American Journal of Analytical Chemistry, 2018, 9, 1-14
http://www.scirp.org/journal/ajac
ISSN Online: 2156-8278
ISSN Print: 2156-8251

Biofunctionality Studies of *Cudrania cochinchinensis* Extracts

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How to cite this paper: Liao, W.C., Lin, C.-F., Lin, C.-C., He, R.-F., Chen, C.-C. and Huang, W.-Y. (2018) Biofunctionality Studies of *Cudrania cochinchinensis* Extracts. *American Journal of Analytical Chemistry*, **9**, 1-14.
https://doi.org/10.4236/ajac.2018.91001

Received: October 3, 2017
Accepted: January 1, 2018
Published: January 4, 2018

Abstract

Cudrania cochinchinensis has been found to show remarkable medicinal values. The total phenolic and flavonoid contents of *C. cochinchinensis* extracts were analyzed, and the antioxidant activity and reducing ability of *C. cochinchinensis* extracts were also evaluated. Tetrahydroxyflavanonol (THF) was isolated from the xylem and pith portions of *C. cochinchinensis* stem; however, the bark portion of *C. cochinchinensis* stem was found to contain no THF. Consequently, solutions extracted from the xylem and pith portions of *C. cochinchinensis* showed good antioxidant activity. The IC_{50} values of pith, xylem, and bark extracts were 0.779, 3.020, and 3.507 mg/mL, respectively. As the pith portion of *C. cochinchinensis* stem contained more THF and had a higher flavonoid content, it exhibited better antioxidant activity and reducing ability. In addition, *C. cochinchinensis* pith extracts reduced tyrosinase activity in a dose-dependent manner with IC_{50} = 16.1 μg/mL. The inhibitory activity was determined to be noncompetitive with K_m = 0.23 mM.

Keywords

Cudrania cochinchinensis, Tetrahydroxyflavanonol, Antioxidant Activity, Reducing Ability, Tyrosinase

1. Introduction

Cudrania cochinchinensis has shown remarkable medicinal values [1] [2] [3]. Because leaf and root extracts of *C. cochinchinensis* have shown good biofunctionality, it has been used as a folk medicine in oriental countries [4] [5]. Al-

though flavonoids, prenylated xanthones, and other active compounds have been isolated from *C. cochinchinensis* [4] [6] [7], studies on *C. cochinchinensis* have focused primarily on its roots. To date, no study has been reported on active compounds found in the xylem and pith portions of *C. cochinchinensis* stem.

Extracts of *C. cochinchinensis* stem have shown good tyrosinase inhibitory ability [8]. This inhibitory activity of tyrosinase (EC 1.14.18.1) has been extensively studied in the past [9] [10] [11] [12] [13]. Tyrosinase catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA), which forms DOPAchrome [14]. These catalyzed reactions result in the formation of melanin, which is responsible for the pigmentation of skin [15]. Natural medical plants are considered to be a good source of tyrosinase inhibitors [16]. Zheng *et al.* (2011) used 95% ethanol to extract *C. cochinchinensis,* and the extracted solutions showed good tyrosinase inhibitory ability [8].

Although *C. cochinchinensis* extracts have shown the ability to inhibit tyrosinase activity, its inhibitory mechanism has not been studied. Generally, enzyme inhibitors are classified into competitive or noncompetitive inhibitors [17]. A Lineweaver-Burk plot (Equation (1)), obtained by plotting the inverse values of reaction rate (V) and substrate concentration [S], can be used to determine the inhibitory activity.

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \times \frac{1}{[S]} + \frac{1}{V_{\max}} \tag{1}$$

The linear regression model applied to the double-reciprocal plot can be used to determine the Michaelis constant (K_m) and maximum velocity ($V_{\max}$). The x-intercept represents $-1/K_m$, the y-intercept represents $1/V_{\max}$, and the slope of the straight line represents $K_m/V_{\max}$. Based on the Lineweaver-Burk plot, the inhibitory activity can be determined to be competitive or noncompetitive.

The aim of this study is to analyze the antioxidant activities and tyrosinase inhibitory activity of bark, xylem, and pith extracts of *C. cochinchinensis*. In addition, the kinetic study on the inhibitory activity of *C. cochinchinensis* extracts was determined based on the Lineweaver-Burk plot.

2. Materials and Methods

2.1. Materials

C. cochinchinensis samples were collected from Taiwanese mountain areas, air dried, and then kept in a cool place for further use. Liquiritin was purchased from ChromaDex (Santa Ana, CA, USA). Methanol, acetonitrile, ethyl acetate (EtOAc), and n-hexane were purchased from Merck (Darmstadt, Germany). DPPH (1, 1-diphenyl-2-picrylhydrazyl), potassium ferricyanide (III) [$K_3Fe(CN)_6$], potassium dihydrogenphosphate, dipotassium hydrogenphosphate, phosphoric acid, trichloroacetic acid, iron (III) chloride, butylated hydroxyanisole (BHA), and ascorbic acid were purchased from Sigma (St. Louis, MO, USA). L-3,4-dihydroxyphenylalanine (L-DOPA), kojic acid, and dimethyl sulfoxide

(DMSO) were purchased from Acros Organics (Fair Lawn, NJ, USA). Sodium phosphate dibasic anhydrous was purchased from J. T. Baker (Petaling Jaya, Selangor, Malaysia).

2.2. Preparation of *C. cochinchinensis* Extracts

A sample of pulverized *C. cochinchinensis* (1.0 g) was sonicated in an ultrasonic bath (Chrom Tech, Taipei, Taiwan) for 20 min with 7 mL of 70% methanol (methanol/H_2O = 7/3, v/v). The suspension was centrifuged at 6000 rpm (HERMLE Z206A, Germany) for 10 min. The supernatant was collected and run through a 0.45 μm filter. The residual solids were extracted with fresh 70% methanol. After the *C. cochinchinensis* sample was extracted three times, all the collected supernatants were mixed together. Subsequently, 70% methanol was used to make up the total volume to 20 mL. Moreover, 1.0 mg liquiritin was dissolved in 10 mL of 70% methanol and used as the internal standard (IS) solution. Before performing the HPLC analysis, 100 μL of the extracted solution was mixed with 100 μL of the IS solution.

Tetrahydroxyflavanonol (THF) was isolated from *C. cochinchinensis* stem by Chen *et al.* [18], based on the modified method described by Kobayashi *et al.* [19]. Dried *C. cochinchinensis* samples were extracted four times by methanol under reflux. The extract was partitioned using a mixture of EtOAc and water (1:1, v/v). The EtOAc extract was run through a silica gel column, and then eluted with a mixture of n-hexane and EtOAc/MeOH. Eight fractions were collected during elution. Fraction number 3 was further separated by high performance liquid chromatography (HPLC) using a Cosmosil 5C18-AR column (Nacalai Tesque, Tokyo, Japan) to obtain THF.

2.3. HPLC Method

HPLC analysis was performed on an Agilent 1200 system with a reverse phase column (Cosmosil 5C18-AR II, 5 μm, 25 cm × 4.6 mm I.D.; Nacalai Tesque, Kyoto, Japan). The detection wavelength was set at 254 nm. The flow rate was 0.8 mL/min with a linear solvent gradient of A-B (A = 10 mM KH_2PO_4, pH 4.6; B = $CH_3CN/CH_3OH/H_2O$, 1.5/2.5/1, v/v/v). as follows: 0 min, 40% B; 10 min, 40% B; 20 min, 60% B; 30 min, 70% B; and 50 min, 100% B.

2.4. Analysis of Total Phenolic and Flavonoid Contents

The total phenolic content was measured following the method described by Singleton *et al.* [20]. An amount of 200 μL of different concentrations of samples was mixed with 200 μL of 0.5 N Folin-Ciocalteu reagent, to which 200 μL of 10% (w/v) Na_2CO_3 and 40 μL of distilled water were added. The mixture was incubated at room temperature for 1 h in the dark. After incubation, the mixture was centrifuged at 5000 rpm for 10 min. An amount of 200 μL of the supernatant was transferred to a 96-well plate and the absorbance of each well was measured using an ELISA reader at a wavelength of 700 nm. Gallic acid was used as a posi-

tive control. Each measurement was performed at least in duplicate.

The flavonoid content was measured according to the method described by Chandra *et al.* [21]. Different concentrations of samples (50 μL) were mixed with 100 μL of 10% (w/v) $AlCl_3$. The mixture was incubated at room temperature for 10 min in the dark. The absorbance of the mixture at 430 nm wavelength was measured using an ELISA reader. Quercetin was used as a positive control. Each measurement was performed at least in duplicate.

2.5. Analysis of Antioxidant Activity

Radical scavenging activities of THF marker standards and *C. cochinchinensis* extracts were measured respectively using the methods of Singh and Rajini and Chan *et al.* and Azman *et al.* [22] [23] [24]. The radical scavenging activity of ascorbic acid, used as a positive control, was also measured. The sample (50 μL) was mixed with 50 μL of freshly prepared 160 μM DPPH in ethanol. The mixture was kept in the dark for 30 min. The absorbance of the mixture at 517 nm wavelength was measured using an ELISA reader (TECAN^R, Austria). Each measurement was performed at least in duplicate. The radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity } (\%) = \left(1 - \frac{A_{\text{Sample}}}{A_{\text{Blank}}}\right) \times 100\% \qquad (2)$$

where A_{Sample} and A_{Blank} represent the absorbance of sample and blank solution, respectively.

The reducing ability of the samples was measured following the method described by Canabady-Rochelle *et al.* [25]. Samples of different concentrations (100 μL each) were individually mixed with 100 μL of 1% (w/v) $K_3Fe(CN)_6$ and 100 μL of 2 mM phosphate buffer (pH 6.6). The mixture was incubated at 50˚C for 20 min. After incubation, 100 μL of 10% (w/v) trichloroacetic acid was added to it, and the mixture was centrifuged at 3000 rpm for 2 min. An amount of 100 μL of the supernatant was transferred to a 96-well plate. Each well contained 100 μL of distilled water and 20 μL of 0.1% (w/v) $FeCl_3$ solution. BHA was used as a positive control. The absorbance of each well was measured using an ELISA reader at 700 nm wavelength. Each measurement was performed at least in duplicate.

2.6. Analysis of Tyrosinase Inhibition Activity

An amount of 20 μL of extracted *C. cochinchinensis* pith solution (500 μg/mL, in 3.3% of DMSO) was placed in a 96-well plate. Then, 40 μL of tyrosinase solutions of various concentrations (0.277, 0.554, 1.662, 3.324, and 6.648 μg/mL) and 0.1 mM of L-DOPA solution (dissolved in a sodium phosphate buffer at pH 6.8) were added to it.

Another 20 μL of extracted *C. cochinchinensis* pith solution (31.25, 62.5, 125, 250, and 500 μg/mL, in 3.3% of DMSO) was placed in a 96-well plate, to which 40 μL of tyrosinase solution (6.648 μg/mL) and 0.1 mM of L-DOPA solution

(dissolved in a sodium phosphate buffer at pH 6.8) were added. These mixed solutions were kept at room temperature (25˚C) for 25 min. The absorbance was measured at 475 nm [12] [26] using the Microplate-Reader (Sunrise Basic, Grödig, Austria). Kojic acid was used as a positive control. The tyrosinase inhibition rate (%) was calculated from the following equation:

$$\text{The inhibition rate } (\%) = \left(1 - \frac{\text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}}\right) \times 100\% \quad (3)$$

The absorbance of sample ($\text{OD}_{\text{sample}}$) and control ($\text{OD}_{\text{control}}$) was measured at 475 nm. The IC_{50} value was determined by regression of a constructing dose-response curve at which 50% target activity was lost.

In a 96-well plate, 20 μL of extracted *C. cochinchinensis* pith solution (7.8125, 15.625, 31.25, and 62.5 μg/mL, in 3.3% of DMSO) was placed, to which 40 μL of tyrosinase solutions (6.648 μg/mL) was added. The substrate was L-DOPA solution, which was prepared by dissolving L-DOPA (0.1, 0.3, 0.5, 0.7, and 1.0 mM) in sodium phosphate buffer at pH 6.8. The Line weaver-Burk plot was obtained by plotting the inverse values of reaction rate (V) and concentration of L-DOPA (Equation (1)).

2.7. Statistical Analysis

Statistical evaluation was performed by running one-way analysis of variance (ANOVA) with SAS^{R} software (version 6.08, SAS Institute Inc., Cary, NC, USA). All data were presented as mean ± standard deviation (SD). Differences were considered to be statistically significant when the p-value was less than 0.05.

3. Results and Discussion

3.1. Total Phenolic and Flavonoid Content Analyses of *C. cochinchinensis* Extracts

The total phenolic content of *C. cochinchinensis* extracts is shown in **Figure 1(a)**. The pith extract contained more phenolic components than the xylem and bark extracts. No significant difference was observed in the total phenolic content between the bark and xylem extracts. Flavonoid contents of *C. cochinchinensis* extracts are shown in **Figure 1(b)**, which were in the order pith > xylem > bark. Thus, the pith portion of *C. cochinchinensis* stem was expected to have better antioxidant activity.

3.2. Antioxidant Activity Analysis of *C. cochinchinensis* Extracts

The DPPH radical scavenging activities of the extracts were measured as the decrease of absorbance at a wavelength of 517 nm, and the results are shown in **Figure 1(c)**. The DPPH radical scavenging activity of the THF standard is shown in **Figure 1(d)**. The IC_{50} value of the THF standard was 0.122 mg/mL and those of the pith, xylem, and bark extracts were 0.769, 2.809, and 3.34 mg/mL, respectively. The pith portion of *C. cochinchinensis* stem showed better DPPH

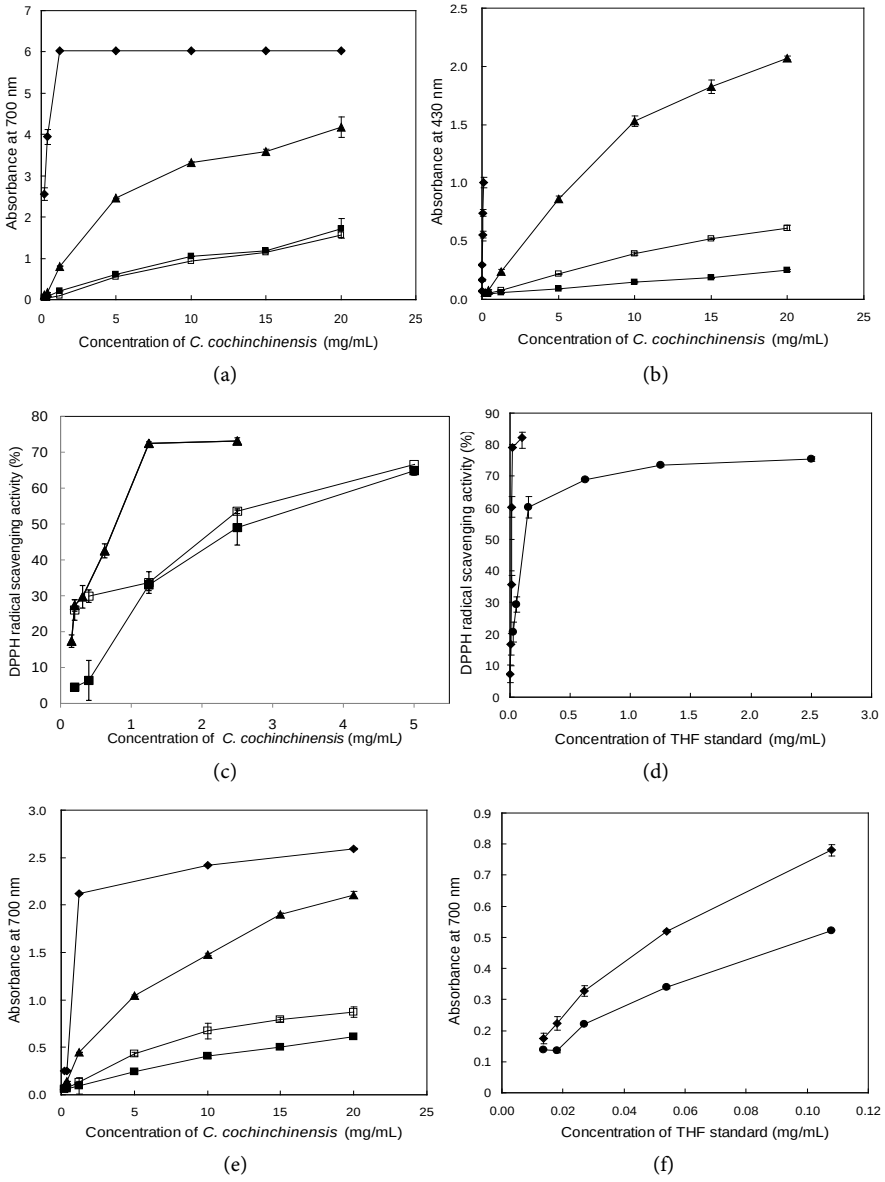


Figure 1. (a) Total phenolic content of *C. cochinchinensis* stem extracts (▪: bark; □: xylem; ▲: pith); (b) Flavonoid content of *C. cochinchinensis* stem extracts (▪: bark; □: xylem; ▲: pith); (c) DPPH radical scavenging activity of *C. cochinchinensis* stem extracts (◆: ascorbic acid; ▪: bark; □: xylem; ▲: pith); (d) DPPH radical scavenging activity of the standard (◆: ascorbic acid; ●: THF); (e) Reducing ability of *C. cochinchinensis* stem extracts (▪: bark; □: xylem; ▲: pith); (f) Reducing ability of the standard (◆: BHA; ●: THF).

radical scavenging activity. The reducing ability was measured as the change in absorbance at a wavelength of 700 nm. **Figure 1(e)** and **Figure 1(f)** show the reducing ability of *C. cochinchinensis* extracts and the THF standard, respectively. Generally, higher flavonoid contents result in better reducing ability. On the other hand, better reducing ability represents stronger antioxidant activity.

Figures 2(a)-(c) show the representative HPLC chromatograms of *C. cochinchinensis* stem extracts from the bark, xylem, and pith portions respectively. THF was well separated by HPLC with a retention time of 15 min. Based on

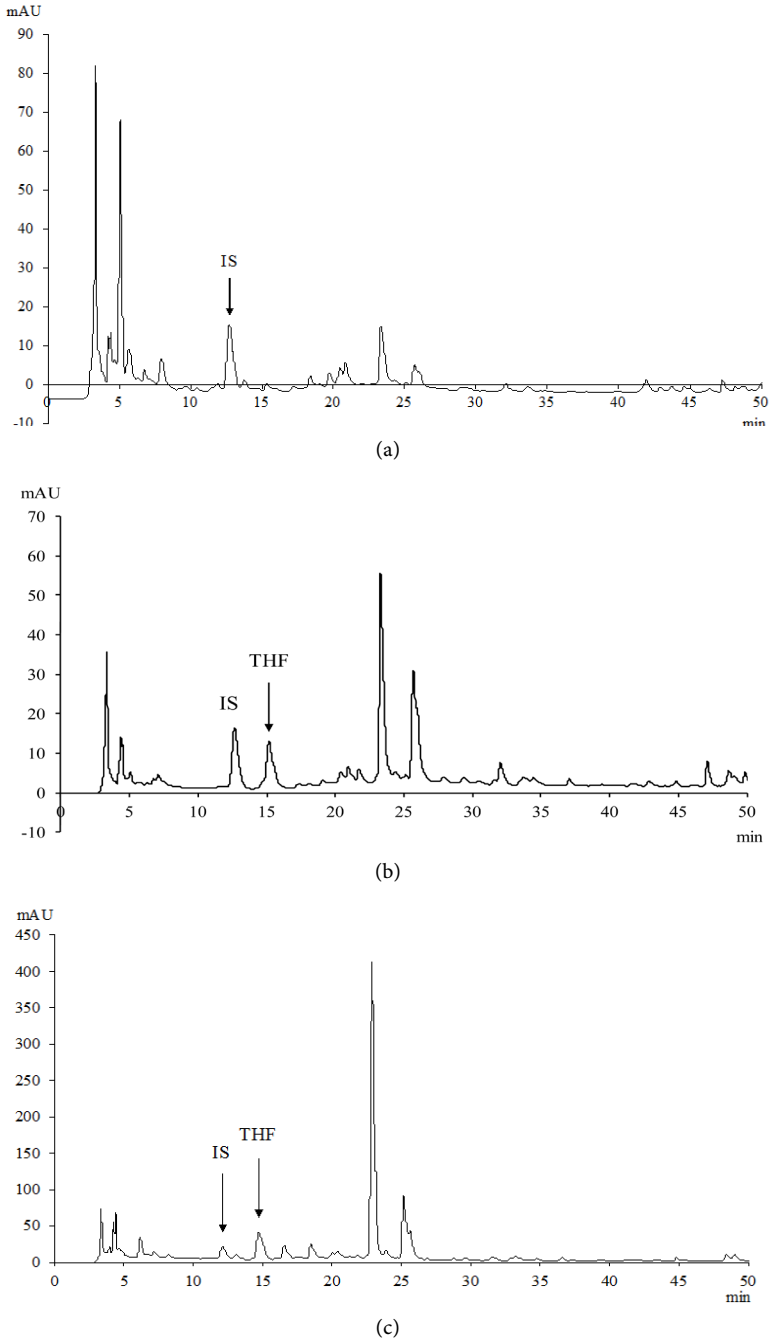


Figure 2. Representative HPLC (high performance liquid chromatography) chromatograms of *C. cochinchinensis* stem extracts: (a) bark; (b) xylem; (c) pith.

the chromatographic analysis results, THF was found only in xylem and pith extracts of *C. cochinchinensis* (691.5 ± 3.2 and 1,489.7 ± 5.5 μg/g, respectively). The THF content of bark extract was not detectable. The pith portion of *C. cochinchinensis* stem contained more THF than the xylem portion. Therefore, the pith extracts showed better radical scavenging activity and reducing ability. Although xylem and bark extracts had similar antioxidant results, only xylem extracts were found to contain THF. Bark extracts may contain some other ingre-

dients which may also provide antioxidant activity. More studies are required to explain why bark extracts had similar antioxidant activity without containing THF.

Derivatives of both flavonol and flavanone have been approved as good antioxidants [27]. Flavonol derivatives such as THF and quercetin have similar chemical structures (**Figure 3**). Multiple hydroxyl groups, especially on the B-ring, improve the antioxidant activity of flavonoids [28]. Likewise, flavanone derivatives such as tetrahydroxyflavanone and luteolin also have similar chemical structures (**Figure 3**). The difference between THF and tetrahydroxyflavanone is that THF has one extra hydroxyl group on the B-ring. Consequently, THF has better antioxidant activity [28]. Because the pith portion of *C. cochinchinensis* extracts contained more THF, pith extracts had a better reducing ability than other extracts. Likewise, the bark portion contained the least THF and had the worst reducing ability.

3.3. Tyrosinase Inhibitory Ability of *C. cochinchinensis* Pith Extracts

Pith extracts of *C. cochinchinensis* showed the ability to inhibit the formation of DOPA chrome, which can be detected with a spectrophotometer at a wavelength of 475 nm. When 0.1 mM of L-DOPA was used as the substrate, tyrosinase activity was increased with the addition of more tyrosinase. A linear relationship of the first-order was observed between the tyrosinase activity and tyrosine concentration (**Figure 4**). However, tyrosinase activity reduced with the addition of

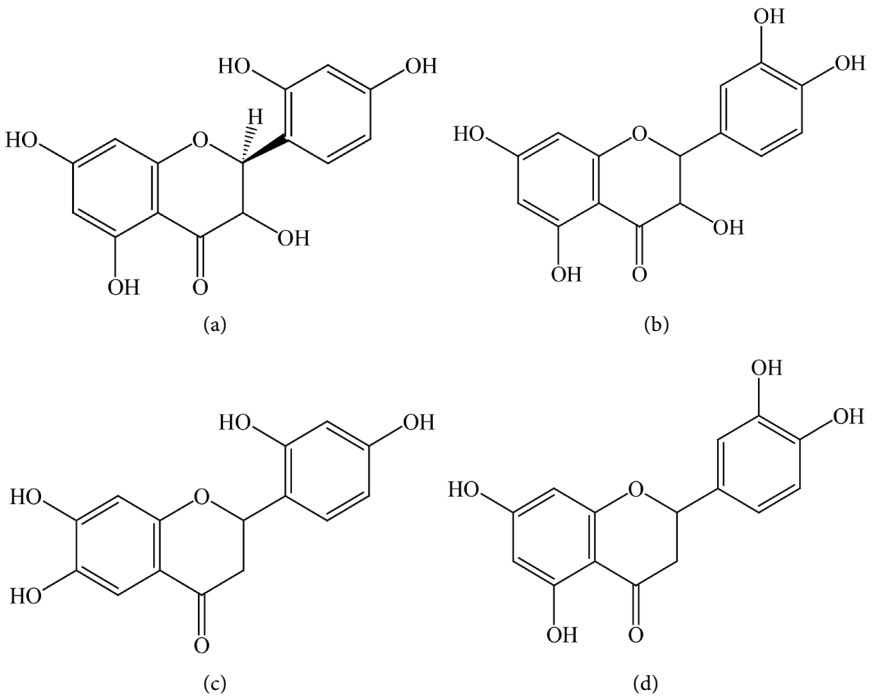


Figure 3. Chemical structures of some flavonoids: (a) THF; (b) quercetin; (c) tetrahydroxyflavanone; (d) luteolin.

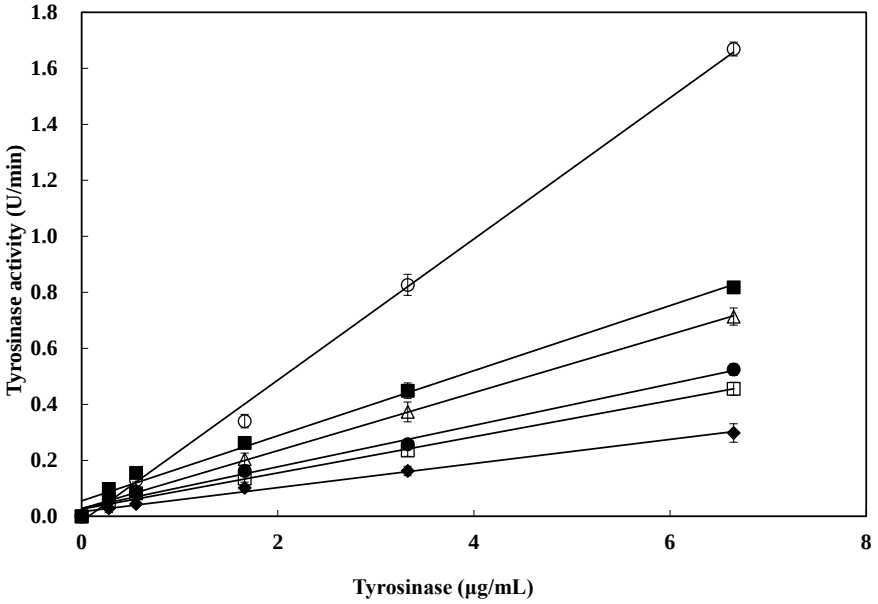
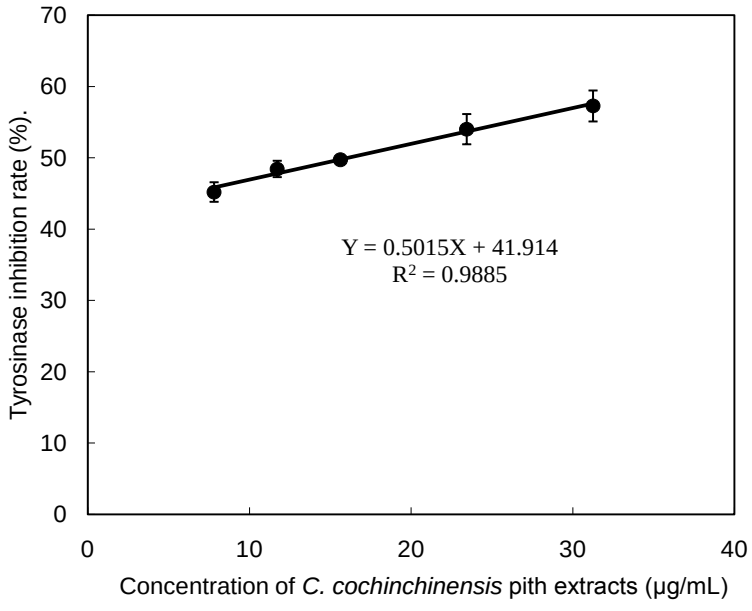


Figure 4. Influence of *C. cochinchinensis* pith extracts on tyrosinase activity when 0.1 mM of L-DOPA was used as the substrate (◯: 0; ▪:31.25; △: 62.5; ●: 125; □: 250; ◆: 500 μg/mL of *C. cochinchinensis* pith extracts).

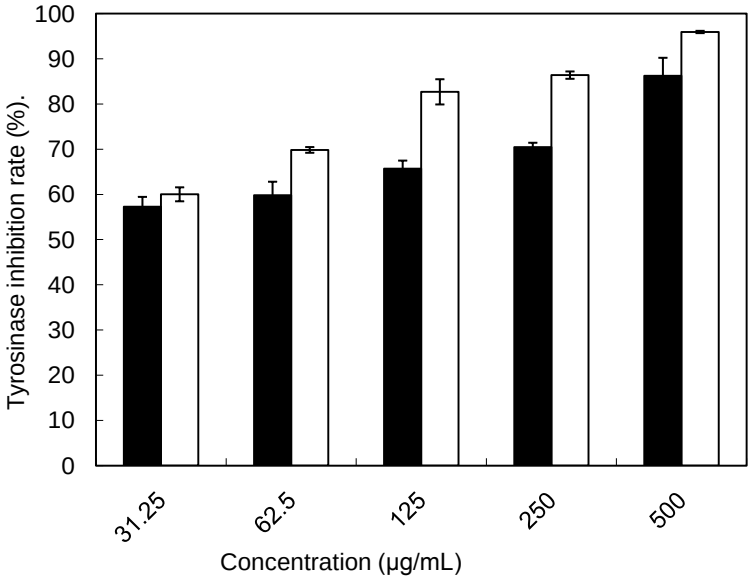
more *C. cochinchinensis* pith extracts. This confirmed that the extracted *C. cochinchinensis* pith solution could inhibit tyrosinase activity. In addition, the slope of the linear lines decreased with the addition of more *C. cochinchinensis* pith extracts (**Figure 4**).

The ability of *C. cochinchinensis* extracts to inhibit tyrosinase activity could be attributed to the presence of phenolics in the extracts. The IC_{50} value (36.3 μg/mL) of ethanol extracted *C. cochinchinensis* stem solution was reported by Zheng *et al.* [8]. In this study, the pith portion of *C. cochinchinensis* stem was further extracted with methanol. **Figure 5(a)** shows the inhibition rate of tyrosinase activity using *C. cochinchinensis* pith extracts, which reduced the tyrosinase activity in a dose-dependent manner. The slope and intercept of the linear regression line were 0.5015 and 41.914, respectively. The IC_{50} value of methanol extracted *C. cochinchinensis* pith solution was calculated to be 16.1 μg/mL. Comparing this result with the results reported by Zheng *et al.*, *C. cochinchinensis* pith extracts were found to exhibit a better inhibitory ability. **Figure 5(b)** shows a comparison of tyrosinase inhibitory rate between *C. cochinchinensis* pith extracts and kojic acid, which was used as a positive control. Based on **Figure 5(b)**, the inhibition rate of tyrosinase activity was 70.4% when 250 μg/mL of *C. cochinchinensis* pith extracts were added. This inhibition rate was close to that of kojic acid (70.5%), at a concentration of 62.5 μg/mL. Although the inhibitory ability of *C. cochinchinensis* pith extracts was approximately 25% of that of kojic acid, *C. cochinchinensis* extracts are natural ingredients and may possibly be used in cosmetic products.

In this study, it was required to determine whether the inhibitory activity of *C. cochinchinensis* pith extracts was competitive or noncompetitive. **Figure 6**



(a)



(b)

Figure 5. (a) Inhibition rate of tyrosinase activity using *C. cochinchinensis* pith extracts as the inhibitor (40 μL of tyrosinase solution (6.648 μg/mL) and 0.1 mM of L-DOPA solution were added for each measurement); (b) Inhibition rate of tyrosinase activity using *C. cochinchinensis* pith extracts as the inhibitor (□: Kojic acid as the positive control; ■: *C. cochinchinensis* pith extracts).

shows the Line weaver-Burk double reciprocal plot of *C. cochinchinensis* pith solutions. The substrate was L-DOPA. Based on **Figure 6**, the x-intercept ($-1/K_m$) remained the same but the y-intercept ($1/V_{max}$) increased with increasing concentrations of *C. cochinchinensis* pith extracts. As a result, K_m remained unchanged (0.23 mM), but V_{max} decreased by the introduction of an inhibitor. The binding of *C. cochinchinensis* pith extracts to tyrosinase had no effect on

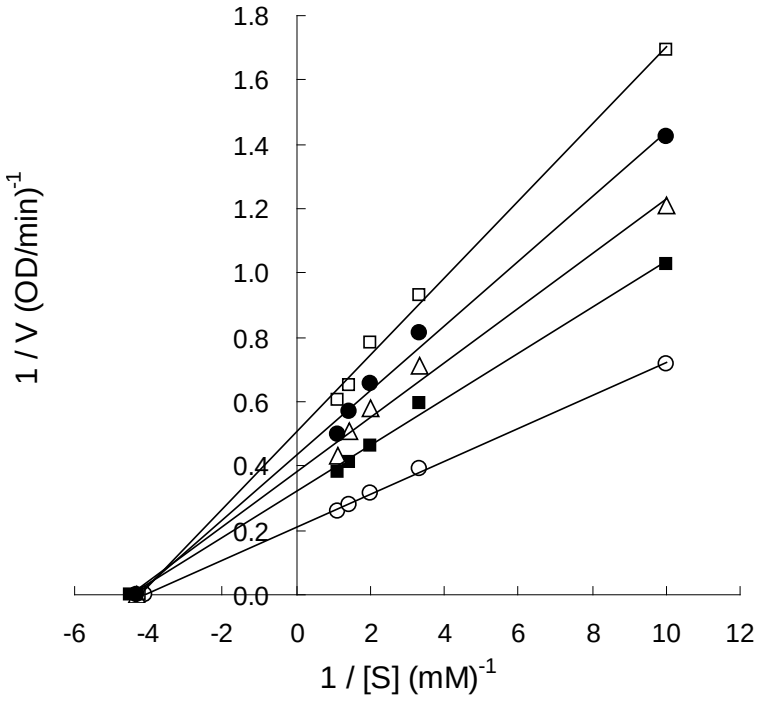


Figure 6. Lineweaver-Burk double reciprocal plot of extracted *C. cochinchinensis* pith solution (◯: 0; ▪:7.8125; △: 15.625; ●: 31.25; □: 62.5 μg/mL of *C. cochinchinensis* pith extracts; V: absorbance change rate, △OD_{475nm}/min; [S]: concentration of L-DOPA).

the binding of L-DOPA to tyrosinase. The binding sites of L-DOPA and *C. cochinchinensis* pith extracts to tyrosinase were different. Based on the Lineweaver-Burk double reciprocal plot shown in **Figure 6**, the inhibitory activity was determined to be noncompetitive.

4. Conclusion

The *C. cochinchinensis* extracts of bark, xylem and pith were shown different antioxidant activities. The pith extracts showed better antioxidant activity and higher reducing ability, which might because of the higher THF content. In addition, *C. cochinchinensis* pith extracts could reduce tyrosinase activity successfully. The IC_{50} value of *C. cochinchinensis* pith extracts was 16.1 μg/mL, and the tyrosinase inhibitory activity was determined to be noncompetitive. *C. cochinchinensis* pith extracts could be used in cosmetic formulations as a natural whitening agent. Based on our studies, the pith extracts of *C. cochinchinensis* stem contained THF and showed good whitening ability. The future study suggests using B16F10 murine melanoma cells to perform *in vivo* tests. Results can be used to verify the whitening ability of the pith extracts in cells.

Acknowledgements

The authors are grateful for the financial support of this study by the Ministry of Science and Technology, Taiwan China, under contract number MOST 104-2622-E-241-003-CC3.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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