured using a spectrophotometer. The cresolase reaction was carried out for 10 minutes in the presence of p-coumaric acid substrate, at wavelength of 288 nm and enzyme concentration of 17.7 μM. The catecholase reaction of MT was performed for two minutes in the presence of caffeic acid substrate, wavelength of 311 nm, phosphate buffer with pH of 6.8, temperature of 293 K and enzyme concentration of 11.8 μM.

3.3. Cresolase and Catecholase Activities of Mushroom Tyrosine in the Presence of Quercetin and Kaempferol

All enzymatic reactions were performed with the abovementioned substrates and enzyme, yet different concentrations of quercetin and keampferol were used. Cresolase reactions were carried out with fixed concentrations of quercetin (0, 0.01, 0.02 and 0.04 mM) and kaempferol (0, 0.1, 0.2 and 0.3 mM) as inhibitors and by using series of p-coumaric acid concentrations (20, 40, 60 and 80 μM), as the substrate. Catecholase reactions were carried out with fixed concentrations of quercetin (0, 0.025, 0.05 and 0.1 mM) and kaempferol (0, 0.1, 0.25 and 0.5 mM) as inhibitors and series of caffeic acid concentrations (30, 60, 90 and 150 μM), as the substrate.

4. Results

The inhibitory type of quercetin and keampferol on the monophenolase activity, during the hydroxylation of p-coumaric acid was determined by Lineweaver–Burk double reciprocal plots. The Michaelis constant (K_m) and maximum velocity (V_{max}) of the tyrosinase were determined by the Lineweaver-Burk plots. The velocity equation for competitive inhibition in reciprocal form is: $1/V = K_m/V_{max} (1 + [I]/K_i) 1/[S] + 1/V_{max}$. The inhibition constants (K_i) of the competitive inhibitors were calculated by the following equation: $K_{mapp} = K_m [1 + ([I]/K_i)]$ where K_{mapp} is the apparent K_m in the presence of an inhibitor. The reciprocal equation for the noncompetitive inhibition is: $1/V = K_m/V_{max} (1 + [I]/K_i) 1/[S] + 1/ V_{max} (1 + [I]/K_i)$. Ki of the noncompetitive inhibitors were calculated using the following equation: $1/V_{maxapp} = (1+ [I]/Ki)/V_{max}$ where V_{maxapp} is the apparent V_{max} in the presence of an inhibitor. In the presence of quercetin and keampferol, the kinetics of the enzyme is shown in Figure 2 and 3, respectively. The plots of 1/v versus 1/[S] gave a series of straight lines with different slopes yet they intersected one another at the Y-axis. The values of V_{max} remained the same and the value of K_m increased with increasing concentrations of the inhibitor, which indicates that they are competitive inhibitors. The results showed that quercetin and keampferol could only bind with the free enzyme. The inhibition constant for the inhibitor binding with the free enzyme (E), K_i, was obtained from the secondary plot of the slope lines in Figures 4A and 4B versus the inhibitor concentration. The inhibition constant (K_i) was 0.016 and 0.06 mM for quercetin and keampferol, respectively. Same pattern of inhibition (competitive) was obtained for the catecholase reaction (Figures 5 and 6). The inhibition constant for the inhibitor binding with the free enzyme (E), K_i, was obtained from the secondary plot of the slope lines (Figures 7A and 7B) versus the inhibitor concentration. The inhibition constant (K_i) was 0.072 and 0.112 mM for quercetin and kaempferol, respectively.

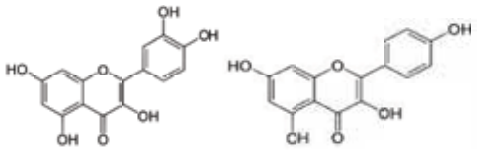
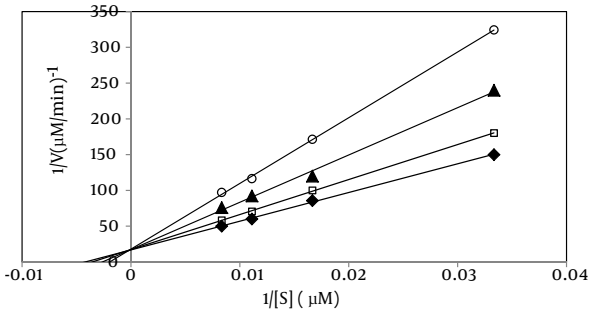


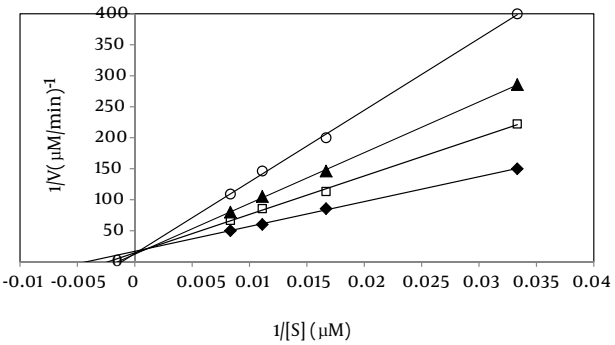
Figure 1. Chemical Structure of Kaempferol and Quercetin as Two Flavonoids

Figure 2. Cresolase Activity of Tyrosinase in the Presence of a Quercetin as a Competitive Inhibitor



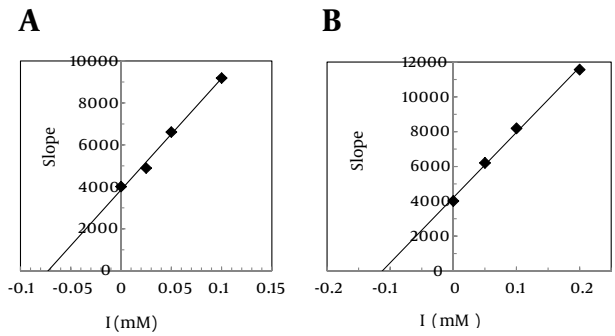
Lineweaver–Burk double reciprocal plots of tyrosinase cresolase activity with a fixed concentration of inhibitor: 0(♦), 0.01(□), 0.02(▲), 0.04(○) mM.

Figure 3. Cresolase Activity of Tyrosinase in the Presence of Kaempferol as a Competitive Inhibitor



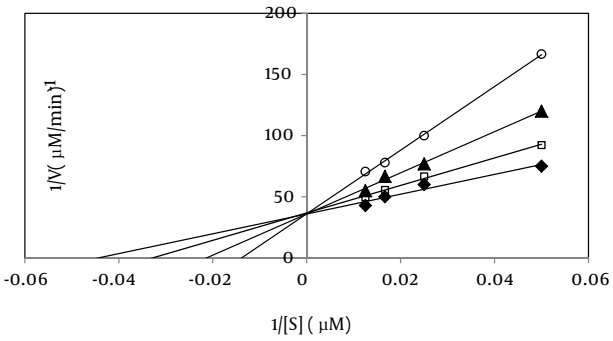
Lineweaver–Burk double reciprocal plots of tyrosinase cresolase activity with a fixed concentration of inhibitor: 0(♦), 0.1 (□), 0.2 (▲), 0.3 (○) mM.

Figure 4. Secondary plots Obtained From Slopes of the Curves in Figures 2 and 3 Against Different Concentrations of Quercetin (A) and Kaempferol (B) as Inhibtors



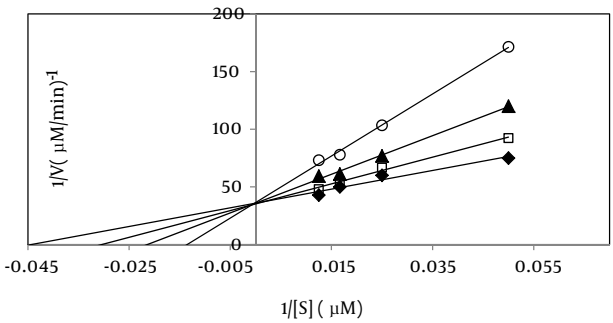
Ki can be obtained from their x-intercepts.

Figure 5. Catecholase Activity of Tyrosinase in the Presence of Quercetin as a Competitive Inhibitor



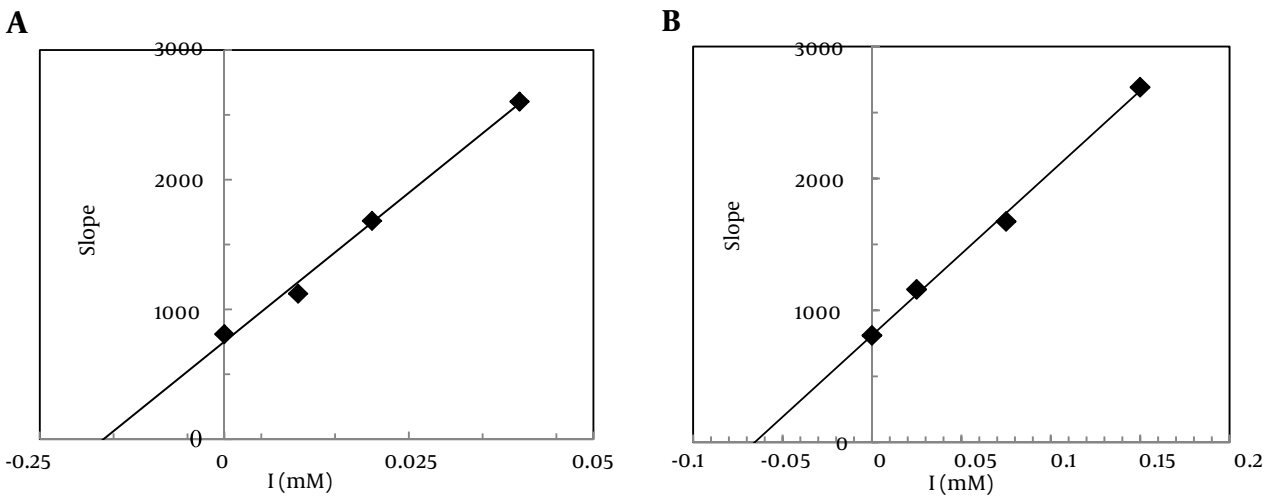
Lineweaver–Burk double reciprocal plots of catecholase activity of tyrosinase with a fixed concentration of inhibitor: 0(♦), 0.025 (□), 0.05 (▲), 0.1(○) mM.

Figure 6. Catecholase Activity of Tyrosinase Enzyme in the Presence of a Kaempferol as a Competitive Inhibitor



Lineweaver–Burk double reciprocal plots of catecholase activity of tyrosinase with a fixed concentration of inhibitor: 0(♦), 0.1 (□), 0.25 (▲), 0.5(○) mM.

Figure 7. Secondary Plots Obtained From Slopes of the Curves of Figures 5 and 6 Against Different Concentrations of Quercetin (A) and Kaempferol (B) as Inhibtors



Ki can be obtained from their x-intercepts.

5. Discussion

According to the results of the Lineweaver–Burk double reciprocal plots of cresolase and catecholase reactions of MT there were competitive modes of inhibition in the presence of quercetin and kaempferol. However, the flavonoids induced tyrosinase inhibition and the K_{mapp} for the substrate increased, while the magnitude of V_{max} remained unchanged. In the process of catalysis, tyrosinase can be found in three emet, eoxy and edeoxy forms. Both the emet and eoxy forms can catalyze the diphenol substrate, but in contrast to the emet, the eoxy form can also catalyze the monophenol substrate. It should be noted that the pre-incubated enzyme was mostly met-tyrosinase, known as the resting form of the enzyme, indicating the quercetin and kaempferol can chelate copper in the met-form of tyrosinase (18).

In conclusion, the competitive mode of inhibition of flavonoids on MT works through their hydroxyl groups that bind to the active site on tyrosinase, resulting in steric hindrance or changed conformation (19). Flavonoids and phenols have shown to possess important antioxidant activities toward free radicals, which are principally based on the redox potentials of their phenolic hydroxyl groups and the structural relationships between different parts of their chemical structure (20). Gallic acid, (GA, 3,4,5-trihydroxybenzoic acid)- epicatechin, procyanidin B2 and epicatechin-3-gallate identified in mushrooms, proved to be effective inhibitors of tyrosinase activity, as reported by many researchers (21-23).

On the other hand, the inhibition of melanogenesis may be related to GA's antioxidant activity in scavenging reactive oxygen species (24). Antioxidants such as ascorbic acid derivatives are used as melanogenesis inhibitory agents (25), and reduced glutathione (GSH) is a well known biological antioxidant that acts as a quencher of oxidative insults, thereby plays a significant role in the inhibition of melanogenesis (26). Many phenolic compounds are known to have potent antioxidant activity (27), and a number of naturally occurring melanogenic inhibitors contain a phenolic structure (28-30).

The inhibitory mode of flavonol inhibitors is usually competitive for the oxidation of L-dopa by tyrosinase, and the 3-hydroxy-4-keto moiety of the flavonol structure acts a key role in copper chelation (20). As previously reported, quercetin inhibitory ability on catecholase activity is 20% that of kojic acid's inhibitory ability, thus it could be applied in whitening and food anti-browning products (31). Researches have shown that all flavonoids inhibition on enzyme by chelating copper at the active site of the enzyme applied. Recently, it has been shown that flavonoids with keto groups specially α-ketos have strong inhibition ability (32).

Overall, the results of this study showed the inhibitory effect of some flavonoids on catecholase and cresolase activity of tyrosinase enzyme. Kaempferol and quercetin inhibition performance on cresolase and catecholase enzyme activity have a competitive inhibition pattern. Comparison of public inhibiting tyrosinase such as phenyl and kojic acid show that the flavonoids can be considered a member of potent inhibitor of tyrosinase. The use of these compounds in medicine, including treatments for hyperpigmentation and other skin imperfections is recommended.

Acknowledgements

This work was financially supported by the deputy of research of Qazvin University of Medical Sciences, Qazvin, Iran.

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