Screening Marker Components Of Tyrosinase Inhibitor From *Xylocarpus Granatum* Stem

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Abstrak

The aim of our research was to screen the marker components of tyrosinase inhibitor from *Xylocarpus granatum* stem collected from Pulau Sebuku, South Kalimantan, Indonesia. The screening method started from selection of part of *X. granatum*, stem or stem bark. Stem and stem bark of *X. granatum* were dried and grounded before submitted to methanol. The stem extracts is more potent as tyrosinase inhibitor (IC_{50} for monophenolase is 45.12 µg/ml and diphenolase is 31.59µg/ml) compared to the bark extracts. The IC_{50} values of kojic acid as positive control are 17.43µg/ml for monophenolase and 20.69 µg/ml for diphenolase. The stem extract then separated with silica gel column chromatography and preparative thin layer chromatography. The results showed that component with Rf 0,25 and 0.63 (TLC analysis with stationary phase silica gel GF_{254} and mobile phase ethyl acetic: methanol (8:2)) are the marker components as tyrosinase inhibitor for *X. granatum*.

Keywords : tyrosinase inhibitor, stem of *Xylocarpus granatum*, marker components

1. INTRODUCTION

Tyrosinase inhibitors have been a great concern solely due to the key role of tyrosinase in both mammalian melanogenesis and fruit or fungi enzymatic browning (Chang 2009). Melanogenesis is a principal parameter of differentiation of melanocytes and melanoma cells. The formation of melanin in human body influenced or reduced by several mechanisms, including anti-oxidation, direct tyrosinase inhibition, melanin inhibition of migration from cell to cell and hormonal activities etc (Pawelek and Korner, 1982).

Tyrosinase or polyphenol oxidase (PPO) (monophenol, o-diphenol; oxygen oxidoreductase; EC 1.14.18.1) is a copper enzyme that catalyzes two different reactions using molecular oxygen; the *ortho* hydroxylation of tyrosine (mono-phenols) to 3,4-dihydroxyphenylalanine or DOPA (o-diphenols) named monophenolase activity and the oxidation of DOPA to dopaquinone (o-quinones) named diphenolase activity. (Sanchez-Ferrer et al 1995). Tyrosinase is responsible for pigmentation of skin, eyes and hair. It made tyrosinase inhibitors have been used frequently in cosmetics and depigmenting agents for hyperpigmentation. Investigation of inhibitors of this enzyme may lead to development of novel skin whitening agents.

A number of tyrosinase inhibitors from both natural and synthetic sources have been identified. However searching the other potential compound as tyrosinase inhibitors from natural sources still need. We screened some Indonesian medicinal plants potency as tyrosinase inhibitors (Batubara et al 2010). We found *Xylocarpus granatum* is one of the potent sample as tyrosinase inhibitor. The aim of this paper is to search the marker components of tyrosinase inhibitor from *Xylocarpus granatum*.

2. MATERIAL AND METHODS

Plant Materials and Preparation

Xylocarpus granatum was collected from Pulau Sebuku, South Kalimantan, Indonesia in 2009. *X. granatum* was separated into bark and stem parts. All the parts were dried and grounded before submitting to methanol extraction. Briefly, 100 gram *X. granatum* meal was macerated with 5 L methanol for 12 h three times. The extracts were filtered using Whatman filter paper (No. 2) and concentrated *in vacuo* at 30°C using a rotary evaporator to obtain extracts. The phytochemistry analysis was preformed to stem and bark extracts.

Marker Determination with Separation Screening Methods

Stem and Bark extracts of *X. granatum* was applied on TLC (Silica gel $G_{60}F_{254}$, Merck). TLC was developed on EtOAc:MeOH (8:2) as developing solvent. The band was detected with UV at 254 and 366 nm. The active extract was then applied on Silica gel column chromatography (30 cm ID 2 cm) using step gradient (EtOAc:MeOH). The fractions were analyzed with TLC with the same condition for the first step. The active fraction was separated further with preparative TLC. The fractions obtained were analyzed with TLC. The correlation between Rf from TLC and the activity resulted the marker.

Tyrosinase activity test

This assay was performed using methods as described earlier (Curto et al., 1999; Nerya et al., 2003). Extracts were dissolved in DMSO (dimethyl sulphoxide) to a final concentration of 20 mg/ml. This extract stock solution was then diluted to 600 µg/ml in 50 mM potassium phosphate buffer (pH 6.5).

The extracts were tested at the concentrations ranged from 7.8125 to 2000 µg/ml. Kojic acid, which were used as positive controls were also tested at concentrations 7.8125 to 500 µg/ml. In 96-well plate, 70 µl of each extract dilution was combined with 30 µl of tyrosinase (333 Units/ml in phosphate buffer) in triplicate. After incubation at room temperature for 5 min, 110 µl of substrates (2 mM L-tyrosine or 12 mM L-DOPA) was added to each well. Incubation commenced for 30 min at room temperature. Optical densities of the wells were then determined at 510 nm with multi-well plate reader. The concentration of plant extract at which half the original tyrosinase activity is inhibited (IC_{50}), was determined for each plant extract.

3. RESULT AND DISCUSSION

The phytochemistry components exist in the bark and stem of *X.granatum* were shown in Table 1. There were no different between the bark extracts and stem extracts. Both were consisted of alkaloid, flavonoid, and tannin.

Table 1 Phytochemistry components exist in the bark and stem of *X. granatum*

Phytochemistry groups	Part of *X granatum*	
	Bark	Stem
Alkaloid	+	+
Flavonoid	+	+
Saponin	-	-
Tannin	+	+
Triterpenoid	-	-
Quinon	-	-
Steroid	-	-

note (-):not detected; (+): detected

Since there were no significant phytochemistry components groups between bark and stem, it is important to search the marker component from *X. granatum* as tyrosinase inhibitor. The tyrosinase activity of bark and stem extracts were different (Table 2). The IC_{50} values of kojic acid as positive control are 17.43µg/ml for monophenolase and 20.69 µg/ml for diphenolase. The stem extracts was more active compared to bark extracts for monophenolase and diphenolase. The differences of tyrosinase activity might be because of the differences of component in each extracts. TLC analysis showed that stem extracts had the band with Rf 0.25 and 0.63 which did not appear in the bark extracts.

Table 2 Tyrosinase inhibition activity (IC_{50}) and TLC analysis of bark and stem extracts of *X. granatum*

X. granatum extracts	TLC analysis	IC_{50} (µg/mL)	
	Rf	Monophenolase	Diphenolase
Bark	0.06; 0.13; 0.19; 0.45; 0.56; 0.76; 0.90	-	-
Stems	0.06; 0.13; 0.20; **0.25**; 0.53; **0.63**; 0.76; 0.85	45.12	31.59
Kojic acid		17.43	20.69

note: (-): inhibition less than 50% at maximum concentration 500 µg/ml

To make sure Rf 0.25 and 0.63 can be used for marker, we separated the stem extracts with silica gel column chromatography resulted 13 frations. Each fraction were analyzed from its tyrosinase activity and TLC analysis. The results showed in Table 3. The most active fraction was Fr. 3 with IC_{50} about 18.02 for monophenolase µg/ml and 21.15 µg/ml for diphenolase. On Fr. 3, we found band with Rf 0.25 and 0.63. This result consistent with Rf on the active stem extract. To make sure about Rf 0.25 and 0.63, we separated Fr.3 with preparative TLC. The results showed that Fr D was the most potent tyrosinase inhibitor (Table 4). Band with Rf 0.25 and 0.63 were also exist in Fr. D.

Table 3. Tyrosinase activity and Rf value of column chromatography fractions

Fractions	IC_{50} (µg/ml)		Rf
	Monophenolase	Diphenolase	
1	-	-	0.89
2	22.45	-	0.63; 0.91
3	18.02	21.15	0.25; 0.63; 0.69; 0.80; 0.89
4	64.78	40.76	0.20; 0.29; 0.60; 0.63; 0.79; 0.88
5	-	-	0.13; 0.24; 0.28; 0.61; 0.70; 0.80; 0.89
6	-	-	0.13; 0.23; 0.29; 0.60; 0.64; 0.79; 0.88
7	-	20.88	0.24
8	-	-	0.13; 0.23; 0.29; 0.59; 0.69 0.80; 0.90
9	-	-	0.09; 0.24; 0.26
10	-	-	0.04; 0.13; 0.23; 0.30
11	-	-	0.04; 0.13; 0.24; 0.29
12	-	-	0.04; 0.13; 0.21
13	-	-	0.05; 0.13

note: (-): inhibition less than 50% at maximum concentration 500 µg/ml

The active compound groups in fraction D was analyzed with chemical determination. The active band color was pale yellow visually, with UV light was dark brown and with amonia the color was yellow. In addition with spectrum of IR data (Fig 1), the active compounds was flavonol group (Fig 2).

Table 4. Tyrosinase activity and Rf value of TLC fractions

Fractions	IC_{50} (µg/ml) Monophenolase	IC_{50} (µg/ml) Diphenolase	Rf
A	-	345.69	0.74; 0.80; 0.86
B	77.09	537.13	0.71; 0.78; 0.84
C	619.66	76.71	0.56; 0.69; 0.76; 0.84
D	18.73	21.92	0.25; 0.63; 0.69; 0.76; 0.84
E	23.71	37.46	0.14; 0.23; 0.63; 0.69; 0.78; 0.85
F	-	-	0.13; 0.18; 0.56; 0.66; 0.75; 0.78
G	-	-	0.10; 0.15; 0.23
H	-	-	0.06; 013

note: (-): inhibition less than 50% at maximum concentration 500 µg/ml

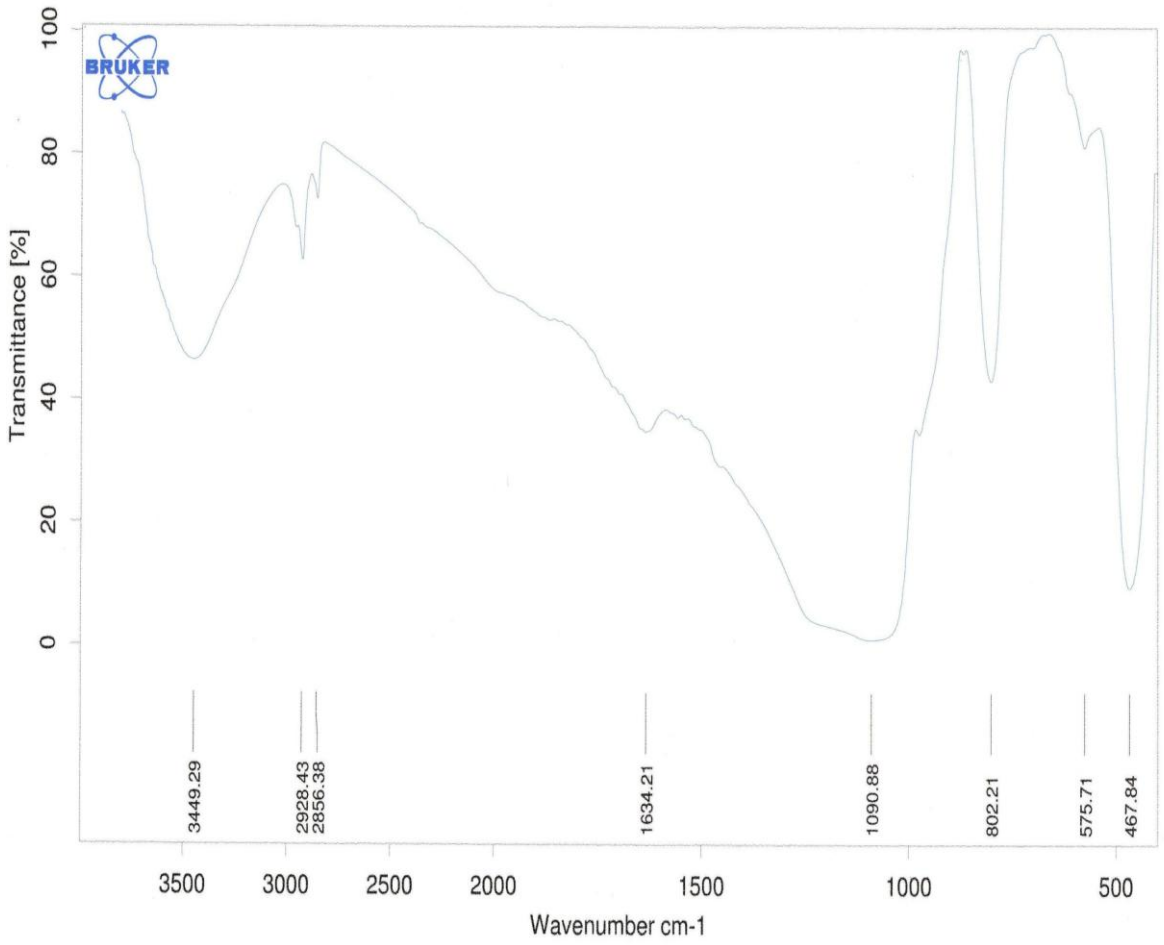

Fig 1. IR Spectrum of Fraction D

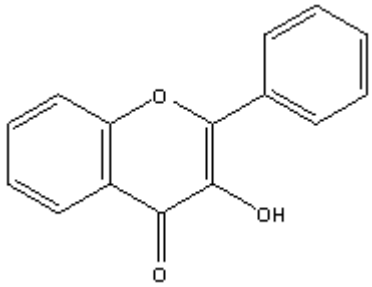

Fig 2. Structure of flavonol

4. CONCLUSION

The stem extracts of *X. granatum* was more potent as tyrosinase inhibitor compared to the bark extracts. Marker components for tyrosinase inhibitor in *X. granatum* stem are band with Rf 0,25 and 0.63 (TLC analysis with stationary phase silica gel GF_{254} and mobile phase ethyl acetic: methanol (8:2)) based on the most active fraction from column chromatography and TLC. The active compound was flavonol groups.

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