Natural Product Sciences
10(2) : 80-84 (2004)

Rosmarinic Acid as a Tyrosinase Inhibitors from *Salvia miltiorrhiza*

Hye Sook Kang, Hyung Rak Kim, Dae Seok Byun, Hye Jin Park[1], and Jae Sue Choi*

Faculty of Food Science and Biotechnology, Pukyong National University; Busan 608-737, Korea
[1]Department of Food Science and Nutrition, Chang Shin College, Masan 630-520, Korea

Abstract – Rosmarinic acid and its methyl ester, isolated from the ethyl acetate soluble fraction of the methanolic extract of *Salvia miltiorrhiza* Bunge (Labiatae), were found to inhibit the oxidation of L-tyrosine catalyzed by mushroom tyrosinase with the IC_{50} values of 16.8 and 21.5 μM, respectively. It was comparable with kojic acid, a well-known tyrosinase inhibitor, with an IC_{50} of 22.4 μM. The inhibitory kinetics analyzed by the Lineweaver-Burk plots, were found rosmarinic acid and its methyl ester to be competitive inhibitors with K_i of 2.4×10^{-5} and 1.5×10^{-5} M, respectively.
Keywords – Rosmarinic acid, Tyrosinase inhibitor, *Salvia miltiorrhiza*, Competitive inhibitor

Introduction

Tyrosinase (EC 1.14.18.1) is a multifunctional, copper-containing oxidase that catalyzes three distinct reactions of melanin synthesis, the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), the oxidation of DOPA to dopaquinone and the conversion of 5,6-dihydroxyindole to melanochrome (Mason, 1956; Wilcox *et al.*, 1985). Tyrosinase also known as polyphenol oxidase (PPO) is ubiquitously distributed in nature such as microorganisms, animals and plants (Mayer, 1987; Whitaker, 1995). The enzyme is known for the browning of some fruits, vegetables and crustaceans, and causes a significant discount in their nutritional and market values. In addition, tyrosinase is a key enzyme in the insect molting process (Anderson, 1979), so that its inhibitors might ultimately provide clues to control insect pests. The enzymatic oxidation of L-tyrosine to melanin is of considerable importance because melanin has many functions, and alterations in melanin synthesis occur in many disease states. Recently, tyrosinase inhibitors have become increasingly important in cosmetic and medical products in relation to hyperpigmentation (Kim *et al.*, 2002; Pérez-Bernal *et al.*, 2000). Furthermore, it has been reported that tyrosinase could be central to dopamine neurotoxicity as well as contributing to the neurogeneration associated with Parkinson's disease (Xu *et al.*, 1997).

Danshen, the rhizome of *Salvia miltiorrhiza* Bunge (Labiatae), has been used in traditional Chinese medicine to treat coronary heart diseases, particularly angina pectoris and myocardial infarction (Tang and Eisenbrand, 1992). Some of the clinical effects of Danshen could be, to some extent, related to its possible antioxidant activity. In the previous study, we had reported antioxidative effects of the EtOAc and the BuOH soluble fractions from MeOH extract of the rhizome of *S. miltiorrhiza* on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and new efficient components isolated from this plant, such as 3-(3', 4'-dihydroxyphenyl)-(2*R*)-lactamide (Kang *et al.*, 1997) and salviamiltamide (Choi *et al.*, 2001). In addition, a furanofuranoid lignan glycoside, showing radical scavenging activities on peroxynitrite, total reactive oxygen (ROS) and DPPH radical was isolated from *S. miltiorrhiza* (Kang *et al.*, 2003). The tyrosinase inhibitory activity from *S. miltiorrhiza* has not been reported. In this study, rosmarinic acid and its methyl ester were isolated from *S. miltiorrhiza* through column chromatographies and established the inhibitory kinetics of the two compounds on the mushroom tyrosinase.

Materials and Methods

Plant material – The rhizome of *S. miltiorriza* was obtained from Chien Yuan Herbal Medicinal Co., Taipei, Taiwan in 1995 and authenticated by Prof. H. J. Chi of the Natural Products Research Institute, Seoul National University. A voucher specimen (No. 950921) has been deposited in the Herbarium of the Natural Products Research Institute.

Chemicals and Reagents – Column chromatography was done with silica gel (Merck, 70-230 mesh). TLC was carried out on precoated Merck Kieselgel 60 F_{254} plate

*Author for correspondence
Fax: +82-51-620-6330; E-mail: choijs@pknu.ac.kr

(0.25 mm) and spots were detected under UV light using 50% H_2SO_4 reagent. L-Tyrosine was purchased from Janssen Chimica (Geel Belgium), and Kojic acid and mushroom tyrosinase (EC 1.14.18.1) from Sigma Chemical Co. (St. Louis, MO). 3-(3',4'-Dihydroxyphenyl)-lactate, its methyl ester and 3-(3',4'-dihydroxyphenyl)-(2*R*)-lactamide used in this study, was isolated from *S. miltiorriza* previously (Kang *et al.*, 1997) K_2HPO_4 was obtained from Junsei Chemical Co. Ltd. (Tokyo, Japan), KH_2PO_4 from Yakuri Pure Chemicals Co. Ltd. (Osaka, Japan). All the solvents for column chromatography were of reagent grade from commercial sources.

Isolation of compounds 1 and 2 – The powdered rhizome (8.5 kg) was refluxed with MeOH (3×9 *l*) for 3 h. The extract (1.03 kg) was suspended in water and partitioned with CH_2Cl_2 (165 g), EtOAc (80 g), *n*-BuOH (80 g), in sequence. The EtOAc (66 g) fraction was applied to a silica gel (Merck, 70-230 mesh, 1.65 kg) column (8×160 cm). The column was eluted using mixtures of EtOAc/MeOH under gradient conditions (20:1-2:1) to yield 12 subfractions, i.e., F1-F4; EtOAc/MeOH, 20:1 (5 *l*), F5-F7; EtOAc/MeOH, 10:1 (5 *l*), F8~F10; EtOAc/MeOH, 5:1 (5 *l*) and F11-F12; EtOAc/MeOH, 2:1 (5 *l*). The F4 (1.65 g) was further silica gel (70-230 mesh, 250 g) column (3×70 cm) chromatography eluted with the CH_2Cl_2/MeOH, 50:1 to give methyl rosmarinic acid (**2**, 260 mg, TLC, CH_2Cl_2/MeOH/H_2O; 7:1:0.1, R_f 0.50,). The F7 (4.5 g) was subjected to silica gel (70-230 mesh, 450 g) column (4×80 cm) chromatography with CH_2Cl_2/MeOH; 17:3 as a solvent and then Sephadex LH-20 column (2.5×50 cm) chromatography with MeOH to obtain the rosmarinic acid (**1**, 140 mg, TLC, CH_2Cl_2/MeOH/H_2O; 10:3:0.3, R_f 0.25). ^{1}H- and ^{13}C-NMR spectra were taken with a JNM ECP-400 spectrometer. The chemical shifts were referenced to residual solvent peaks (3.0 ppm in ^{1}H-NMR, 49.0 ppm in ^{13}C-NMR), and were recorded in values. Multiplicities of ^{1}H-NMR signals were indicated as s (singlet), d (doublet), and dd (double doublet).

Rosmarinic acid (1) – $[\alpha]_D^{20}$: +84.8°(*c* 0.006, MeOH), ^{1}H-NMR (MeOH-d_4) δ: 3.00 (1H, dd, *J* = 8.4, 14.3 Hz, H-7'), 3.09 (1H, dd, *J* = 4.3, 14.4 Hz, H-7'), 5.17 (1H, dd, *J* = 4.3, 8.4 Hz, H-8'), 6.26 (1H, d, *J* = 15.9 Hz, H-8), 6.60 (1H, dd, J= 2.1, 8.1 Hz,H-6'), 6.69 (1H, d, *J* = 8.1 Hz, H-5'), 6.74 (1H, d, *J* = 2.0 Hz, H-2), 6.77 (1H, d, *J*= 8.2 Hz, H-5), 6.94 (1H, dd, *J* = 2.0, 8.3 Hz, H-6), 7.03 (1H, d, *J* = 2.1 Hz, H-2'), 7.54 (1H, d, *J* = 15.9 Hz, H-7). ^{13}C-NMR (MeOH-d_4) δ: 38.7 (C-7'), 75.4 (C-8'), 115.2 (C-2), 116.0 (C-8), 117.1 (C-5'), 117.3 (C-5), 118.4 (C-2'), 122.6 (C-6'), 123.9 (C-6), 128.4 (C-1), 130.0 (C-1'), 146.1 (C-4'), 146.9 (C-3'), 147.6 (C-3), 148.5 (C-7), 150.5 (C-4), 169.2 (C-9), 174.3 (C-9'). ^{1}H and ^{13}C NMR spectral data were agreed well with literatural data (Kohda *et al.*, 1989).

Methyl rosmarinic acid (2) – $[\alpha]_D^{20}$: +32.4° (*c* 0.007, MeOH), ^{1}H-NMR (MeOH-d_4) δ: 3.02 (1H, dd, *J* = 14.4, 6.9 Hz, H-7'), 3.03 (1H, dd, *J* = 14.4, 4.7 Hz, H-7'), 3.69 (3H, s, $COOCH_3$), 5.18 (1H, dd, *J* = 5.3, 7.5 Hz, H-8'), 6.26 (1H, d, *J* = 16 Hz, H-8), 6.69 (1H, d, *J* = 8.2 Hz, H-5'), 6.70 (1H, d, *J* = 2.5 Hz, H-2'), 6.77 (1H, d, *J*= 8.2 Hz, H-5), 6.95 (1H, dd, *J* = 2.1, 8.2 Hz, H-6), 7.04 (1H, d, *J* = 2.1 Hz, H-2), 7.55 (1H, d, *J* = 16 Hz, H-7). ^{13}C-NMR (MeOH-d_4) δ: 38.7 (C-7'), 53.5 ($COO\underline{C}H_3$), 114.9 (C-2), 116.0 (C-8), 117.1 (C-5'), 117.3 (C-5), 118.3 (C-2'), 122.6 (C-6'), 124.0 (C-6), 128.4 (C-1), 129.6 (C-1'), 146.2 (C-4'), 147.0 (C-3'), 147.6 (C-3), 148.7 (C-7), 150.6 (C-4), 169.1 (C-9), 173.0 (C-9').

Enzyme assay – Tyrosinase activity using L-tyrosine as a substrate was spectrophotometrically determined a method (No *et al.*, 1999) described previously with a little modification. Ten μl of each sample solution with different concentrations (1-500 μg/ml) and 20 μl of mushroom tyrosinase (1000 units/ml) in a 50 mM phosphate buffer (pH 6.5) were added to 170 μl of a assay mixture containing with the ratio 10:10:9 of 1 mM L-tyrosine solution, 50 mM potassium phosphate buffer (pH 6.5) and distilled water in a 96-well microplate. One unit (U) of enzyme activity was defined as the absorbance change at 280 nm of 0.001 per min at pH 6.5 at 25°C in 3 ml reaction mixture containing L-tyrosine by the supplier. The samples dissolved in DMSO were diluted to 30 times with distilled water before experiment. After incubation of the reaction mixture at 25°C for 30 min, the absorbance of the mixture was determined at 490 nm ($\varepsilon = 3.3\times10^3$ $M^{-1}cm^{-1}$) in a microplate reader (VERSA max, Molecular Device, CA). The extent of inhibition by the addition of samples is expressed as a concentration necessary for 50% inhibition (IC_{50}). The percent inhibition of tyrosinase activity was calculated by the following equation: the % Inhibition = $[\{1-(A_a-A_b)/A_c\}\times100]$ where A_a is absorbance at 490 nm with test sample and enzyme, A_b is absorbance at 490 nm with test sample and without enzyme, and A_c is absorbance at 490 nm with enzyme and without test sample.

Kinetic analysis – The reaction mixture consisted of four different concentrations of L-tyrosine (0.5 to 2 mM) as a substrate and mushroom tyrosinase in 50 mM potassium phosphate buffer. Each sample of several concentrations was added to the reaction mixture, respectively. Michaelis constant (K_m) and maximal velocity (V_{max}) of the tyrosinase were determined by Lineweaver-Burk plots. The velocity equation for the competitive inhibition in reciprocal form is: $1/V = K_m/V_{max}\ (1 + [I]/K_i)\ 1/[S] + 1/V_{max}$. Inhibition

constants (K_i) of the compatitive 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 any inhibitor concentration.

Results and Discussion

The MeOH extract of *S. miltiorrhiza* was partitioned into CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O fractions. To identify the active principles, we evaluated the tyrosinase inhibitory activities of these organic solvent soluble fractions. The EtOAc fraction of them showed the inhibitory activity with IC_{50} value of 323.6 μg/ml, while the other fractions observed inhibition of below fifty percent at the highest concentration of 500 μg/ml, in order of CH_2Cl_2 fraction, MeOH extract, *n*-BuOH fraction, and H_2O fraction with 34%, 30%, 23% and 10%, respectively. Bioassay-guided fractionation of the ethyl acetate soluble fraction of the methanolic extract of danshen, led to the isolation of tyrosinase inhibitory compounds **1** and **2** (Fig. 1). The two compounds were identified as rosmarinic acid and its methyl ester on the basis of chemical and physicochemical evidence, and compared with those in the literatures as previously reported (Kohda *et al.*, 1989). The fact that the known depsides from this plant possess an *R*-(+)-β-(3,4-dihydroxyphenyl) lactic moiety suggested that the C-8' atoms of **1** and **2** may also have an *R* configuration. This was supported by dextrorotatory optical rotation values of the compounds. Methyl rosmarinic acid was considered the artifact of rosmarinic acid occurred on the way of extracting procedure with MeOH.

Rosmarinic acid and its methyl ester showed strong inhibitory activity on mushroom tyrosinase using L-tyrosine as a substrate with IC_{50} values of 16.8 and 21.5 μM, respectively. This result was comparable that of kojic acid (22.4 μM), a well-known tyrosinase inhibitor (Table 1). Lineweaver-Burk plots as shown in Fig. 2 analyzed the inhibitory kinetics of compounds **1** and **2**, respectively. The lines, obtained from the uninhibited enzyme and from the two or three different concentrations of the test compounds, intersected on the vertical axis. The result demonstrates that compounds **1** and **2** exhibited competitive inhibitors for the oxidation of L-tyrosine catalyzed by mushroom tyrosinase. K_i values of **1** and **2** were estimated to be 2.4×10^{-5} and 1.5×10^{-5} M, respectively.

A competitive inhibitor is a substance that combines with

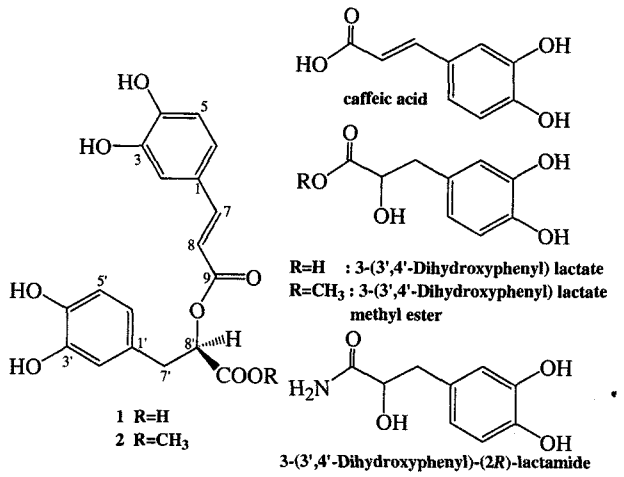

Fig. 1. The structures of the compounds.

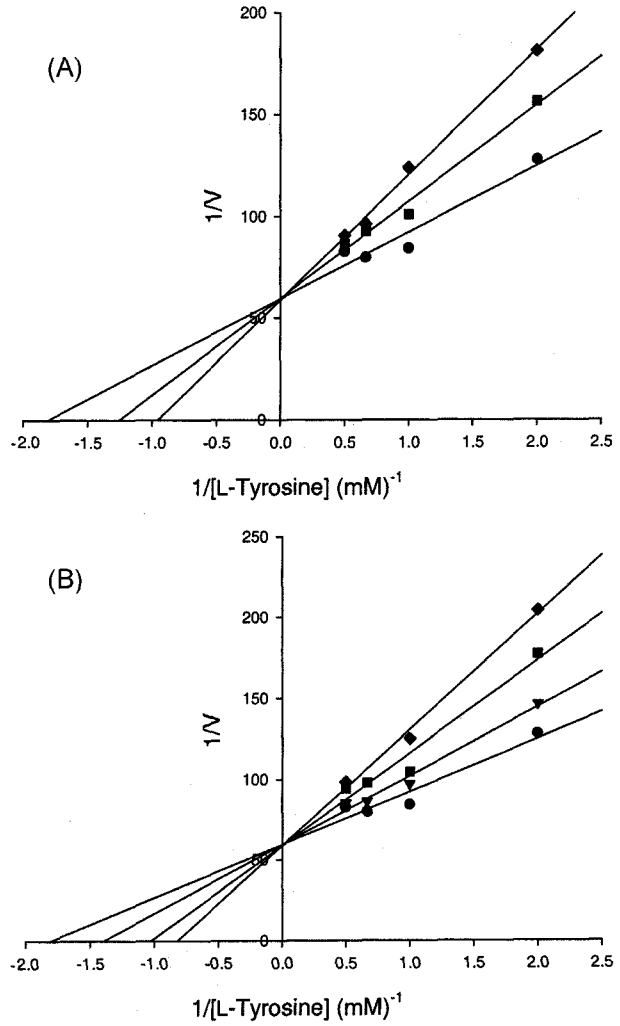

Fig. 2. Lineweaver-Burk plots for the inhibition of mushroom tyrosinase by rosmarinic acid (A) or methyl rosmarinic acid (B) using L-tyrosine as a substrate. The reaction was done in the presence of rosmaric acid (**1**) [0 (●), 10 (■) and 20 μM (◆)] or its methyl ester (**2**) [0 (●), 5 (▼), 10 (■) and 20 μM (◆)]. V_{max} = 0.02 ΔOD_{490}/min, $K_m = 5.6\times10^{-4}$ M. 1/V: 1/(ΔOD_{490}/min). The inhibitory constants (K_i) of the compounds **1** and **2** were determined as 2.4×10^{-5} and 1.5×10^{-5} M, respectively.

Table 1. Inhibitory activities of mushroom tyrosinase by rosmarinic acid and related compounds

Compounds	IC_{50} (μM)[a]
Rosmarinic acid (**1**)	16.8
Methyl rosmarinic acid (**2**)	21.5
Caffeic acid[b]	–[c]
3-(3',4'-Dihydroxyphenyl)-lactate	–[c]
3-(3',4'-Dihydroxyphenyl)-lactate methyl ester	–[c]
3-(3',4'-Dihydroxyphenyl)-(2*R*)-lactamide	–[c]
Kojic acid	22.4

[a]Inhibitory activity was expressed as the arithmetic mean value of 50% inhibitory concentrations of triplicate determines, obtained by interpolation of concentration-inhibition curve.
[b]The result cited from the reference 16.
[c]– showed that the compounds didn't inhibit the mushroom tyrosinase at the same conditions

free enzyme in a manner that prevents substrate binding. That is, the inhibitor and the substrate are mutually exclusive, often because of true competition for the same site. A competitive inhibitor might be a nonmetabolizable analog or derivative of the true substrate, or alternate substrate of the enzyme, or a product of the reaction (Segel, 1976).

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphlenyl lactic acid. As shown in Table 1, 3-(3',4'-dihydroxyphenyl)-lactate didn't exhibit mushroom tyrosinase inhibitory activity using L-tyrosine as substrate, also caffeic acid was oxidized by tyrosinase as substrate (Lim *et al.*, 1999). To clarify the effect of side chain of the diphenols on the mushroom tyrosinase, the derivatives of the phenolic acids, such as 3-(3',4'-dihydroxyphenyl)-lactate methyl ester and 3-(3',4'-dihydroxyphenyl)-(2*R*)-lactamide, also tested, however, they had no inhibitory effect (Table 1). The results showed that the substituted moieties at the side chain in these compounds such as methyl or amide group did not influence the activity of monophenolase activity of tyrosinase. In addition, it reported that tyrosinase accepts many phenols and catechols as substrates, and produces only one type of oxidation product using catechol, 4-methylcatechol, 3,4-dihydroxyphenyl acetic acid, and caffeic acid as substrates, respectively (Madani *et al.*, 1999).

No *et al.* (1999) reported that gallic acid produced only marginal inhibitory effect but three major components of green tea, (–)-epicatechin 3-*O*-gallate (ECG), (–)-gallocatechin 3-*O*-gallate (GCG), and (–)-epigallocatechin 3-*O*-gallate (EGCG) showed the most potent inhibitory activity. Based on that, they concluded that the structural requirement for the inhibitory potency needs additional structures besides gallic acid moiety. The well-known tyrosinase inhibitors, tropolone and kojic acid were assumed that these inhibitors complex the two copper atoms, which are presented in the active site of the enzyme (Kahn *et al.*, 1995). The most of the competitive inhibitors have the ability to chelate copper in this enzyme (Nerya *et al.*, 2003).

As mentioned above, both subunits of rosmarinic acid, similar to L-tyrosine or L-DOPA did not show inhibitory activity but substrate-like behavior, while compounds **1** and **2** exhibited competitive inhibitory activities. Therefore, the fact may suggest that additional structure is to be the potent inhibitory requirement as steric hindrance of substrate or chelator of copper ion at the active center of the tyrosinase. The methyl group of compound **2** on tyrosinase activity using the L-tyrosine as a substrate did not affect the IC_{50} value, significantly. The results obtained so far may hint to their interaction with the enzyme but this remains unclear, since the structure of mushroom tyrosinase used for this study has not been established yet.

Salvia genus is a rich source of polyphenols, with an excess of 160 polyphenols having been identified, some of which are unique to the genus. A large number of these polyphenolic compounds are apparently constructed from the caffeic acid building block *via* a variety of condensation reactions (Lu and Foo, 2002). Rosmarinic acid, the most abundant caffeic acid dimer, has been reported to be the major phenolic compound responsible for the high antioxidant activity of *Salvia* sample (Cuvelier *et al.*, 1996). The presence of rosmarinic acid in medicinal plants, herbs and spices has beneficial and health promoting effects. This chemical is rapidly eliminated from the blood circulation after intravenous administration and shows a very low toxicity with a LD_{50} in mice of 561 mg kg^{-1} after intravenous application (Petersen and Simmonds, 2003).

The lack of an efficient whitening agent which does not suffer from low activity, high cytotoxicity and mutagenecity, poor skin penetration, or low stability in formulation encourages a continuation of research for new skin-lightening agents to meet medical needs. Safety is a primary consideration for tyrosinase inhibitors, especially for those used in food and cosmetic products, as these may be regularly utilized in unregulated quantities. Rosmarinic acid has been shown to inhibit adenylate cyclase (Kohda *et al.*, 1989), aldose reductase (Kasimu *et al.*, 1998), complement (Sahu *et al.*, 1999), nitric oxide (Yokozawa and Chen, 2000), HIV-intergrase (Kim *et al.*, 1999) and lipid peroxidation (Huang and Zhang, 1992), and to have antithrombotic and antiplatelet effects (Zou *et al.*, 1993). This paper showed that rosmarinic acid and its methyl ester have a bright prospect of cosmetic and medical utility on the whitening effect, as they inhibit the tyrosinase activity using L-tyrosine as a substrate *in vitro*, for the first time.

Acknowledgement

This work was supported by Pukyong National University Research Foundation Grant in 2002.

References

Andersen, S.O., Biochemistry of insect cuticle. *Annu. Rev. Entomol.* **24**, 29-61 (1979).

Choi, J.S., Kang, H.S., Jung, H.A., Jung, J.H., and Kang, S.S., A new cyclic phenyllactamide from *Salvia miltiorrhiza. Fitoterapia* **72**, 30-34 (2001).

Cuvelier, M.E., Richark, H., Berset, C., Antioxidative activity and phenolic composition of pilot-plant and commercial extracts of sage and rosemary. *J. Am. Oil Chem. Soc.* **73**, 645-652 (1996).

Huang, Y.S., and Zhang, J.T., Antioxidative effect of three water-soluble components isolated from *Salvia miltiorrhiza in vitro. Acta Pharm. Sinica* **27**, 96-100 (1992).

Kahn, V., Lindner, P., and Zaka, V., Effect of Kojic acid on the oxidation of *o*-dihydroxyphenols by mushroom tyrosinase. *J. Food Biochem.* **18**, 253-271 (1995).

Kang, H.S., Chung, H.Y., Jung, J.H., Kang, S.S., and Choi, J.S., Antioxidant effect of *Salvia miltiorrhiza. Arch. Pharm. Res.* **20**, 496-500 (1997).

Kang, H.S., Chung, H.Y., Byun, D.S., and Choi, J.S. Further isolation of antioxidative (+)-1-hydroxypinoresinol-1-O-β-D-glucoside from the rhizome of *Salvia miltiorrhiza* that acts on peroxynitrite, total ROS and 1,1-diphenyl-2-picrylhydrazyl radical. *Arch. Pharm. Res.* **26**, 24-27 (2003).

Kasimu, R., Tanaka, K., Tezuka, Y., Gong, Z.N., Li, J.X., Basnet, P., Namba, T., and Kadota, S., Comparative study of seventeen *Salvia* plants: aldose reductase inhibitory activity of water and MeOH extracts and liquid chromatography-mass spectrometry (LC-MS) analysis of water extracts. *Chem. Pharm. Bull.* **46**, 500-504 (1998).

Kim, H.K., Lee, H.K., Shin, C.G., and Huh, H., HIV integrase inhibitory activity of *Agastache rugosa. Arch. Pharm. Res.* **22**, 520-523 (1999).

Kim, Y.M., Yun J., Lee, C.K., Lee, H.H., Min, K.R., and Kim, Y.S., Oxyresveratrol and hydroxystilbene compounds. Inhibitory effect on tyrosinase and mechanism of action. *J. Biol. Chem.* **277**, 16340-16344 (2002).

Kohda, H., Takeda, O., Tanaka, S., Yamasaki, K., Yamashita, A., Kurokawa, T., and Ishibashi, S., Isolation of inhibitor of adenylate cyclase from Dan-shen, the root of *Salvia miltiorrhiza. Chem. Pharm. Bull.* **37**, 1287-1290 (1989).

Lim, J.Y., Ishiguro, K., and Kubo, I., Tyrosinase inhibitory p-coumaric acid from ginseng leaves. *Phytother. Res.* **13**, 371-375 (1999).

Lu, Y., and Foo, L.Y., Polyphenolics of Salvia-a review. *Phytochemistry*, 59, 117-140 (2002).

Madani, W., Kermasha, S. and Versari, A., Chararacterization of tyrosinase- and polyphenol esterase-catalyzed end products using selected phenolic substrates. *J. Agric. Food Chem.* **47**, 2486-2490 (1999).

Mason H.S., Structures and functions of the phenolase complex. *Nature* **177**, 79-81 (1956).

Mayer, A.M., Polyphenol oxidase in plants-Recent progress. *Phytochemistry* **26**, 11-20 (1987).

Nerya O., Vaya J., Musa R., Izrael A., Ben-Arie R., Tamir S., Glabrene and isoliquiritigenin as tyrosinase inhibitors from Licorice roots. *J. Agric. Food Chem.* **51**, 1201-1207 (2003).

No, J.K., Soung, D.Y., Kim, Y.J., Shim, K.H., Jun, Y.S., Rhee, S.H., Yokozawa, T., and Chung, H.Y., Inhibition of tyrosinase by green tea components. *Life Science* **65**, PL 241-246 (1999).

Pérez-Bernal, A., Muñoz-Pérez, M.A., and Camacho, F., Management of facial hyperpigmentation. *Am. J. Clin. Derm.* **1**, 261-268 (2000).

Petersen M., Simmonds M.S.J., Molecules of interest rosmarinic acid. *Phytochemistry* **62**, 121-125 (2003).

Sahu, A.; Rawal, N.; Pangburn, M.K., Inhibition of complement by covalent attachment of rosmarinic acid to activated C3b. *Biochem. Pharmacol.* **57**, 1439-1446 (1999).

Segel, I.H., Enzymes, in Segel, I.H. (ed.) *Biochemical calculations, How to solve mathematical problems in general biochemistry.* John Wiley & Sons Inc., New York, 1976, pp. 246-252.

Tang, W. and Eisenbrand, G., *Chinese Drugs of Plant Origin. Springer-Verlag*, New York, 1992, pp. 891.

Whitaker, J.R., Polyphenol oxidase: in Wong, D.W.S. (ed.). *Food Enzymes, Structure and Mechanism.* Chapman & Hall, New York, pp. 271-307 (1981).

Wilcox D.E., Porras A.G., Hwang Y.T., Lerch K., Winkler M.E., Solomon E.I., Substrate analogue binding to the coupled binuclear copper active site in tyrosinase. *J. Am. Chem. Soc.* **107**, 4015-4027 (1985).

Xu Y., Stokes A.H., Freeman W.M., Kumer S.C., Vogt B.A., Vrana K.E., Tyrosinase mRNA is expressed in human substantia nigra. *Mol. Brain Res.* **45**, 159-162 (1997).

Yokozawa, T., and Chen, C.P., Role of *Salviae miltiorrhizae* radix extract and its compounds in enhancing nitric oxide expression. *Phytomedicine* **7**, 55-61 (2000).

Zou, Z.W., Xu, L.N., and Tian, J.Y., Antithrombotic and antiplatelet effects of rosmarinic acid, a water-soluble component isolated from radix *Salviae miltiorrhizae* (Danshen). *Acta Pharm. Sinica* **28**, 241-245 (1993).

(Accepted April 6, 2004)