Inhibitory Effect of the Ethyl Acetate Fraction of Ethanol Extract from *Rhus verniciflua* Stokes Wood on the Activity of Mushroom Tyrosinase

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Solvent extracts of *Rhus verniciflua* Stokes wood were made using decompressing inner ebullition, and a Box-Behnken design was used to optimize extraction conditions to produce an extract that inhibited tyrosinase activity. The chemical compositions and inhibition rates were determined in extracts made with petroleum ether, ethyl acetate, n-butanol, and an aqueous fractionation. The ethyl acetate fraction had the highest total phenolic content and inhibition rates. The main flavonoids in this fraction were 0.531% fisetin, 7.582% fustin, 0.848% sulfuretin, and 0.272% butein. The effects of the extract on the monophenolase and diphenolase activity of mushroom tyrosinase were studied using the Lineweaver-Burk equation to determine the effect of the extract on inhibition of tyrosinase activity. The results showed that the extract inhibited both the monophenolase and diphenolase activity of the enzyme. The IC_{50} of the ethyl acetate extract was 308 μg/mL, with the lag period of the enzyme being obviously lengthened; it was estimated to be 2.45 min in the absence of the inhibitor and extended to 9.63 min in the presence of 500 μg/mL of extract. The ethyl acetate extract acted as a mixed type inhibitor. The K_I was less than the K_{IS}, which demonstrates that the [ESI] is less stable than [EI], suggesting that the extract could easily combine with free enzyme in the enzyme catalysis system, thus affecting enzyme catalysis on the substrate.

Keywords: Rhus verniciflua Stokes; Ethyl acetate extract; Tyrosinase; Inhibition rate; Inhibition mechanism

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INTRODUCTION

Rhus verniciflua Stokes is a deciduous tree of the Anacardiaceae family in the toxicodendron category (Zheng and Min 1980). It is an important economic forest species in China. Raw lacquer from this species as a material for traditional paint has been used in China, Japan, and Korea for thousands of years (Fu *et al*. 2005). It also is a traditional medicinal wood; the leaf, flower, roots, bark, fruit, and raw lacquer can be used as a medicine, for coughs, to eliminate addiction, act as an insecticidal compound, and to combat tumours. Recently, it has been found that phenolic extracts of *R. verniciflua* Stokes bark act as marked antioxidants (Lim *et al*. 2001), anti-microbials (Kim *et al*. 2010), anti-tumour compounds (Lee *et al*. 2004), and function in immunoregulation (*Kang et al.* 2012). The primary bioactive compounds are polyphenols, such as fisetin, fusin, butein, and sulphuretin (Kim *et al*. 2006a).

Tyrosinase (EC 1.14.18.1) is a phenoloxidase and a copper metal oxidase, which exists widely in microorganisms, plants, animals, and in the human body (Sanchez *et al*. 1995). In organisms, tyrosinase plays an important role as a catalyst in the metabolism of tyrosine and in the synthesis of melanin (Fenoll *et al*. 2004). In the process of melanin synthesis, tyrosinase is mainly involved in two types of reactions: one is catalyzing the tyrosine to o-diphenols (monophenols activity), and the other is catalyzing o-diphenols to o-quinones (diphenolase activity) (Cooksey *et al*. 1997). Overproduction of melanin is believed to result in skin conditions such as freckles, chloasma, and age spots. Thus, the search for natural chemicals to inhibit tyrosinase activity is of great interest.

Rhus verniciflua Stokes resources in China are abundant in the provinces of Jiangxi, Hunan, Yuannan, and Hubei. Most research and applications have focused on the raw lacquer and seeds. It is known that *Rhus verniciflua* Stokes extracts have marked antioxidant, anti-tumor, and anti-inflammatory effects, but there is little research on natural tyrosinase inhibitors. In the present study, we optimized the extraction of tyrosinase inhibition compounds from the wood using the method decompressing inner ebullition by a Box-Behnken design through extraction with petroleum ether, ethyl acetate, n-butanol, and aqueous fractionation. The ethyl acetate fraction had the highest total phenolic content and inhibition rate. The inhibitory efficacy of the ethyl acetate extract on tyrosinase was evaluated, and the mechanism underlying this interaction was extensively explored. These findings will be useful for the exploration of tyrosinase inhibitors from the *Rhus verniciflua* Stokes, which has high bioactivity and low toxicity.

EXPERIMENTAL

Materials

Rhus verniciflua Stokes wood was harvested from HuBei Province (China), dried at room temperature for two weeks, crushed into a powder, sieved to select particles smaller than 1 mm, and stored in desiccators. Ethanol, Folin-denis, phosphate buffer solution (0.1mmol/L; pH 6.8), L-tyrosine, and L-dopa were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Tyrosinase (EC 1.14.18.1) from mushroom and gallic acid was purchased from the Sigma-Aldrich Chemical Co. (China). The specific activity of the enzyme was 3130 U/mg.

Methods

Extraction and isolation of bioactive compounds

A total of 5 g of *Rhus verniciflua* Stokes bark powder was transferred into an extraction flask with 70% to 90% (by volume) ethanol-water solution (liquid-solid ratio 20:1 mL/g), and the vacuum was controlled (0.07 MPa to 0.09 MPa) to maintain the solution boiling. The extraction was performed two times, and the time varied from 0.5 h to 1.5 h. Solids were separated by filtration and the filtrate was dried by rotary evaporator to calculate the extract yield and inhibition rate.

Under the optimal extraction conditions, the ethanolic extract was obtained, then partitioned with petroleum ether, ethyl acetate, and n-butanol. Each partitioned portion was vacuum dried to yield petroleum ether (6.6%), ethyl acetate (60.1%), n-butanol (3.64%), and an aqueous fraction (29.6%).

Assay of inhibition of tyrosinase activity

Inhibition of tyrosinase activity was tested according to the method of Prasad (Prasad *et al*. 2009), with minor modifications. First, 100 μL of L-tyrosine (1 mM) was added to three wells of a 96-well plate. A total of 70 μL of solvent sample was added to the first well, 70 μL of the sample solution was added to the second and third wells, and 30 μL of mushroom tyrosinase was added to the first and second mixture solution. The enzyme concentration of the solution was 234.75 U/mL. After 30 min of incubation at 30 °C, the absorbance was recorded at 492 nm using a microplate reader. The percentage inhibition (IR) of tyrosinase activity was calculated *via* Eq. 1,

$$\mathrm{IR} = \left(1 - \frac{A_2 - A_3}{A_1}\right) \times 100\% \quad (1)$$

where A_1 is the absorbance at 492 nm with enzyme, but without test sample; A_2 is the absorbance at 492 nm, with test sample and enzyme; and A_3 is the absorbance at 492 nm, with test samples but without enzyme

Optimization of decompressing inner ebullition conditions

A Box-Behnken design study of the effects of the three main factors (*i.e.*, vacuum degree, ethanol concentration, and time) on the inhibition rate was tested. The experimental design and data analysis were performed by using Design-Expert software version 7.1.3 (Stat-Ease, Inc., USA). Analysis of variance (ANOVA) of the response Y was used to determine the significance of the models, pure error, and the variables, followed by a *F*-test. Values of $*P<0.05$ were considered significant.

Total phenol analysis

Total phenolic content (TPC) was measured by the Folin-Ciocalteu method (Qiu *et al*. 2013). The reaction mixture contained 100 μL of bark extract solution, 500 μL of the Folin-Ciocalteu reagent, and 2.5 mL of sodium carbonate (20%, w/v). Tubes were incubated in darkness at 25 °C for 1 h, and the absorbance of the mixture was read at 760 nm. The concentration of total phenolics in bark extract solution was calculated on the basis of a standard curve generated with gallic acid. The calibration formula was,

$$Y = 0.06895\,X + 0.0465 \qquad \mathrm{R}=0.9998 \quad (2)$$

where *Y* denotes the absorbance and *X* denotes the concentration of the standard solution.

High performance liquid chromatography analysis

The extractions were analyzed with high performance liquid chromatography (HPLC). Chromatographic analyses were carried out on Shimadzu HPLC system (Japan) with an RP-18 endcapped column (4.6 × 250 mm, 5 μm). The mobile phase was methanol (A) / 0.5% acetic acid-water (B), from 0.1 to 10 min, 65% B; 10 to 25 min, 50% B; 25 to 50 min, and 25% B; 50 to 60 min, 10% B. The flow rate was maintained at 1 mL/min. Injection was performed *via* a 20-μL injector. Sample separation was monitored using a PDA detector at 280 nm and 360 nm.

Assay of the monophenolase activity and diphenolase activity

The extract was dissolved in dimethyl sulfoxide solution at 50, 200, or 500 μg/mL. Then, 100 μL of L-tyrosine (1 mM) or L-dopa (1 mM) were added to wells of a 96-well plate, and 70 μL of different concentrations of extract solution were added. The reaction was incubated for 2 min at 30 °C; 30 μL of mushroom tyrosinase was added to the first and second mixture solutions, and the mixture was monitored with a microplate reader at 492 nm from 1 to 30 min.

Kinetic analysis for inhibition

A Lineweaver–Burk plot (Lineweaver *et al*. 1934) was drawn using Eq. 3. With different concentrations of substrate, the reaction velocities were determined by using 1/[*S*] as the abscissa and 1/*V* as the ordinate to produce a straight line, from which was then calculated the K_m and V_{max} values. Thus, the inhibition mode was assayed.

$$\frac{1}{V} = \frac{K_m}{V_m} \times \frac{1}{S} + \frac{1}{V_m} \quad (3)$$

In Eq. 3, V_m is the biggest reaction velocity of enzyme, *S* is the concentration of substrate, K_m is the Michaelis constant, and *V* is the initial velocity of the enzymatic reaction.

UV-visible spectroscopy measurements

A total of 100 μL of the inhibition samples was added to three wells of a 96-well plate: 100 μL of mushroom tyrosinase was added to the first well, 100 μL of $CuSO_4$ (0.125 mM) was added to the second well, and 100 μL of the phosphate buffer solution as added to the third well. The reaction was incubated for 10 min at 30 °C, then monitored with a microplate reader at 230 to 600 nm.

RESULTS AND DISCUSSION

Response Surface Analysis of the Decompressing Inner Ebullition Conditions

Box-Behnken design (BBD) (Ferreira *et al*. 2007) is an analytical method for the optimization of processes. Decompressing inner ebullition (Chen *et al*. 2012) is a new extract method that occurs under decompressing conditions. By reducing the extraction liquid boiling point, the oxidation of active ingredients and macromolecular substances in the extraction process can be reduced.

Using the tyrosinase activity inhibition rate (*Y*) of extracts from the *Rhus verniciflua* Stokes bark as the evaluation index, Box-Behnken design was used to optimize the major extract parameters: vacuum degree (X_1), ethanol concentration (X_2), and time (X_3). The experimental matrix for the three factors consisted of 17 experiments, as shown in Table 1. The experimental results calculations were performed using Design-Expert software version 7.1.3. A classical second-degree model was obtained, as follows. According to the regression model equations, the fitting coefficient of the three variables show values of 1.21 > 0.55 > 0.011, implying that the ethanol concentration and vacuum degree were the main variables in the tyrosinase activity inhibition rate of extract by decompressing inner ebullition.

$$Y = 41.65 + 0.55X_1 + 1.21X_2 + 0.011X_3 + 1.33X_1X_2 - 0.19X_1X_3 - 1.80X_2X_3\text{-}1.09X_1^2 - 5.08X_2^2 - 1.22X_3^2 \quad (4)$$

Table 1. Response Surface Design and Experimental Results

Experiment No.	Vacuum degree, MPa(*X*1)	Ethanol concentration, %(*X*2)	Times, hours(*X*3)	*Y*
1	0.08	90	0.5	38.50
2	0.08	80	1	41.40
3	0.09	80	0.5	39.5
4	0.07	90	1	34.70
5	0.08	70	1.5	35.80
6	0.07	80	1.5	39.58
7	0.08	80	1	42.00
8	0.08	70	0.5	32.8
9	0.08	90	1.5	34.3
10	0.09	80	1.5	39.76
11	0.08	80	1	41.20
12	0.08	80	1	42.15
13	0.07	70	1	34.62
14	0.07	80	0.5	38.55
15	0.09	90	1	39.00
16	0.08	80	1	41.50
17	0.09	70	1	33.61

The statistical parameters obtained from the analysis of variance for the reduced models are given in Table 2. For the models, $p < 0.001$, imply that the models are significant and that the models can predict the real experimental data. For the pure error, $p > 0.05$, imply that the calculated values fit with the experimental values. The X_1 and X_2 variables had a significant effect on inhibition rate. For the X_1X_2 interaction, $p < 0.05$, imply that the vacuum degree was closely related to ethanol concentration in inhibition rate. For the X_2X_3 interaction, $p < 0.05$, imply that ethanol concentration was closely related to the time in inhibition rate. The results were in agreement with the response surface figure (Fig. 1). When the vacuum degree was low, increasing ethanol concentration could effectively improve the inhibition rate.

After applying the BBD to determine the best inhibition rate by the decompressing inner ebullition, the optimal extraction conditions were a vacuum degree of 0.084 MPa, an ethanol concentration of 82%, and a time of 60 min. Under these conditions, the inhibition rate was 41.5%.

Table 2. Analysis of Variance (ANOVA) of the Response Y of the Box-Behnken Design

Source of variation	Sum of squares	df	Mean square	*F* value	*P* value	Significance test
model	161.09	9	17.9	56.44	<0.0001	**
X1	2.44	1	2.44	7.70	0.0275	*
X2	11.69	1	11.69	38.86	0.0005	*
X3	1.013E-003	1	1.013E-003	3.193E-003	0.9565	
X12	7.05	1	7.05	22.23	0.0022	*
X13	0.15	1	0.15	0.48	0.5162	
X23	12.96	1	12.96	40.87	0.0004	*
X12	4.96	1	4.96	15.63	0.0055	*
X22	108.77	1	108.77	342.98	<0.0001	**
X32	6.24	1	6.24	19.68	0.003	*
Residual	2.22	7	0.32			
Lack of fit	1.56	3	0.52	3.15	0.1483	
Pure error	0.66	4	0.16			
C or total	163.31	16				

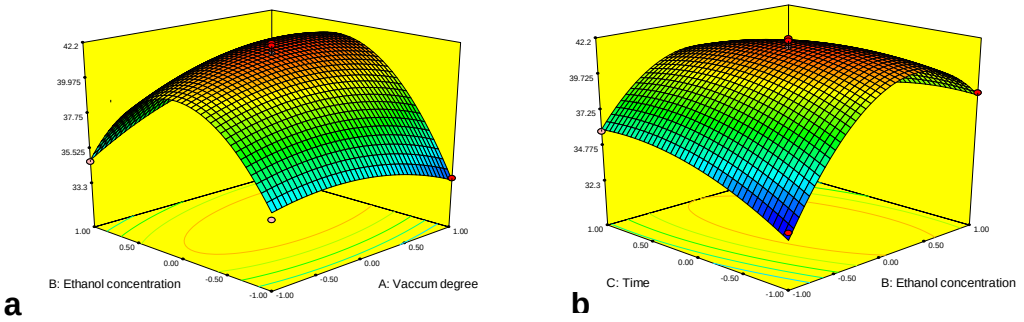

Fig. 1. The response surface figure of the extract in the tyrosinase activity inhibition rate; (a) is X_1X_2, and (b) is X_2X_3)

Composition and the Inhibition Rate of the Extracts

Since the chemical compositions have different solubilities in the solvent, partitioning was carried out with petroleum ether, ethyl acetate, and n-butanol, obtaining a four-partitioned portion. The total phenolic content, main chemical composition, and inhibition rates are shown in Table 3. The ethyl acetate fraction exhibited the highest total phenolic content and inhibition rate. The petroleum ether and aqueous fraction showed the lowest total phenolic content and inhibition rate. The polyphenolic components, which can be considered as derivatives of phenol, are known to have many physiological functions, and they are also sources of natural tyrosinase inhibitors. They are abundant in the ethyl acetate fractions of plant extracts (Rao *et al*. 2008; Kang *et al*. 2012). Polyphenolic constituents in the ethyl acetate fractions may contribute to the high anti-tyrosinase activity. Flavonoids are one of the most numerous and best-studied groups of plant polyphenols; they appear to be the main natural source of the tyrosinase inhibitor (Kubo and Kinst 1999). In the present study, the main flavonoids of the ethyl acetate fraction as determined by HPLC (Fig. 2) were 0.531% fisetin (eluting at 32 min), 7.582% fustin (eluting at 12 min), 0.848% sulfuretin (eluting at 36 min), and 0.272% butein (eluting at 38 min). Fisetin is a typical active of the *Rhus verniciflua* Stokes, which displays a variety of biological effects

including antioxidant, anti-flammatory (Woodman *et al*. 2004), anti-carcinogenic, and *in vitro* antiangiogenesis (Fotsis *et al*. 1997).

Table 3. Total Phenolic Content, Main Flavonoid Content, and Inhibition Rate of Extracts from *Rhus verniciflua* Stokes Bark

Sample	Extract (%)	Petroleum ether (%)	Ethyl acetate (%)	N-butanol (%)	Aqueous (%)
Total phenolic	30.46	2.54	53.43	15.48	9.20
Fisetin	0.396	0.062	0.531	0.059	-
Fustin	3.378	0.567	7.528	0.494	-
Sulfuretin	0.475	0.152	0.848	0.074	-
Butein	0.143	0.869	0.272	0.047	-
Inhibition rate	40.8	12.82	63.4	30.0	3.0

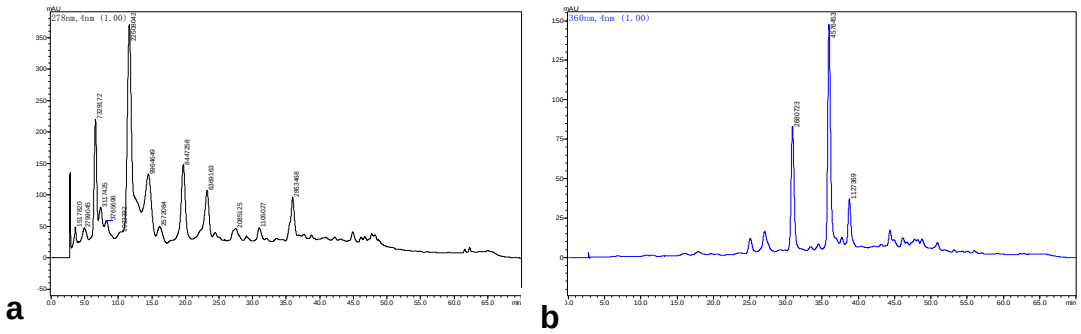

Fig. 2. The ethyl acetate extract from *Rhus verniciflua* Stokes by HPLC; (a) 280 nm and (b) 360 nm

Inhibitory Effects of the Ethyl Acetate Extract from *Rhus verniciflua* Stokes on Tyrosinase

Figure 3 shows the inhibitory rate of the different concentrations of the ethyl acetate extract. The inhibitory effects depend on the concentration of the ethyl acetate extract; when the concentration increased, the inhibition rate also increased. Through linear fitting, the fitting equation was obtained in Eq. 5,

$$Y = 0.001X + 0.192 \quad (5)$$

where Y is the inhibition rate of the ethyl acetate extract from *Rhus verniciflua* Stokes (%) and X is the concentration of the ethyl acetate extract (μg/mL).

The coefficient of determination was 0.995, showing that linear fitting was effective. According to the equation, the calculated value of IC_{50} of the ethyl acetate extracts was 308 μg/mL.

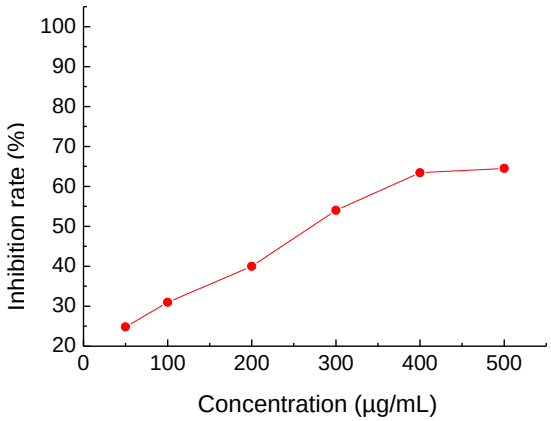

Fig. 3. Inhibitory effects of the ethyl acetate extract from *Rhus verniciflua* Stokes on tyrosinase

The reaction with 500 μg/mL of the extract, 100 μL of L-tyrosine (1mmol/L), and 30 μL of mushroom tyrosinase was monitored with a microplate reader at 230 to 600 nm from 2 to 12 min at 30 °C to produce the UV-vis spectra shown in the Fig. 4. In the process of melanin synthesis, tyrosinase is mainly involved in two types of reactions: the hydroxylation of tyrosine by monophenolase action and the oxidation of 3,4-dihydroxyphenylalanine (L-DOPA) to o-dopaquinone by diphenolase action. The peak at 320 nm is dopaquinone, and that at 492 nm is dopachrome. From the figure, one can find that the dopachrome increased with time. With the added the extract, the dopachrome generated more slowly, indicating that the ethyl acetate extract had an inhibitory effect on the formation of the dopachrome.

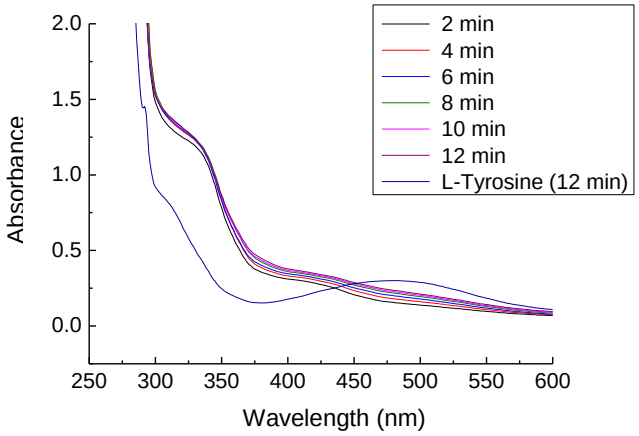

Fig. 4. Consecutive UV-vis spectra obtained in the oxidation of 1 mM L-tyrosine by mushroom tyrosinase in the absence and presence of 200 μg/mL extract

Effects of Extract on the Monophenolase Activity of Mushroom Tyrosinase

During enzyme catalysis, monophenolase exhibits a hysteresis effect due to the larger steric hindrance in the hydroxylation process on the monophenolase by the tyrosine. In order to study the effects of the extracts on the monophenolase activity, the inhibition kinetics of mushroom tyrosinase by extract were studied. Figure 5 shows the kinetic progression of L-tyrosine oxidation by tyrosinase in the presence of different

concentrations of extract. Monophenolase activity is typically characterized by a lag time which is dependent on factors such as substrate and enzyme concentrations, and presence of a hydrogen donor (Cooksey *et al*. 1997; Sanjust *et al.* 2003). The lag period increased markedly with an increase in the concentration of extract, as shown in Fig. 6. The lag period was estimated to be 2.45 min in the absence of this inhibitor and extended to 9.63 min in the presence of 500 μg/mL of extract. After the lag time, the system reached a steady state. The steady-state rate (V_{ss}) was observed to decrease with increasing extract concentration. If the ethyl acetate extract is present in the catalytic system, it will make it difficult for the substrate to combine with the active site of the enzyme. The results showed that the ethyl acetate extract had a dose-dependent inhibitory effect on monophenolase.

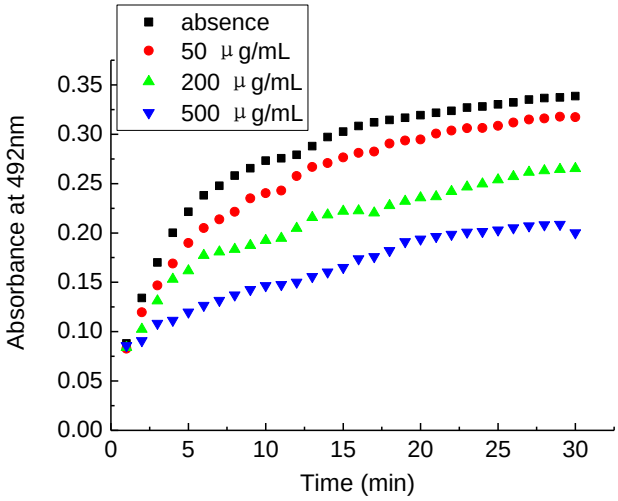

Fig. 5. Catalytic reaction progress of monophenolase of tyrosinase at various concentrations of extract

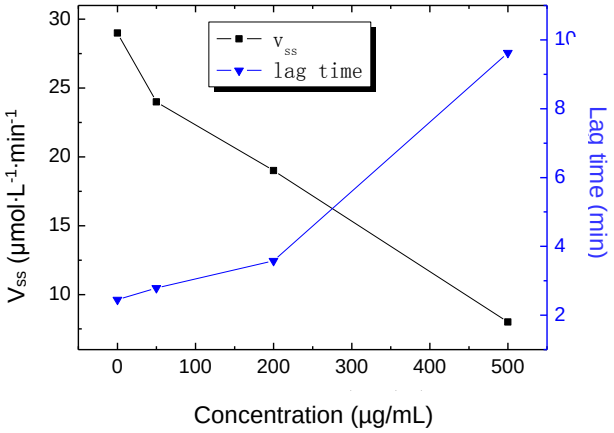

Fig. 6. Effect of extract on catalytic lag time and steady state velocity of monophenolase of tyrosinase

Effects of Extract on the Diphenolase Activity of Mushroom Tyrosinase

According to the tyrosinase inhibitor impact on the enzyme active site, the type of inhibiting can be divided into competitive inhibition, noncompetitive inhibition, and hybrid inhibition (Fang *et al*. 2011; Kim *et al*. 2005; Ohguchi *et al.* 2003). The inhibitory effects

of the different concentrations of the ethyl acetate extract on the oxidation of L-DOPA by tyrosinase were studied to determine inhibition type. The inhibitory type of the extract on the diphenolase activity was determined from Lineweaver-Burk plots. In the presence of extract, the kinetics of the enzyme are shown in Fig. 6 and Table 4. The plots of 1/V *versus* 1/[S] with the application of linear regression are shown in Fig. 7. The K_m and V_{max} values can be obtained from the slopes and the intercepts, respectively. The value of K_m increased and the value of V_{max} decreased with increasing concentration of the extract. The results demonstrated that the extract behaved as a mixed type inhibitor through kinetics studies.

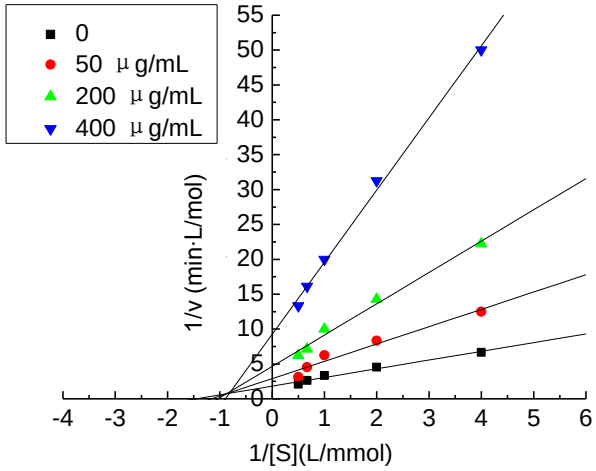

Fig. 7. Lineweaver-Burk plot for inhibition to diphenolase of tyrosinase by extract

The equilibrium constants for the inhibitor binding with the free enzyme (K_I) and with enzyme substrate complex (K_{IS}) were obtained from the slope or the vertical intercept versus the inhibitor concentration, respectively (Table 5). In this study, the K_I and K_{IS} of the ethyl acetate extract were determined to be 75.94 and 137.29 μg/mL, respectively. The value of K_I was less than that of K_{IS}, which demonstrated that the [ESI] exhibited lower stability than [EI]. In the enzyme catalytic reaction, the extract easily combined with free enzyme, which affected the enzyme catalysis on the substrate. Plant polyphenols are referred to as a diverse group of compounds containing multiple phenolic functionalities. Some flavonoids, such as kaempferol, quercetin, morin, and fisetin, show the inhibitory activity of tyrosinase, act as cofactors or substrates of tyrosinase (Kubo *et al*. 1999, 2000; Kermasha *et al*. 2001; Xie *et al*. 2003). They have the ability to chelate copper in the active center of the enzyme.

Table 4. Lineweaver-Burk Equation for Inhibition to Tyrosinase by Extract

Extract concentration (μg/mL)	Fitting equation	R^2
0	Y=1.248x+1.813	0.979
50	Y=2.482x+2.895	0.960
200	Y=4.489x+4.647	0.988
500	Y=10.34x+9.248	0.996

Table 5. Kinetic Parameters of Inhibition to Tyrosinase by Extract

Extract concentration (μg/mL)	K_m (μg/mL)	V_m (μmol/mL/min)	K_I (μg/mL)	K_{IS} (μg/mL)
0	0.69	0.55	75.94	137.29
50	0.87	0.35		
200	0.99	0.22		
500	1.14	0.11		

Tyrosinase on the UV Visible Spectrum of the Ethyl Acetate Extract

Tyrosinase is a copper II metalloproteinase, with two dinuclear copper ions, located on the spiral beam of the active center. The region is highly conserved, and plays an important role in the tyrosinase catalysis process (Matoba *et al*. 2006). If the inhibitors chelate Cu^{2+}, it can enhance the conjugative effect of the inhibitor and provide a low energy trajectory. This is enabled through the combination of the molecular orbital and the decrease in total energy of the molecule. The energy difference between the low energy orbital also decreases. In the ultraviolet chromatogram, the wavelength of maximum absorption of the inhibitors will exhibit redshift. The excessive Cu^{2+} added in the flavonol, the inhibitor chelated with Cu^{2+}, the characteristic absorption peaks of the flavonol were moved toward longer wavelength (Kim *et al*. 2006b). According to this feature, the effect of Cu^{2+} and tyrosinase on the UV-visible spectrum of the ethyl acetate extract are shown in Fig. 8. The maximum absorption peaks of the ethyl acetate extract were at 320 nm and 420 nm. After adding excess Cu^{2+} and tyrosinase, the characteristic absorption peak did not change, demonstrating that the extract did not chelate Cu^{2+}.

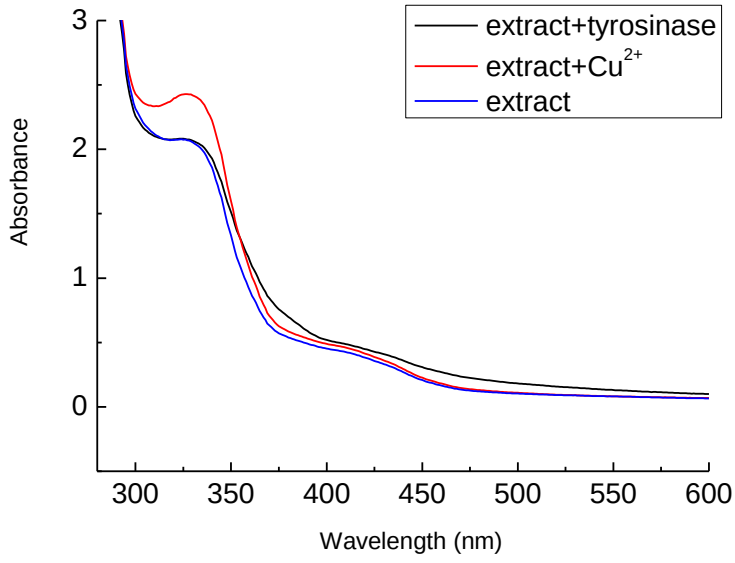

Fig. 8. Effect of Cu^{2+} and tyrosinase on the UV-visible spectrum of the ethyl acetate extract

CONCLUSIONS

1. The optimal extraction conditions using decompressing inner ebullition by a Box-Behnken design were a vacuum of 0.084 MPa, an ethanol concentration of 82%, and a time of 60 min. Under these conditions, the inhibition rate was 41.5%.
2. The ethyl acetate fraction had the highest total phenolic content (53.43%) and inhibition rates (63.4%). The main flavonoids in this fraction were 0.531% fisetin, 7.582% fustin, 0.848% sulfuretin, and 0.272% butein.
3. The extract inhibited both the monophenolase and diphenolase activity of the enzyme. The IC_{50} of the ethyl acetate extract was 308 μg/mL. The extract acted as a mixed type inhibitor by Lineweaver-Burk plots. An extract of 500 μg/mL resulted in the extension of lag time from 2.45 min to 9.63 min.
4. From this work, it can be concluded that there are active compounds in the ethyl acetate extract, which had high anti-tyrosinase activity. Therefore, we can isolate the compounds in future studies and work to develop the natural tyrosinase inhibitor.

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