Note

Tyrosinase Inhibitor Isolated from the Leaves of *Zanthoxylum piperitum*

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Two flavonols, quercetin (1) and quercitrin (2), were isolated from the leaves of *Zanthoxylum piperitum*. Their structures were established by UV, one- and two-dimensional NMR, and mass spectroscopic methods. Quercetin showed significant inhibition against mushroom tyrosinase with an IC_{50} value of 3.8 μg/ml, and appeared to inhibit the polyphenol oxidase activity of tyrosinase in a competitive manner (Ki = 10 ± 0.20 μM) when L-tyrosine was used as a substrate, although it did not inhibit the melanin production of *Streptomyces bikiniensis*.

Key words: *Zanthoxylum piperitum; quercetin; quercitrin; tyrosinase; Streptomyces bikiniensis*

Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions of melanin synthesis, the hydroxylation of a monophenol and the conversion of an O-diphenol to the corresponding O-quinone. In these oxidation reactions, three different forms of binuclear copper are involved in the active site.1,2

Tyrosinase is also known as a polyphenol oxidase (PPO),3,4 and the browning of some fruits, vegetables and crustaceans due to tyrosinase causes a significant decrease in their nutritional and market values. This unfavorable darkening due to the enzymatic oxidation of phenols has therefore been of great concern,5 and tyrosinase inhibitors should have broad applications. In addition, tyrosinase inhibitors have also become increasingly important in medicinal6 and cosmetic7 products in relation to hyperpigmentation. There is therefore a concerted effort to search for naturally occurring tyrosinase inhibitors from plants, because plants constitute a rich source of bioactive chemicals, many of them being largely free from harmful adverse effects.8–10

*Zanthoxylum piperitum* DC (Korean name: Chopi) are deciduous shrubs of Rutaceae and plants native to the Japanese islands, mainland China, and the Korean peninsula. Fresh young leaves and dried fruits of Japanesees pepper are used as a spice to impart a fresh flavor or to suppress any unpleasant fishy and meaty odor in dishes, and as diuretics and stomachics in traditional Chinese and Japanese medicines.11,12 We investigate in this study the mushroom tyrosinase inhibitor from the leaves of *Zanthoxylum piperitum*.

During the preliminary screening with mushroom tyrosinase, we observed that the methanol extract of *Z. piperitum* leaves showed a significant inhibitory effect on L-DOPA oxidation. Fractionation guided by the tyrosinase inhibitory activity indicated the butanol fractions from the extract to have inhibitory activity against mushroom tyrosinase. Little or no activity was produced from the chloroform and water fractions. The biologically active compounds from the butanol fraction were purified by silica gel column chromatography, and the isolated compounds were bioassayed. Two active compounds were finally isolated, their structures being elucidated as quercetin and quercitrin through the results of spectroscopic analyze by IR, MS and NMR. We examined the inhibitory effects of quercetin and quercitrin on the mushroom tyrosinase activity by using L-tyrosine as the substrate. A bioassay was also performed with arbutin and kojic acid as positive controls. The tyrosinase activity in the mushroom tyrosinase solution was inhibited by all tested agents in a concentration-dependend manner. Quercetin and quercitrin are flavonoids abundantly found in the leaves of *Z. piperitum*; they have been reported to have antioxidative,13 anti-cancer,14 antimicrobial,15 antiviral,16 and other activities.17 The bioassay with pure quercetin and quercitrin showed a dose-dependent inhibitory effect on L-DOPA oxidation by mushroom tyrosinase. Quercetin and kojic acid were more potent than arbutin and quercitrin, with respective IC_{50} values of 3.8, 10, 15 and >50 μg/ml (Fig. 1).

The inhibition kinetics of quercetin were analyzed by a Lineweaver-Burk plot (Fig. 2) The three lines obtained from the uninhibited enzyme and from different concentrations of quercetin intersected on the vertical axis. The result for the enzyme remain to be resolved, because the structure of mushroom tyrosinase has not yet been established. The inhibition kinetics analyzed by the Lineweaver-Burk plot indicate quercetin to be a competitive inhibitor of tyrosinase when L-tyrosine was the
The result indicates quercetin to be a competitive inhibitor of the L-DOPA oxidation by mushroom tyrosinase. Additionally, pre-incubating the enzyme in the presence of 10 μM of quercetin and in the absence of the substrate shows that quercetin did not directly inactivate the enzyme, since it did not significantly decrease the enzyme activity. The foregoing data confirm that quercetin had a strong inhibitory effect on the action of mushroom tyrosinase. Quercetin could be utilized as a natural inhibitor of browning of mushroom and other food sources such as pre-peeled potato, and in juice manufacturing from various plants and vegetables. However, neither quercetin nor quercitrin showed any inhibitory activity at a 30-μg/disk concentration against S. bikiniensis.

**Experimental**

*Instruments.* IR spectra were obtained with a Hitachi 270-50 spectrophotometer, and MS data were measured
with a Jeol JMS-700 spectrometer in the EI mode. 

Inhibition of tyrosinase. To a 96-well microplate was added 150 μl of a 0.1 M phosphate buffer at pH 6.5, 25 μl of a 1.5 mM L-tyrosine solution and 7 μl of 2,100 unit/ml of mushroom tyrosinase (Sigma, 0.05 mM phosphate buffer at pH 6.5). After incubating at 30°C for 10 min, the amount of dopa produced in the reaction mixture was determined with a microplate reader (Bio-Rad 3550) as the optical density at 490 nm. The inhibitory activity of the sample is expressed as the concentration at which 50% of the enzyme activity was inhibited (IC50). The Michaelis constant (Km) and inhibitor constant (Ki) of the tyrosinase were determined by a Lineweaver-Burk plot with various concentrations of L-tyrosine.18)

Inhibition of the melanin production of S. bikiniensis. A preserved culture of S. bikiniensis (63 mg) was isolated by recrystallization with MeOH. The homogeneity of Compound 1 (quercetin) was demonstrated by TLC in a developing solvent system of CHCl3–MeOH (3:1) (Rf = 0.65).

Fraction 8 (6.4 g) was combined and applied to a silica gel column, eluting with a chloroform–methylene mixture of increasing polarity (49/1 → 1/1), to give five major subfractions (F8-1 through F8-5). F8-4 was purified by Sephadex LH-20 resin column chromatography with MeOH. Compound 2 (108 mg) was isolated by recrystallization with MeOH. The homogeneity of 2 was demonstrated by TLC in a developing solvent system of CHCl3–MeOH (3:1) (Rf = 0.27).

Compound 1 (quercetin). IR νmax (KBr) cm⁻¹: 3426, 1664, 1611, 1521; 1H-NMR (500 MHz, DMSO-d₆) δ: 6.22 (1H, d, J = 2.0 Hz, H-6), 6.44 (1H, d, J = 2.0 Hz, H-8), 6.92 (1H, d, J = 8.5 Hz, H-5), 7.57 (1H, dd, J = 8.5, 2.2 Hz, H-6), 7.71 (1H, d, J = 2.2 Hz, H-2); 13C-NMR (125 MHz, DMSO-d₆) δ: 92.2, 97.1, 101.9, 114.0, 114.5, 118.9, 120.9, 134.6, 143.6, 145.7, 146.6, 155, 159.6, 162.8, 174.7.

Compound 2 (quercitrin). IR νmax (KBr) cm⁻¹: 3378, 2932, 1651, 1605; 1H-NMR (500 MHz, methanol-d₄) δ: 0.94 (d, J = 6.0 Hz, 3H), 3.31 (dd, J = 3.5, 2.0 Hz, 1H), 3.43 (m, 1H), 3.76 (brd, J = 7.5 Hz, 1H), 4.22 (s, 1H), 5.34 (s, 1H), 6.13 (s, 1H), 6.28 (s, 1H), 6.90 (d, J = 6.5 Hz, 1H), 7.27 (d, J = 7.5 Hz, 1H), 7.33 (s, 1H); 13C-NMR (125 MHz, methanol-d₄) δ: 18.1, 72.3, 72.4, 72.6, 73.8, 96.1, 101.5, 103.9, 105.2, 116.8, 117.3, 123.2, 123.4, 136.4, 147.0, 150.5, 159.2, 159.3, 163.4, 170.2, 179.6.

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References


