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Research Article

STUDY OF TYROSINASE INHIBITORY ACTIVITY AND PHYTOCHEMICAL SCREENING OF *CASSIA FISTULA* L. LEAVES

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ABSTRACT

Objective: This study was carried out to evaluate the phytochemical constituents and tyrosinase inhibitory activity of *Cassia fistula* leaves.

Methods: A tyrosinase inhibitory activity assay was performed by measuring the decrease in the intensity of color suggestive of the inhibition of dopachrome formation resulting from the L-DOPA-tyrosinase reaction.

Results: The test results showed that the tyrosinase inhibitory activity of the water fraction of *C. fistula* leaf extract had the highest IC_{50} value (152.031 µg/mL) among other fractions (n-hexane, ethyl acetate, and n-butanol). An enzyme kinetic assay showed that the water fraction of *C. fistula* leaf extract inhibited tyrosinase with mixed-type inhibition. Phytochemical screening showed that the water fraction of *C. fistula* leaf extract contained alkaloids, flavonoids, glycosides, phenols, and tannins.

Conclusion: The current study indicated that *C. fistula* leaves possess significant tyrosinase inhibitory activity.

Keywords: *Cassia fistula,* L-DOPA, Phytochemical screening, Tyrosinase inhibitor.

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INTRODUCTION

Tyrosinase is a critical enzyme that has many catalytic functions in pigment production. It catalyzes the hydroxylation of monophenols to *o*-diphenols (cresolase or monophenolase activity) and *o*-diphenols to reactive *o*-quinones (catecholase or diphenolase activity) [1]. The activity of this enzyme is responsible for the pigmentation of skin and the browning of fruit and vegetables [2]. Although melanin has mainly a photoprotective function in human skin, a hyperpigmentary condition in visible parts of the skin presents esthetic issues [1]. Synthetic skin whitening agents, such as hydroquinone, arbutin, and kojic acid, are not sufficiently safe to use due to their adverse effects including irreversible cutaneous damage and ochronosis. These adverse effects have led to a search for skin whitening agents from natural sources [3].

Cassia fistula L. (family: Fabaceae), commonly known as the golden shower tree, is distributed in various regions including Asia, South Africa, China, the West Indies, and Brazil. It grows in open forests and grasslands in lowlands. On Java, it is often cultivated as an ornamental plant, while in the Philippines, it is grown as a medicinal and ornamental plant; it is also cultivated throughout New Guinea. This small to medium-sized tree is generally deciduous or semi-deciduous in summer, with spread branches and bare twigs [4,5]. Various parts of *C. fistula* are known to be important sources of secondary metabolites, mainly phenolic compounds. The leaves contain (−) epiafzelechin, (−)epiafzelechin-3-O-glucoside, (−)epicatechin, procyanidin B2, bioflavonoids, triflavonoids, rhein, rhein glucoside, sennoside A, sennoside B, chrysophanol, and physcion [6,7]. The roots contain 7-methylphyscion, betulinic acid, and β-sitosterol. The bark of the stem contains two flavonol glycosides and a xanthone glycoside [8]. The presence of kaempferol, leucopelargonidin tetramer (with free glycol unit), rhein, fistulin, alkaloids, and triterpenes in *C. fistula* flowers has been documented [9,10]. The plant has been shown to possess wound healing, anti-inflammatory, antioxidant, hepatoprotective, antibacterial, and antifungal activities [11-15]. The focus of the current study is to evaluate the phytochemical constituents and the tyrosinase inhibitory activity of methanolic leaf extracts of *C. fistula* and its fractions.

MATERIALS AND METHODS

Plant material

C. fistula leaves were collected from Citeureup Medicinal Plants Garden, Directorate of Indonesian Indigenous Medicine, National Agency of Drug and Food Control of the Republic of Indonesia in October 2012, and the plant determination was conducted at the Research Center for Biology, Indonesian Institute of Sciences.

Preparation of extract and fractions

Air-dried and powdered leaves (880 g) were macerated with 80% methanol for 72 h. The extract was filtered and the solvent was recovered using a rotary evaporator with a vacuum pump at 40–50°C. This process was repeated another 6 times and the combined extract was kept in a vacuum oven at 40°C to achieve a paste-like consistency (yield 20.398% w/w). The extract was dissolved in water for successive fractionation with n-hexane, ethyl acetate, and n-butanol. Then, 80% methanol extract, n-hexane fraction, ethyl acetate fraction, n-butanol fraction, and aqueous fraction of *C. fistula* leaves were used as test samples along with positive controls (kojic acid and ascorbic acid) for the enzyme inhibition assay.

Phytochemical screening

The phytochemical screening of the methanolic extract of *C. fistula* leaves and its fractions was carried out using standard procedures to identify the constituents such as alkaloids, flavonoids, glycosides, terpenoids, phenolics, saponins, tannins, and anthraquinones.

Alkaloids

Bouchardat, Mayer, and Dragendorff tests [16,17] were used to perform the alkaloid identification.

Flavonoids

To 100 mg of sample was added 10 mL of hot water, followed by boiling for 5 min and filtering under hot temperature. As much as 5 mL of filtrate was pipetted, followed by the addition of 100 mg of magnesium powder, 1 mL of concentrated hydrochloric acid, and 2 mL of amyl alcohol, after

which vigorous shaking was performed. If the amyl alcohol layer was orange or red, this meant that the sample contained flavonoids [17].

The sample was spotted on silica gel plates and eluted with ethyl acetate:methanol (1:9) and, then, sprayed with 0.5% AlCl3. Flavonoids were detected as yellow fluorescence in UV light at a wavelength of 365 nm. Silymarin was used as a positive control.

Glycosides

The identification of glycoside compounds was determined using Kedde, Lieberman, and Keller–Kiliani tests [18].

Terpenoids

To 500 mg of sample was added 2 mL of chloroform, after which 3 mL of concentrated H_2SO_4 was added carefully to form a layer. A reddish-brown color on the surface indicated the presence of terpenoids [18].

Phenolics

To 100 mg of sample was added 10 mL of 80% methanol, followed by sonication for 20 min and filtering with cotton. As much as 1 mL of filtrate was supplemented with 1 mL of Folin–Ciocalteu reagent, followed by boiling for 5 min in the dark. A greenish-yellow or green color indicated the presence of phenolic compounds [19].

Saponins

To 100 mg of sample was added 10 mL of hot water, followed by cooling down, after which vigorous shaking was performed for 10 s. If froth formed and was maintained for 10 min and, then, still remained after the addition of one drop of 2 N HCl, this indicated that the sample contained saponins.

Tannins

To 100 mg of sample was added 100 mL of hot water, followed by boiling for 5 min. After filtration, part of the filtrate was supplemented with two drops of 1% $FeCl_3$. The formation of a green, blue, or blackish color represented a positive result [17]. Second, to 1 mL of filtrate was added 3 mL of a 10% solution of gelatin, where white precipitation indicated a positive result regarding the presence of tannins.

Anthraquinones

To 2 mL of methanolic extract was added 2 mL of 10% NH_4OH. A bright pink color indicated the presence of anthraquinones [20].

Determination of total polyphenolic content (TPC)

The TPC was determined by spectrophotometry, using gallic acid as a standard, in accordance with the method described by the International Organization for Standardization 14502-1 [21]. Briefly, 50 mg of sample was dissolved in 0.5 mL of dimethyl sulfoxide (DMSO), followed by the addition of demineralized water up to the limit of a 5.0-mL volumetric flask. 1 ml of sample solution was transferred in duplicate to separate tubes containing 5.0 mL of a 1/10 dilution of Folin–Ciocalteu reagent in water. Then, 4.0 mL of a sodium carbonate solution (7.5% w/v) was added. Next, the tubes were allowed to stand at room temperature for 60 min before the absorbance at 765 nm was measured against water. The TPC was expressed as gallic acid equivalents (GAE) in mg/g material. The concentration of polyphenols in samples was derived from a standard curve of gallic acid ranging from 10 to 50 µg/mL.

Tyrosinase inhibition assay

Tyrosinase inhibition was determined using the modified dopachrome method with L-DOPA as a substrate. The samples were dissolved in 1.25% DMSO. Assays were conducted in a 96-well microtiter plate with each well containing 40 µl of sample with 80 µl of phosphate buffer (0.1 M, pH 6.8), 40 µL of tyrosinase (31 U/mL), and 40 µl of L-DOPA (20 mM). The mixture was incubated for 10 min at 37°C and absorbance was measured at 490 nm. Each sample was accompanied by a blank that had all components except tyrosinase. Ascorbic acid and kojic acid were used as positive controls. The results were compared with a control consisting of 1.25% DMSO in place of the sample. The percentage of tyrosinase inhibition was calculated as follows:

$$\text{Tyrosinase inhibition } (\%) = \frac{(A-B) - (C-D)}{(A-B)} \times 100$$

Where,
A = Absorbance of the blank solution with enzyme
B = Absorbance of the blank solution without enzyme
C = Absorbance of the sample solution with the enzyme
D = Absorbance of the sample solution without enzyme.

Determination of tyrosinase kinetic inhibition

The determination of tyrosinase kinetic inhibition was performed on the sample that had the smallest IC_{50}. The type of inhibition was determined by making a curve from the data of Lineweaver–Burk inhibition reactions with various concentrations of substrates and comparing it with the Lineweaver–Burk curves regarding the influence of inhibitors on the rate of reaction. Substrate at a concentration of 20, 10, 5, or 2.5 mM was supplemented with enzyme solution (31 U/mL). The Michaelis–Menten kinetic constant (K_m) was calculated based on the regression equation y=a+bx, where x is the number of substrates 1/[S] and y is 1/V.

RESULTS AND DISCUSSION

C. fistula leaves were dried in the shade due to the fact that some phenolic compounds are unstable and degrade under direct sunlight due to enzymatic reactions. Moreover, oven drying was not recommended because it can reduce the extractability of certain polyphenols (such as catechin), which may be related to fiber and protein [22]. In addition, thermal degradation can be occurred in oven drying. The loss on drying result of the dried leaves was 72.19%. The dried leaves were resorted to remove impurities and then powdered with an electric mill.

A sample of approximately 880 g of dried leaves was macerated with 80% methanol for 72 h, which was repeated 7 times. Extraction with maceration using alcoholic solvent denatures plant enzymes that will prevent problems associated with plant enzyme activity [23]. Methanol was chosen for this purpose because it is commonly used. Methanol at 70–80% gave good results for extracting hydrocynamate, flavone, flavonol, and catechin derivatives [24,25]. In the extraction of *C. fistula* leaves using 80% methanol, a viscous extract weighing about 179.5 g was obtained, giving a yield of 20.398%.

Then, the viscous extract was fractionated using a variety of solvents with increasing polarity, namely n-hexane, ethyl acetate, and n-butanol. Here, the aim was to separate the chemical compounds that were present based on the level of polarity to obtain the active compound suspected of having inhibitory activity against tyrosinase.

Preliminary phytochemical screening of the methanolic extract of *C. fistula* leaves and its fractions revealed the presence of various bioactive components (Table 1). Specifically, it revealed the presence of phenols and flavonoids in the methanolic extract of *C. fistula* leaves and in all of its fractions.

Table 2 shows the TPC of the methanolic extract of *C. fistula* leaves and its fractions. The results obtained showed that the TPC varied from 0.808±0.050 to 39.428±0.041 mg GAE/g (Table 2).

Tyrosinase inhibitory activity assay was conducted using ascorbic acid and kojic acid as positive controls because they are widely used in commercial skin whitening preparations. Table 3 showed the results. The IC_{50} value of ascorbic acid did not differ markedly from the value reported by Patel and Zaveri [26], where the IC_{50} was 6.38 µg/mL. Meanwhile, the IC_{50} value of kojic acid did not differ markedly from the value [27-30], where the IC_{50} was 1.7 µg/mL. The aqueous fraction showed the highest tyrosinase inhibitory activity, while the ethyl acetate fraction that contained the

Table 1: Phytochemical analysis of the methanolic extract of *C. fistula* leaves and its fractions

Tests	Methanolic extract	n-hexane fraction	Ethyl acetate fraction	n-butanol fraction	Aqueous fraction
Alkaloids	+	-	-	+	+
Flavonoids	+	+	+	+	+
Glycosides	+	-	+	+	+
Terpenoids	-	+	+	-	-
Phenolics	+	+	+	+	+
Saponins	+	-	-	+	-
Tannins	+	-	+	+	+
Anthraquinones	+	-	+	-	-

C. fistula: Cassia fistula

Table 2: TPC of the methanolic leaf extract of *C. fistula* and its fractions

Parameter	Methanolic extract	n-hexane fraction	Ethyl acetate fraction	n-butanol fraction	Aqueous fraction (residue)
Polyphenol content*	2.026±0.011	0.808±0.050	39.428±0.041	20.872±0.034	6.392±0.012

*Expressed as mg GAE/g of dry plant material, *C. fistula: Cassia fistula* . The data are displayed as mean ± standard deviation of triplicate experiments

Table 3: IC_{50} values of tyrosinase inhibition assay of the methanolic extract of *C. fistula* and its fractions

Extract	IC_{50} (µg/mL)	Score*
Methanolic extract	392.764±0.008	7
n-hexane fraction	190.375±0.026	7
Ethyl acetate fraction	535.937±0.010	6
n-butanol fraction	347.281±0.008	7
Aqueous fraction (residue)	152.031±0.027	7
Ascorbic acid	6.879±0.010	8
Kojic acid	2.094±0.010	8

*According to the scoring system of Batubara *et al*. for mushroom tyrosinase inhibitory activity. *C. fistula: Cassia fistula.* . The data are displayed as mean ± standard deviation of triplicate experiments

Table 4: Michaelis–Menten (K_m) and V_{max} constants

Inhibitor concentration	a	B	r	K_m (b/a)	V_{max} (1/a)
Blank	1.6229	8.6072	0.9997	5.303	0.616
125 µg/mL	1.8191	8.57	0.9985	4.711	0.55
250 µg/mL	1.855	9.1374	0.9977	4.926	0.539

highest level of polyphenols showed the lowest inhibitory activity. It can be concluded from these results that there is a possibility of there being a tyrosinase inhibitory compound not from the polyphenol class that has significant inhibitory activity. In contrast, in the ethyl acetate fraction that had the highest polyphenol content, there may have been compounds that interfered with the activity of polyphenols in inhibiting tyrosinase so that the ethyl acetate fraction was less significant at inhibiting tyrosinase.

Batubara *et al.* classified mushroom tyrosinase inhibitory activity using a scoring system, in which a score of 6–8 indicates potent tyrosinase inhibition [31]. Based on this scoring system, it can be concluded that the methanolic extract of *C. fistula* leaves and its fraction have potency as a tyrosinase inhibitor.

As Fig. 1 shows, the nature of the tyrosinase inhibition by the methanolic extract of the aqueous fraction of C. fistula leaves can be revealed by measuring the enzyme inhibition kinetics using Lineweaver–Burk plots with varying concentrations of L-DOPA as the substrate [3]. Based on the obtained equation, the V_{max} and K_m values can be determined (Table 4). In a system without inhibitor, the obtained equation is as follows: y=1.6229+8.6072x, with V_{max} of 0.616 µmol/mL min and K_m of 5.303 µmol/mL. Meanwhile, in a system with an inhibitor at a concentration of 125 µg/mL, the following equation was obtained: y=1.8191+8.57x, with V_{max} of 0.55 µmol/mL min and K_m of 4.711 µmol/mL. In a system with an inhibitor at a concentration of

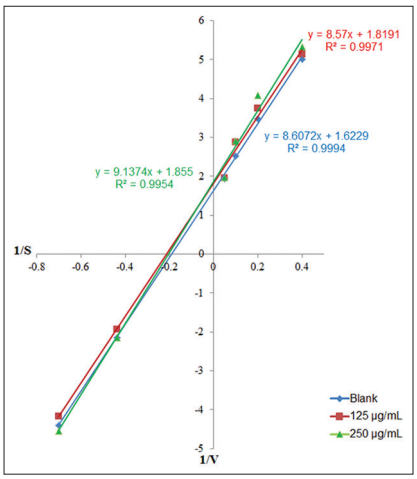

Fig. 1: Tyrosinase inhibition kinetics of the aqueous fraction of *Cassia fistula*

250 µg/mL, the equation y=1.855+9.1374x was obtained, with V_{max} of 0.539 µmol/mL min and Km of 4.926 µmol/mL. The results showed that the V_{max} in the system with an inhibitor was lower than the V_{max} in the system without an inhibitor, and there was an increase of K_m score, indicating that the aqueous fraction contained mixed-type tyrosinase inhibitor. This may have been because the aqueous fraction still consisted of many compounds.

The data are displayed as mean ± standard deviation of triplicate experiments

CONCLUSION

The current study indicates that *C. fistula* leaves possess significant tyrosinase inhibitory activity. Further studies are needed to isolate and identify the bioactive compounds responsible for tyrosinase inhibition activity.

CONFLICTS OF INTEREST

None declared.

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