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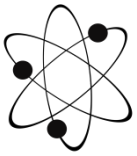

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Tyrosinase Inhibition Type of Isolated Compounds Obtained from *Pachyrhizus erosus*

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Abstract - In Indonesia, Bengkoang (*Phacyrhizus erosus*) have been used as one of cosmetics especially as sun screening and skin whitening materials. Six active compounds in Bengkoang with antioxidant and skin whitening activities have been isolated, namely daidzein, daidzin, genistin, (8,9)-furanyl-pterocarpan-3-ol, 4-(2-(furane-2-yl)ethyl)-2-methyl-2,5-dihydro-furane-3-carbaldehyde and 2-butoxy-2,5-bis(hydroxymethyl)-tetrahydrofurane-3,4-diol. According to literatures, the type of their tyrosinase inhibitory activity has not yet reported. The determination of whitening activity of each compound was evaluated by the evaluation of Lineweaver-Burk plot. The result showed that five compounds had competitive inhibitory activity and 8,9-furanyl-pterocarpan-3-ol showed a non-competitive inhibition.
Keywords: *Pachyrhizus erosus*, tyrosinase, competitive inhibition and non-competitive inhibition

Introduction

A number of tyrosinase inhibitors from natural sources that inhibited monophenolase, diphenolase or both, have been identified (Lee *et al.*, 2007). Some inhibitors from higher plants have been found and can be classified into two groups, polyphenol and aldehyde derivatives (Parvez, 2007). Flavonoids as one of polyphenol groups have strong tyrosinase inhibiton activity, due totheir 3-hydroxyl group of the isoflavonoid is free (Kim *et al.,* 2004). Tannic acid and gallic acid have been investigated by Kubo *et al.* (2003) and the results indicated that the tyrosinase inhibitory activity of tannic acid is more potent than gallic acid. Gilly *et al.* (2001) have reported that resveratrol found in Carignan Grape juice has tyrosinase inhibition activity. A large number of aldehydes and other derivatives were also characterized as tyrosinase inhibitory (Abdel-Hakim, 2008). Its inhibitory effect is due to the formation of a Schiff base with the primary amino group of the enzyme. Joung-Ha *et al.* (2005) reported the tyrosinase inhibitory activity of anysaldehyde in the seed of *Pimpinella anisum.*

Bengkoang is a species of genus Pachyrizus and grows in Indonesia, and has been used as one of traditional cosmetics for sunscreen and whitening cosmetics (Lukitaningsih *et al.*, 2013). This plant content 86-90% water and phenolic compound (Lukitaningsih, 2009). In addition, Bengkoang also contains saponin serving as natural "sunscreen" in preventing skin damage caused by free-radical excitement as the result of absorption of ultra-violet rays (Sandler, 2005). According to Wang *et al.* (2006), phenolic compounds may be used as depigmenting agents, because they have a similar chemical structure to tyrosine (the substrate of the tyrosinase). This enzyme is an important enzyme that plays a major role in melanin synthesis being responsible as a dark pigment in human and animals.

Materials and Methods

Chemicals and solvent

The chemicals used in the detection and isolation methods were glacial acetic acid, hydrochloric acid and concentrated sulphuric acid (Merck, Darmstadt, Germany), mushroom tyrosinase 4187 IU/mg, Folin-Ciocalteau's phenol reagent, *L*-DOPA (dihydroxy phenyl alanine), kojic acid (Fluka, Seelze, Germany), Dulbeco's phosphate buffered saline (purchased from Sigma Aldrich, Steinheim Germany), ethyl acetate (Fisher Scientific, Leichestershire, UK), methanol (Merck, Darmstadt, Germany), chloroform, dichloromethane and n-butanol (Fluka, Seelze, Germany).

Instruments

Cary 50 Bio UV-Visible spectrophotometer (Varian, California, USA, Thermo Mixer Comfort 5355 V.2.12 Eppendorf (Hamburg, Germany), ALPHA II-12 Freeze dryer (Osterode, Germany), analytical balance (Sartorius, Japan)

Determination of the tyrosinase inhibition type

All compounds used in this experiment have been isolated according to the procedure in Lukitaningsih (2009). The determination of the tyrosinase inhibition type of isolated compounds was carried out according to Chen and Kubo (2002) with a little modification. Enzyme activity was determined at 25° C by following the increase in absorbance at λ 475 nm accompanying the oxidation of the substrate (*L*-DOPA). In this condition, one unit (U) of enzymatic activity was defined as the amount of enzyme that increasing 0.001 absorbance at λ 475 nm. The progress of substrate reaction was applied to the current study of the inhibition kinetics of mushroom tyrosinase by isolated compounds. In this method, the mushroom tyrosinase (1.0 mg/mL in 0.1 M phosphate buffer pH 6.8) was first diluted 50 times with water, and then 50 µl of the solution was added to 200 µl of an assay substrate solution with 25 µl DMSO containing different concentrations of the isolated compounds. The increasing UV/Vis absorbance of this mixture was immediately measured at λ 475 nm for 20 min for detection of dopachromed formed. The concentrations of substrate solutions (*L*-DOPA solution in phosphate buffer at pH 6.8) used in this experiment were 0.6; 0.8; 1.0; 1.5 and 2.0 mM, respectively.

The progress curve of substrate reaction was analysed to obtain a rate constants of reaction (V) and was expressed in (unit/min). The reaction rate constant (V) was the slope of the plots of the absorption value (as Y axis) and time (as X axis). The Lineweaver-Burk plot: The correlation between 1/[concentration of the *L*-DOPA solution] versus 1/V in the presence of different concentrations of the isolated compounds, was performed to evaluate the type of the tyrosinase inhibition of the isolated compounds. The compound will be classified into a group of competitive inhibitor, if the increase of the compound concentration produced a series of lines with a common intercept on the 1/V axis but with different slopes. If the increase of the compound concentration produced a series lines with the same intercept on the 1/S axis with also different slopes, the compounds will be assigned to be a non-competitive inhibitor.

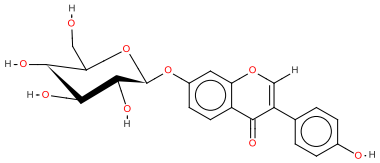
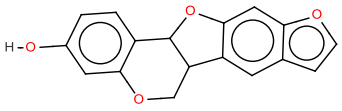
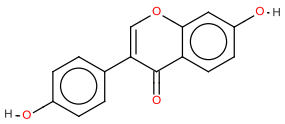
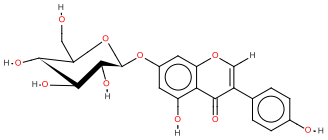
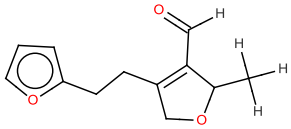
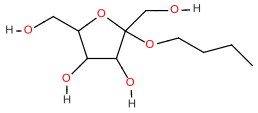
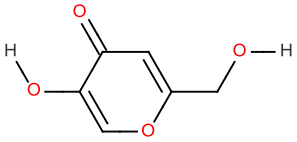
Results and Discussion

Determination on inhibition type of isolated active compounds in Bengkoang has been conducted. Table 1 demonstrated the tyrosinase inhibition type and IC_{50} values of each compound. Only *(8,9)-Furanylpterocarpan-3-ol* compound belongs to the non-competitive enzyme category. It means, this compound can bind with the enzyme on a site that is not an active site (an allosteric site). The presence of non-competitive inhibitor changes the nature and shape of the enzyme so that its catalytic properties are lost. Additionally, there are five other compounds are included in competitive inhibitor type. Competitive inhibition takes place when a molecule that is structurally similar to the substrate for a particular reaction competes for a position at the active site on the enzyme. Competitive inhibition can be reversed by raising the concentration of substrate to sufficiently high levels while the concentration of the inhibitor is held constant. The presence of the competitive inhibitor can reduce the maximum rate of a chemical reaction (V_{max}) without changing the apparent binding affinity of the active site to substrate. This can happen in two ways. Either the non-competitive inhibitor itself physically blocks the access to the active site, or it causes a conformational change in the protein, thus inactivating the active site. Because the substrate molecules cannot reverse the binding of a non-competitive inhibitor, increasing the concentration of substrate will not reverse the inhibition.

To analyze the inhibition type of the present isolated compounds for tyrosinase, a steady-state analysis was performed. Lineweaver-Burk plots for the inhibition of tyrosinase by isolated compounds were obtained with variable concentrations of them and substrate (*L*-DOPA). The Lineweaver-Burk plots of the isolated compounds were displayed in Figure 1. The intersections of the lines on the vertical axis in Lineweaver-Burk plots of daidzein, 4-(2-(furane-2-yl)ethyl)-2-methyl-2,5-dihydrofurane-3-carbaldehyde, daidzein-7-O-ß-glucopyranose, 2-butoxy-2,5-bis(hydroxymethyl)-tetrahydrofurane-3,4-diol and 5-Hydroxy-daidzein-7-O-ß-glucopyranose indicates that these compounds could be included in the group of competitive inhibitors. Meanwhile, the Lineweaver-Burk plot of (8,9)-furanyl-pterocarpan-3-ol produced the lines which had the same intersection on the horizontal axis indicating that this compound had to be included in the group of non-competitive type.

The presence of hydroxyl (-OH) at position C-4' in the isoflavonoid compounds and aldehyde in the furane carbaldehyde compounds might be responsible for the competitive inhibition activity. The compound (8,9)-furanyl-pterocarpan-3-ol didn't have hydroxyl (-OH) at position C-4', therefore it was not able to bind with the active site of tyrosinase enzyme. The inhibition activity of (8,9)-furanyl-pterocarpan-3-ol might be due to its ability to bind with tyrosinase at a site other than the active site of enzyme (at allosteric site). This fact was in agreement with Khatib *et al.* (2005) showing that the position of hydroxyl group in the molecule played an important role in the type inhibition activity rather than the number of hydroxyl group. Chang (2007) has reported that the isoflavone's skeleton was absolutely necessary for the compounds to suicide substrates of mushroom tyrosinase (a competitive tyrosinase's inhibitor).

Table 1. Chemical structures of isolated compounds in Bengkoang and their tyrosinase inhibition activities (IC_{50} values and type of inhibitions)

Code	Chemical Structure Compound name	IC_{50} (Lukitaningsih *et al.*, 2013)	Type of inhibition
Wu1a	 *Daidzein-7-O-ßglucopyranose*	22.20± 0.27 mM	Competitive
A182	 *(8,9)-Furanylpterocarpan-3-ol*	7.19 ± 0.11 mM	Non Competitive
C1	 *Daidzein*	5.35 ± 0.03 mM	Competitive
Wu3a	 *5-Hydroxyldaidzein-7-O-ßglucopyranose*	4.38 ± 0.01 mM	Competitive
HWu10	 *4-(2-(Furane-2-yl)ethyl)-2-methyl-2,5-dihydrofurane-3-carbaldehyde*	0.198± 0.004mM	Competitive
WuBuOH	 *2-Butoxy-2,5-bis(hydroxymethyl)-tetrahydrofurane-3,4-diol*	1.21± 0.02 mM	Competitive
Std	 *Kojic acid*	1.070± 0.001 mM	Competitive

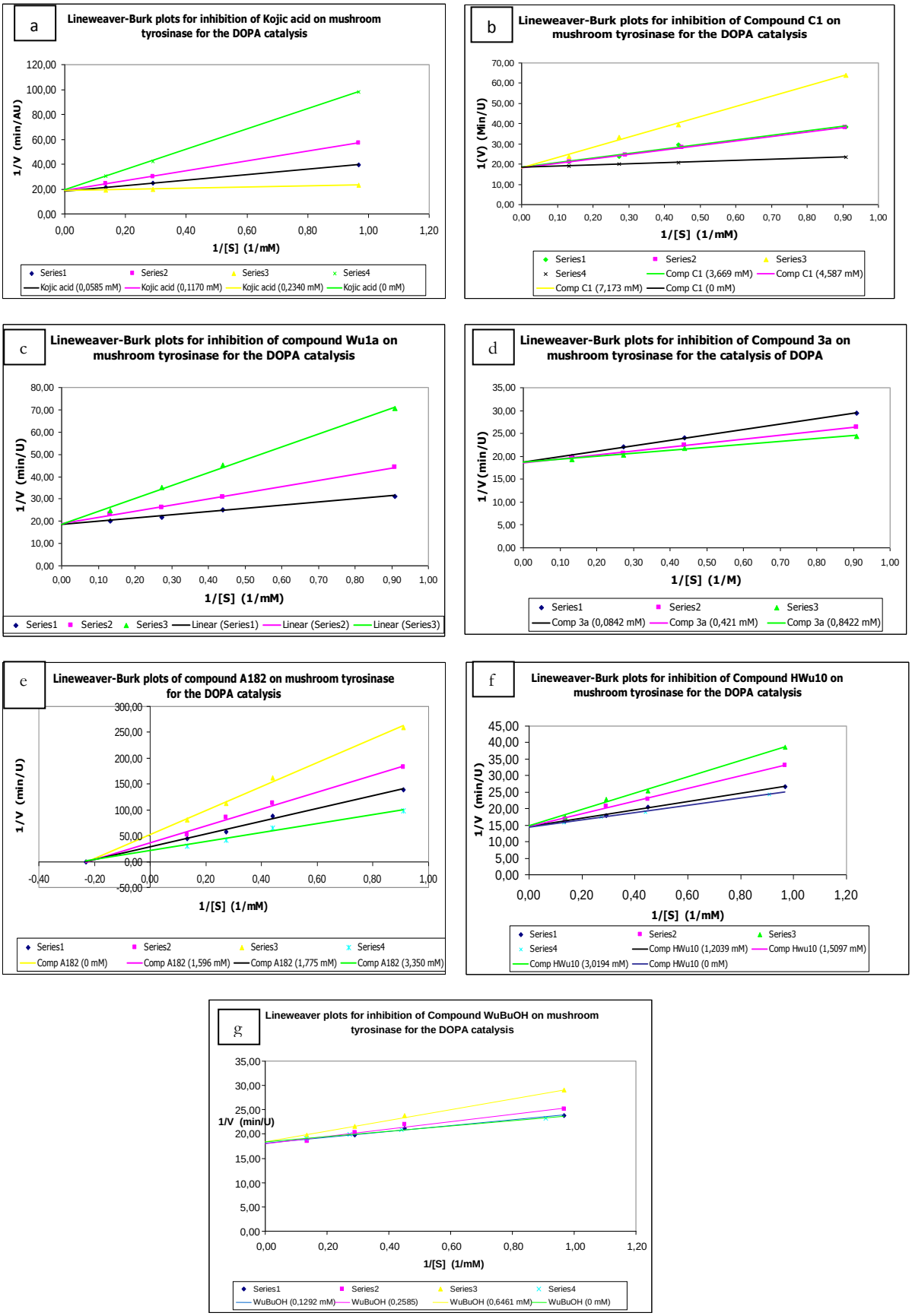


Figure 1. The Lineweaver-Burk plots of standard kojic acid (a) and isolated compounds (b-f). Figure 1g (A182 or *(8,9)-Furanylpterocarpan-3-ol)* was a representative type of the non-competitive inhibitor in this study.

Conclusions

All compounds showed the tyrosinase inhibitory activity. The type of inhibition showed a competitive inhibitory type, except compound 8,9-furanyl-pterocarpan-3-ol. Compound 8,9-furanyl-pterocarpan-3-ol did not have two hydroxyl groups at *ortho* position. Therefore, compound 8,9-furanyl-pterocarpan-3-ol was classified as the non-competitive inhibitor.

Acknowledgements

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