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Inhibitory effects of the water extracts of *Lavendula* sp. on mushroom tyrosinase activity

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Abstract

This study aimed to evaluate the inhibition properties of six lavender species, including *Lavendula angustifolia*, *Lavandula angustifolia* "Vera", *Lavendula X allardii*, *Lavendula stoechas*, *Lavendula viridis* and *Lavendula X heterophylla*, toward the activity of mushroom tyrosinase. When using L-3,4-dihydroxyphenylalanine (L-Dopa) as the substrate for mushroom tyrosinase, the water extracts of leaves and stems from *L. stoechas* and *L. angustifolia* "Vera" showed strong inhibitory effects against the activity of mushroom tyrosinase (70% and 66.4% inhibition, respectively). Oven-drying the leaves and stems or free-drying the water extracts significantly decreased the inhibitory abilities of the water extracts from all lavender species. The water extract from *L. stoechas* decreased the V_{max} values when using L-Dopa, catechol and 3,4-dihydroxyphenylacetic acid (DHPAA) as the substrates. It increased the value of K_m when L-Dopa and catechol were the substrates but it decreased the K_m when DHPAA was used. It behaved as a mixed-type inhibitor toward mushroom tyrosinase.

Keywords: Lavender; Mushroom tyrosinase; Mixed-type inhibitor; L-3,4-Dihydroxyphenylalanine; Catechol, 3,4-Dihydroxyphenylacetic acid

1. Introduction

Lavenders (*Lavandula* sp.) belong to the family Labiatae (Lamiacae) and fall into four main categories: *Lavandula latifolia*, a Mediterranean grass-like lavender; *Lavandula angustifolia*, commonly known as English lavender; *Lavandula stoechas*, known as French lavender; and *Lavandula X intermedia*, which is a sterile cross between *L. latifolia* and *L. angustifolia*. They have been used either dried or as an essential oil for centuries for a variety of therapeutic and cosmetic purposes (Saeki, 2000; Nagai, Wada, Usui, Tanaka, & Hasebe, 2000; Morris, 2002). The oil is most often used in aromatherapy or incorporated into soaps and other products as a pleasant fragrance or as an antimicrobial agent (Vokou, Vareltzidou, & Katinakis, 1993). The main constituents of lavender oil are linalool, linalyl acetate, 1,8-cineole, β-ocimene, terpinen-4-ol and camphor. Each of these constituents can vary significantly in oils derived from different cultivars (Cavanagh & Wilkinson, 2002). However, there is still considerable debate about whether lavender oils do have a significant clinical potential, either in their own right, or as additives to other products (Cavanagh & Wilkinson, 2002). In this study, we evaluated the inhibitory effect of lavender toward tyrosinase activity to determine the potential of using lavender as a potential bleaching agent in food systems.

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase EC 1.14.18.1) is a multifunctional copper-containing enzyme and commonly present in microorganisms, plants and animals. Tyrosinase is responsible

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for enzymatic browning in plants, and it may cause undesirable changes in colour, flavour and nutritive values of plant-derived foods and beverages (Sánchez-Ferrer, Rodríguez-López, García-Cánovas, & García-Carmona, 1995; Friedman, 1996). This enzyme is mainly involved in the first two steps of the melanin biosynthesis, in which L-tyrosine (monophenolase activity) is hydroxylated and the hydroxylation product, L-Dopa (diphenolase activity), is further oxidized into the corresponding *o*-quinone (Seo, Sharma, & Sharma, 2003). Tyrosinase catalyzes the reaction of melanin biosynthesis in human skin and the epidermal hyperpigmentation results in various dermatological disorders, such as melasma, freckles and age spots (Mosher, Pathak, & Fitzpatrick, 1983). Recently, safe and effective tyrosinase inhibitors have become important for their potential applications in improving food quality and preventing pigmentation disorders and other melanin-related health problems in human beings (Seo et al., 2003; Mosher et al., 1983; Maeda & Fukuda, 1991). Furthermore, tyrosinase inhibitors are also important in cosmetic applications for skin whitening effects, because many men and women prefer lighter skin colour (Dooley, 1997). Since plants are a rich source of bioactive chemicals, and mostly free of harmful side effects, there is an increasing interest in finding natural tyrosinase inhibitors from them. Some potent tyrosinase inhibitors, such as cuminaldehyde (Kubo & Kinst-Hori, 1998), oxyresveratrol (Shin et al., 1998), kaempferol (Kubo & Kinst-Hori, 1999), quercetin (Chen & Kubo, 2002) and gallic acid derivatives (No et al., 1999), have been isolated from various plants. Besides, some fungal metabolites, such as kojic acid [5-hydroxy-2-(hydroxymethyl)-*r*-pyrone] have been demonstrated to be potent tyrosinase inhibitors and are extensively used as cosmetic agents with an excellent whitening effect (Chen et al., 1991; Kahn, Ben-Shalom, & Zakin, 1997).

Researches have demonstrated that the biological activities (e.g., antioxidant activity) of extracts from plants depend on the extraction solvents and extraction techniques used (Chen, Shi, & Ho, 1992; Kramer, 1985; Chevolleau, Mallet, Ucciani, Gamisans, & Gruber, 1992). Dapkevicius, Venskutonis, van Beek, and Linssen (1998) showed that aromatic herbs (*L. angustifolia* was included) extracted with polar solvents possessed higher antioxidant efficiency (BHT-equivalent yields) than did essential oil, and *L. angustifolia*, extracted with water, gave a 3.2 times higher antioxidant efficiency than did essential oil. So far, little attention has been paid to the possibility that water extract of lavender may inhibit browning and become a potential bleaching agent in food systems. This study was aimed to evaluate the inhibitory properties of water extracts from six different species of *Lavandula* genus (including *L. angustifolia*, *L. angustifolia* "Vera", *Lavendula X allardii*, *L. stoechas*, *Lavendula viridis* and *Lavendula X heterophylla*) toward the activity of mushroom tyrosinase, and the effects of extraction methods were evaluated as well.

2. Materials and methods

2.1. Lavender materials

Six lavender species were used in this study; they were *L. angustifolia*, *L. angustifolia* "Vera", *L. X allardii*, *L. stoechas*, *Lavendula viridis*, *L. X heterophylla.*

2.2. Chemicals

Mushroom tyrosinase (6050 units/mg), 3,4-dihydroxyphenylalanine (L-Dopa), 3,4-dihydroxyphenylacetic acid (DHPAA), 1,2-dihydroxybenzene (pyrocatechol, catechol) and 5-hydroxy-2-hydroxymethyl-4H-4-pyranone (kojic acid) were purchased from Sigma chemicals Co. (St. Louis, Mo). All other chemicals were reagent grade or purer.

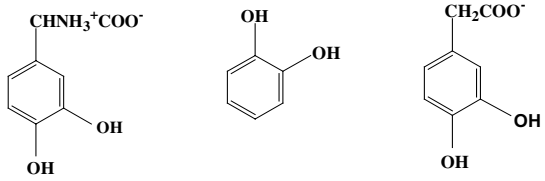
2.3. Preparation of the water extracts of lavenders

The stems and leaves (fresh or oven-dried) of the lavenders were extracted with distilled water in a commercial mixer (Health CLY-5168, Tao-Ya, Taiwan) for 1 min, and then the mixture was stirred at 4 °C for 18 h. After filtering the mixture with four layers of cheesecloth, the mixture was centrifuged at 8500*g* for 30 min with a centrifuge (Beckman Avanti^{TM} J-25, Palo, CA, USA) and then the supernatants from fresh material and oven-dried material were defined as WF and WO, respectively. Both WF and WO were further freeze-dried by a vacuum freeze-dryer (Kingmech FD 24-3s-6P, Keen-Lung, Taiwan) and were defined as WFF and WOF, respectively. Concentrations of all tested extracts were expressed as equivalent weight (mg, dry weight basis) of fresh material per ml of solvent (distilled water).

2.4. Enzymatic assay of tyrosinase

The tyrosinase activity, using L-Dopa as substrate, was measured according to the method of Kubo and Kinst-Hori (1998) with slight modifications. First, 0.29 ml of 4.5 mM L-Dopa solution (the substrate for tyrosinase) was mixed with 0.3 ml of 25 mM phosphate buffer (pH 6.8) and incubated at 25 °C for 10 min. Then, 0.3 ml of each lavender extract with four different concentrations (0.01, 0.02, 0.03 and 0.04 mg/ml) was added to the mixture, followed by adding 0.01 ml of 4000 units/ml of mushroom tyrosinase. The formation of dopachrome was immediately monitored by measuring the linear increase in optical density at 475 nm. Triplicate measurements were recorded. The increased absorbance at 475 nm (ΔA_{sample}) was recorded during 5 min at room temperature. Deionized water was used instead of lavender extracts for the blank (ΔA_{blank}). Kojic acid was used as a positive control for tyrosinase inhibitor (0.01, 0.02, 0.04 and 0.2 mg/ml). One unit (U) of enzymatic activity was defined as the amount of enzyme needed for increasing 0.001 absorbance per min at 475 nm under the experimental conditions. Inhibitory effects of the tested samples on the enzyme activity were represented as % of

inhibition $= (1 - B/A) \times 100$, where $A = \Delta OD_{475}$/min without tested sample and $B = \Delta OD_{475}$/min with tested sample. Concentrations of tested samples that inhibited 50% of tyrosinase activity under the experimental conditions were defined as the IC_{50} values. When DHPAA and catechol were used as the substrates for tyrosinase, the absorbance was measured at 400 nm.



DOPA Catechol DHPAA

2.5. Determination of kinetic parameters

Mushroom tyrosinase (0.03 ml, 400 units/ml) was incubated with various concentrations of 0.27 ml enzyme substrates (L-Dopa, DHPAA and catechol, 2.5–25 mM) in 0.3 ml 25 mM phosphate buffer (pH 6.8) at room temperature, and tested lavender samples (0.3 ml) were simultaneously added to the reaction mixture. Kojic acid, a known tyrosinase inhibitor, was served as a positive control. Kinetic parameters, K_m and $V_{max,}$ of the tyrosinase activity were calculated by linear regression from Lineweaver–Burk plots. The inhibition constant (K_i) of an inhibitor was obtained from the secondary plot of Lineweaver–Burk plots, the slope for the vertical axis, and inhibitor concentration for the horizontal axis. The intercept on the horizontal axis, (I), was the absolute value of the K_i.

2.6. Effect of L. stoechas WF on the oxidation of L-Dopa, DHPAA and catechol

Mushroom tyrosinase (0.03 ml, 400 units/ml) was incubated with 0.27 ml of 4.5 mM L-Dopa, DHPAA and catechol in 0.3 ml of 25 mM phosphate buffer (pH 6.8) at room temperature, and 0.3 ml WF of *L. stoechas* was simultaneously added to the reaction mixture. After 10 min of reaction at room temperature, the absorbance (L-Dopa at 475 nm, but DHPAA and catechol at 400 nm) of the reaction mixture was recorded. Relative oxidation of L-Dopa, DHPAA or catechol catalyzed by mushroom tyrosinase was calculated as % relative oxidation $= B/A \times 100$, where $A = \Delta OD_{475}$ in 10 min without *L. stoechas* WF and $B = \Delta OD_{475}$ in 10 min with *L. stoechas* WF.

3. Results and discussion

3.1. Moisture contents of fresh lavenders

The moisture contents of *L. angustifolia* "Vera", *L. X allardii*, *L. X heterophylla L. angustifolia*, *L. stoechas* and *Lavendula viridis* were 71.0, 72.2, 72.5, 75.2, 75.5, and 76.3, respectively. *L. viridis* had the highest moisture content, but *L. angustifolia* Vera showed the lowest moisture content.

3.2. Inhibitory effects of lavenders on mushroom tyrosinase activity

Caffeic acid, L-Dopa, DHPAA, 4-methylcatechol and catechol are endogenous phenolic substrates for polyphenol oxidase from apple and potato sources (Ndubizu, 1976). In this study, the inhibitory effects of water extracts from lavenders toward mushroom tyrosinase, a polyphenol oxidase, were evaluated. Mushroom tyrosinase catalyzed pigment formation from oxidation of caffeic acid and 4-methylcatechol at a very slow reaction rate (data not shown); therefore, neither caffeic acid nor 4-methylcatechol was a good substrate for mushroom tyrosinase under our experimental conditions. L-Dopa is an intermediate product during oxidation of L-tyrosine and is used commonly as an enzyme substrate of tyrosinase (Sánchez-Ferrer et al., 1995; No et al., 1999; Madani, Kermasha, & Bisakowaki, 1999; Kubo & Kinst-Hori, 1999; Chen & Kubo, 2002). In this study, L-Dopa was used as the substrate of mushroom tyrosinase to screen the inhibitory activities of tested extracts.

Water extracts of lavenders were prepared with four different methods and their effects on mushroom tyrosinase activity were evaluated during the oxidation of dihydroxyphenylalanine (L-Dopa). The inhibitory effects of lavenders on tyrosinase activity depended, not only on species, but

Table 1
Inhibitory effects of the water extracts of leaves and stems from six lavender species against the activity of mushroom tyrosinase

Species	Inhibition[a] (%)				ΔIC_{50}[b]
	WF	WFF	WOF	WO	WF
Lavendula angustifolia	0	0	0	0	>0.04
Lavendula angustifolia "Vera"	70.0 ± 3.3	0	0	0	0.03 ± 0.01
Lavendula stoechas	66.4 ± 3.4	20.8 ± 3.4	0	0	0.04 ± 0.01
Lavendula X allardii	44.0 ± 3.3	10.5 ± 0.9	2.5 ± 0.9	0	> 0.04
Lavendula X heterophylla	15.0 ± 1.9	0	0	0	>0.04
Lavendula viridis	0	0	0	0	>0.04

WF: water extract of fresh leaves and stems; WFF: water extract of fresh leaves and stems and then immediately freeze-drying; WO: water extract of oven-dried leaves and stems; WOF: water extract of oven-dried leaves and stems, and then immediately freeze-drying.

[a] Percent inhibition of the reaction rate of tryosinase activity when 0.04 mg/ml of the extracts was used and values are given as means ± S.D. ($n = 3$).

[b] Concentration (mg/ml) of WF required to reach 50% inhibition on the reaction rate of tryosinase activity toward dihydroxyphenylalanine (L-Dopa).

Table 2
Effect of the water extract of leaves and stems from *Lavendula stoechas* on the kinetic parameters of mushroom tyrosinase

Substrate	No inhibitor	Kojic acid (mg/ml)				*Lavendula stoechas* (mg/ml)		
		0.01	0.02	0.04	0.20	0.01	0.02	0.04
K_m *(mg/ml)*								
L-Dopa	7.1	10.8	11.1	11.7	12.8	7.5	8.8	10.0
DHPAA	10.3	4.0	4.9	5.2	7.0	7.5	8.1	9.0
Catechol	1.9	3.4	4.3	5.1	7.0	2.0	1.9	2.5
$V_{max}(U^a)$								
L-Dopa	38	31	30	28	25	37	38	33
DHPAA	90	48	34	25	23	67	62	58
Catechol	143	98	92	88	76	141	132	104

WF: water extract of fresh leaves and stems.
L-Dopa: dihydroxyphenylalanine.
DHPAA: 3,4-dihydroxyphenylacetic acid.
[a] 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.

also the preparation methods (Table 1). Two of the six tested lavenders, *L. angustifolia* and *L. viridis*, showed no inhibition of tyrosinase activity under the tested conditions, no matter which method was applied to prepare the extract; on the other hand, the remaining lavenders inhibited tyrosinase activity. Among tested extracts, WF of *L. angustifolia* "Vera" and *L. stoechas* showed the greatest inhibitions of tyrosinase activity, with the IC_{50} values of 0.03 and 0.04 mg/ml, respectively.

For the same lavender species, such as *L. stoechas* and *L. X allardii*, ranking of inhibition of tyrosinase activity followed the order: WF >WFF> WOF > WO. Oven-drying destroyed lavenders' inhibitory activities and freeze-drying also weakened Lavenders' effects on tyrosinase. Interestingly, the water extract of freshly prepared (WF) *L. angustifolia* "Vera" showed the strongest inhibition; however, no inhibitory effect was observed in the water extract from the oven-dried leaves and stems. To utilize natural herb materials, the drying procedure is a convenient way to store the materials before further processing. However, our results demonstrated that fresh preparation was required for the water-soluble components of lavenders to exhibit their inhibitory activities toward tyrosinase. Since crude extracts were used in this study, the key compounds performing the inhibitory role in the WF of *L. angustifolia* "Vera" are still unknown. Our study indicated that the compounds were unstable to oven or freeze-drying; therefore, we suspect that the compounds might be proteins. Madani et al. (1999) reported that a polyphenol esterase might show inhibitory effect against mushroom tyrosinase.

Water extract of freshly prepared *L. stoechas* (*L. stoechas* WF) was used for the rest of the study, to evaluate the kinetic parameters of the inhibition and the inhibitory effect on tyrosinase using different enzyme substrates. The selection of *L. stoechas* instead of *L. angustifolia* "Vera" was because the former was more stable to freeze-drying.

3.3. Kinetic study of *L. stoechas* WF on mushroom tyrosinase activity

The kinetic behaviour of mushroom tyrosinase was studied during the oxidation of L-Dopa, DHPAA and catechol. Among the tested substrates, catechol exhibited the lowest K_m and highest V_{max} (Table 2). Presence of either kojic acid or *L. stoechas* WF increased the K_m of both L-Dopa and catechol. But the K_m of DHPAA decreased when kojic acid or *L. stoechas* WF was added to the reaction system. Both kojic acid and *L. stoechas* WF slowed down the oxidation rate, no matter which substrate was used for tyrosinase, indicated by lower V_{max} values. As a tyrosinase inhibitor, kojic acid showed a greater effect on the activity of mushroom tyrosinase than did *L. stoechas*

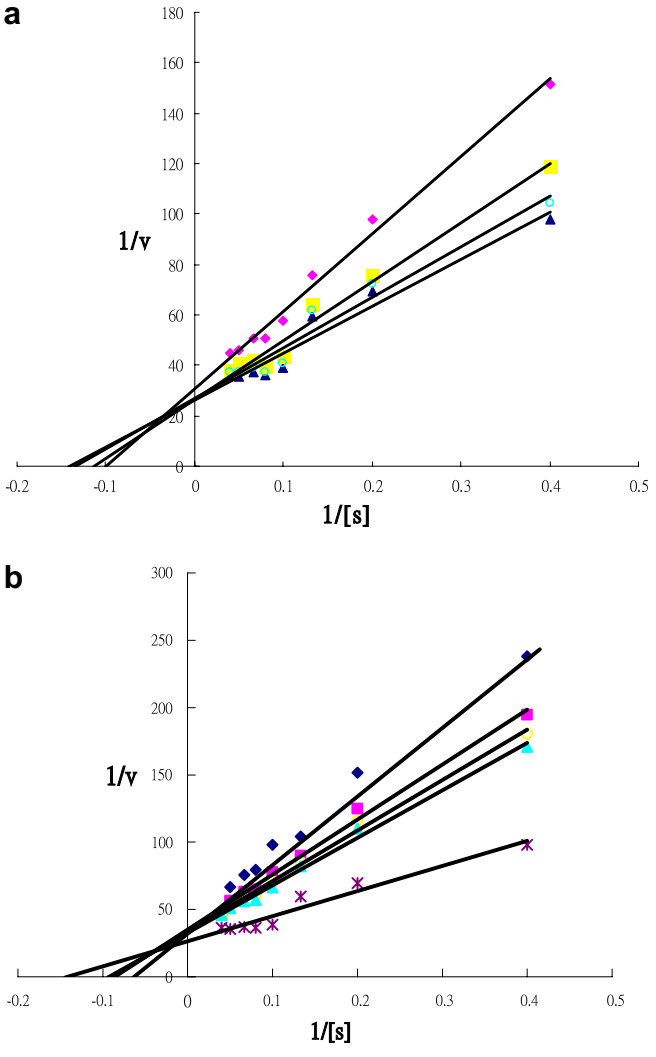


Fig. 1. Lineweaver–Burk plot of 1/V versus 1/[S] with (a) water extract of fresh *Lavendula stoechas* and L-Dopa. *L. stoechas* conc.-◆- (0.04 mg/ml), -■-(0.02 mg/ml), -○- (0.01 mg/ml), -▲- control (no *L. stoech*) (b) Kojic acid and L-Dopa. Kojic acid conc. -◆- (0.2 mg/ml), -■- (0.04 mg/ml), -○- (0.02 mg/ml), -▲- (0.02 mg/ml), control -✱- no kojic acid. The enzymatic assay was performed with mushroom tyrosinase (13 units/ml) at room temperature for 10 min.

Table 3
K_i values and the type of mushroom tyrosinase inhibitor of kojic acid and the water extract of leaves and stems from *Lavendula stoechas*

Substrate	Kojic acid (mg/ml)		*Lavendula stoechas* (mg/ml)	
	K_i (mg/ml)	Inhibition type	K_i (mg/ml)	Inhibition type
L-Dopa	0.046	Mixed	0.183	Mixed
DHPAA	0.152	Mixed	0.227	Mixed
Catechol	0.024	Mixed	0.027	Mixed

WF: water extract of fresh leaves and stems.
L-Dopa: dihydroxyphenylalanine.
DHPAA: 3,4-dihydroxyphenylacetic acid.

WF. In other words, to reach a similar extent of tyrosinase inhibitory effect, a higher concentration of *L. stoechas* WF was needed than that of kojic acid. Although the anti-tyrosinase ability of *L. stoechas* WF was significantly less than that of kojic acid, it was evident that *L. stoechas* WF did have potent tyrosinase inhibitory activity.

Both kojic acid and *L. stoechas* WF worked as mixed type inhibitors of tyrosinase activity with all tested substrates, as shown in Fig. 1 as an example and, again, kojic acid had smaller K_i values than had *L. stoechas* WF (Table 3). The results of kinetic study suggested that kojic acid and *L. stoechas* WF reduced the affinity of the substrate for mushroom tyrosinase, yet did not bind to the active site, and also indicated that the dissociation of the enzyme–substrate complex was prevented (Segal, 1976). Compared to L-Dopa and DHAPP, catechol bound to mushroom tyrosinase was inhibited to a greater extent when either kojic acid or *L. stoechas* WF was present in the reaction system, based on the results of K_i determination. From the K_i value shown in Table 3, kojic acid exerts much more effective inhibition of tyrosinase than does *L. stoechas* WF. Because the most effective inhibitory component of *L. stoechas* WF has not yet been isolated, it is apparent that the crude *L. stoechas* WF did not exhibit better activity than did kojic acid.

Many studies have tested the activity of mushroom tyrosinase by the oxidation of L-Dopa; however, kinetic values, such as K_m, V_{max} and K_i, varied in the different studies. In general, K_m, V_{max} and K_i obtained in this study were smaller

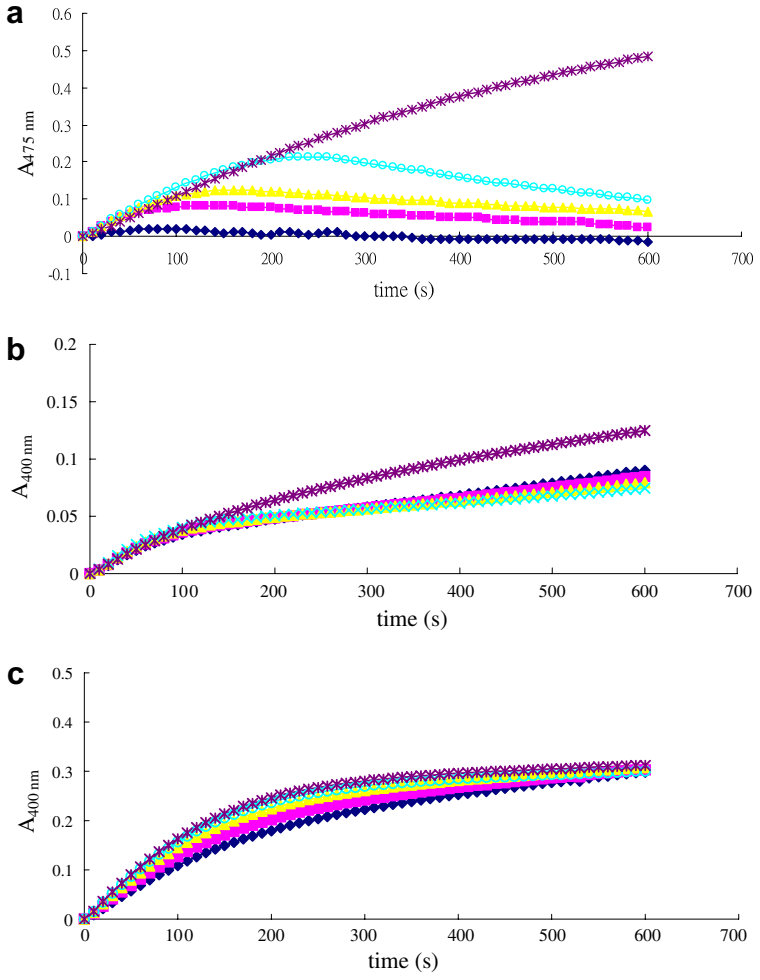


Fig. 2. Effect of water extract of fresh *Lavendula stoechas* WF on mushroom tyrosinase activity with different substrates. Mushroom tyrosinase (44 u/ml) reacted at room temperature for 10 min. (a) *Lavendula stoechas* WF and L-Dopa (b) *L. stoechas* WF and DHPAA (c) *L. stoechas* WF and catechol. *L. stoechas* WF conc. -◆- (0.04 mg/mL), -■- (0.03 mg/mL), -▲- (0.02 mg/mL), -○- (0.01 mg/mL), -✳- no *L. stoechas* WF.

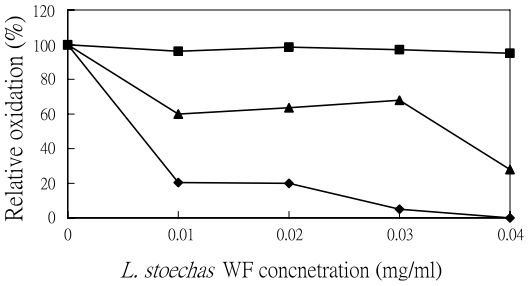


Fig. 3. Relative oxidation of L-Dopa, DHPAA and catechol catalyzed by mushroom tyrosinase (44 u/ml) for 10 min w/o the water extract of leaves and stems from *Lavendula stoechas* . -♦- L-Dopa, -▲- DHAPP, and -■- catechol.

than the reports of others (Winder & Harris, 1991; Madani et al., 1999; Jones, Hughes, Hong, Jia, & Orndorff, 2002; Xie et al., 2003; Huang, Chen, Chang, & Chou, 2005). The reason for the inconsistency of the kinetic values, among different studies, is that the oxidation activity of tyrosinase is affected by enzyme and substrate concentrations, pH of reaction buffer, as well as the types of hydrogen donors, such as Dopa (Pomerantz & Warner, 1967).

3.4. Inhibitory effect of *L. stoechas* WF on the oxidation of L-Dopa, DHPAA and catechol, catalyzed by mushroom tyrosinase

Fig. 2. shows the relative oxidation of L-Dopa, DHPAA and catechol, catalyzed by mushroom tyrosinase in the presence of *L. stoechas* WF. Without *L. stoechas* WF, tyrosinase had the fastest reaction rate in oxidizing catechol but a slow reaction rate toward DHPAA . When comparing the oxidation of L-Dopa, DHPAA and catechol under conditions of w/o *L. stoechas* WF, the relative oxidation is shown in Fig. 3. Addition of *L. stoechas* WF did not significantly decrease the oxidation of catechol catalyzed by mushroom tyrosinase, and this might due to tyrosinase-catalyzed oxidation of catechol at a high initial reaction rate. When *L. stoechas* WF was added in the reaction mixture, the oxidation of L-Dopa was negligible. Although the anti-tyrosinase abilities of *L. stoechas* WF were significantly lower than that of kojic acid, it was evident that the extracts did have potent tyrosinase inhibitory activity.

4. Conclusions

This study showed that the water extracts from lavenders had potent inhibitory effects against the activity of mushroom tyrosinase and they might be used as bleaching agents in food systems. The inhibition was species-dependent. Among six tested extracts, the water extracts from *L. angustifolia* "Vera" and *L. stoechas* showed the greatest inhibitions. Both oven drying and freeze drying could destroy the inhibitory activity of lavenders to a certain extent. A study of the kinetics of the inhibition showed that the water extract from *L. stoechas* served as a mixed-type inhibitor of mushroom tyrosinase.

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