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## Indonesian *Sidaguri* (*Sida rhombifolia* L.) as Antigout and Inhibition Kinetics of Flavonoids Crude Extract on the Activity of Xanthine Oxidase

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**Abstract:** *Sida rhombifolia* L., or *Sidaguri* in Indonesian, is a traditional medicinal plant with potential as a remedy against gout. The earlier research reported that flavonoids crude extract from this plant could *in vitro* inhibit the activity of Xanthine Oxidase (XO) (xanthine: oxygen oxidoreductase EC 1.2.3.2) up to 55% and could be antigout. It is a need to investigate type of inhibition kinetic of *Sida rhombifolia* L.'s extract and the effects of fractionation on the inhibitory effect by *in vitro* method. The results showed that the flavonoids crude extract yielded approximately 12% with  $LC_{50}$  of  $501 \text{ mg L}^{-1}$  and its inhibitory effect from 48 to 71% ( $100\text{-}800 \text{ mg L}^{-1}$ ). The kinetic study resulted that the type of flavonoids crude extract inhibition was a competitive inhibition with inhibitor affinity ( $\alpha$ ) of 2.32 and  $p < 0.01$ . The fractionation yielded 11 fractions and the fraction 4 has highest activity, i.e., 79%. The fractions did not increase the inhibition effect significantly. GC-MS analysis of fraction 4 showed that there were 39 organic compounds and flavonoid fragments with retention times of 4.14, 6.53 and 6.74 that have resemble fragmentation of benzoic acid compound. By phytochemical assay, fraction 4 contain flavonoid.

**Key words:** *Sida rhombifolia* L., flavonoids, xanthine oxidase, inhibition kinetics, antigout

### INTRODUCTION

Gout results from overproduction or underexcretion of uric acid and is associated with nucleic acid rich diets and the catalysis of xanthine by Xanthine Oxidase (XO) which produces uric acid. Allopurinol, an important treatment for gout in modern medicine (Connor, 2009), acts as a substrate for and competitive inhibitor of XO and as a noncompetitive inhibitor at higher concentrations (Hardman and Limbird, 1975). XO inhibitors have been found in a wide variety of plants used in traditional medicines for the treatment of gout and rheumatism from South America (Theoduloz *et al.*, 1991; Hayashi *et al.*, 1989; González *et al.*, 1995) and northeastern North America (Owen and Johns, 1999). *Sida rhombifolia* L., known as *Sidaguri* in Indonesian, is used in Indonesian herbal medicine for treatment of gout with proven uric acid prohibitive action (Dharma, 1985) and anti-inflammatory action (Soedibyoy, 1998).

Several natural compounds such as flavonoids have been reported as inhibitors of XO and this class of

compounds has promise for greater use in gout treatment. Allopurinol, theaflavin, theaflavin-3-gallic theaflavin-3-3'-digallic(-)-epigallocatechin-3-gallic and gallic acid have been reported as inhibitors for XO through the competitive inhibitor mechanism (O'Driscoll *et al.*, 1999; Lin *et al.*, 2000), while flavonol crisin, luteolin, caempferol, kuercetin, myricetin and isorhamnetin, as well as (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin gallic from tea are reported to inhibit XO by the noncompetitive inhibitor mechanism (Aucamp *et al.*, 1997). The *Materia Medica* Indonesia (Departemen Kesehatan Indonesia, 1995) records the presence of the alkaloid rhombifolina in *Sida rhombifolia* L. which could inhibit activity of the XO. The flavonoids crude extract of *Sida rhombifolia* L. was shown to inhibit XO by up to 55% and to lower uric acid (Iswantini and Darusman, 2003; Iswantini *et al.*, 2005), however, there was no experiment conducted to determine the inhibition kinetics of the extract or the potential inhibitory effects of purified fractions of the extract. Therefore, this research was conducted to

determine the inhibition kinetics of the flavonoids crude extract, the inhibition effects of the different fractions and to identify the compounds of the active fraction.

## MATERIALS AND METHODS

**Plant materials:** *Sida rhombifolia* L. plants were collected from its natural habitat in Bogor, East Java, Indonesia.

Preparation of flavonoid crude extract. The herb (stem and leaf) was cleaned, sun-dried, ground into powder and extracted using modified Markham method (Markham, 1988). Approximately 200 g sample was treated with hexane to remove the lipid constituent and macerated using methanol: water (MeOH:H<sub>2</sub>O 9:1 and 1:1) mixtures until the residue was free of flavonoids and then evaporated at 35°C. The extract was freeze-dried and tested qualitatively for flavonoids (Harborne, 1987). The toxicity assay was performed on *Artemia salina* (Finney, 1971).

**The inhibitory assay on XO:** The optimum conditions for the assay were obtained from earlier study by Tamta *et al.* (2005) with modifications. We measured the inhibitory effect of the crude extract in concentrations ranging from 100 to 800 mg L<sup>-1</sup>, while for the fractioned flavonoids we employed concentrations based on the toxicity test results. Into each extract, potassium phosphate buffer 50 mM pH 7.5 was added until the volume reached 1.9 mL. One milliliter xanthine 2.1 mM and 0.1 mL XO of 0.1 unit mL<sup>-1</sup> were added. The solution was incubated for 45 min at 20°C and then 1 mL HCl 0.58 M was added to terminate the reaction. The absorbance of the mixed solution was measured at 262 nm.

**The kinetics inhibition assay on XO:** The assay was conducted on the first concentration of the flavonoids crude extract to exhibit an inhibition effect greater than 50%. Lineweaver-Burk plot analysis was performed to determine the mode of inhibition by the active compounds. This kinetics study was carried out in the absence and presence of active compounds with varying concentrations of xanthine as the substrate.

**Fractionation of flavonoid crude extract:** Fractionations were performed using column chromatography with silica gel column G-60 as solid adsorbent and the best eluent (CHCl<sub>3</sub>:MeOH), in a gradient of polarity level. Flavonoids crude extract (4.609 g) were fractionated twice, consisted of 1.000 g on 2.4×33 cm column (first column) and 3.608 g on 3.0×76.5 cm (second column) with a constant flow rate

of 1-1.54 mL min<sup>-1</sup>. Eluates from fractionations were collected for every 5 mL and the eluates with similar Rf and chromatogram pattern were combined and viewed using analytical TLC (Thin Layer Chromatography). The fractions were freeze-dried, the yields were measured and the cytotoxicity was tested on *Artemia salina*. The cytotoxicity values were subsequently used to determine the maximum concentration limited tolerance in the inhibitory effect assay on XO.

**Compound identification of active fraction:** Identification was performed on the fraction showing the highest inhibition power. All fractions were identified using a Fourier transformed infrared (FTIR) spectrometer instrument. To identify the purity of the fraction of crude extract including flavonoid and component fragmentation pattern, we conducted GC-MS analysis using an Agilent Technologies 6890 Gas Chromatograph with Auto Sampler and 5973 Mass Selective Detector and Chemstation data system with 17 m×0.25 mm i.d. HP Ultra 2 with 0.25 µm film thickness. Initial oven temperature was held at 70°C for 1 min, then increased at 5°C min<sup>-1</sup> to 150°C, held for 4 min, then increased at 10°C min<sup>-1</sup> to 260°C and held for 35 min, with helium carrier gas and constant column flow (0.9 µL min<sup>-1</sup>), injection volume 5 µL and split 10:1.

## RESULTS AND DISCUSSION

The potency of flavonoids crude extract against gout could be determined by examining the inhibition effect of the extract on the XO. The dried sample yielded 11.85% crude extract and LC<sub>50</sub> of 501.23 mg L<sup>-1</sup> using probit analysis. Furthermore, assay of inhibitory effect of the crude extract have been performed with concentration less than its LC<sub>50</sub> value.

Table 1 shows the inhibitory effect increasing with increasing extract concentration, except for a decrease at 300 mg L<sup>-1</sup>. The decreasing of inhibitory effect at that concentration may be caused by various components in the sample extracted by methanol-water. Compounds

Table 1: Inhibitory effect of flavonoid crude extract of *Sida rhombifolia* at various concentrations

Extract concentration (ppm)	Activity (mM/L/min)	Inhibitory effect (%)
100	74.54	47.71
200	65.25	54.23
300	68.31	52.08
400	60.55	57.53
500	57.11	59.94
600	55.21	61.27
700	49.10	65.56
800	41.75	70.71
Control	142.56	-

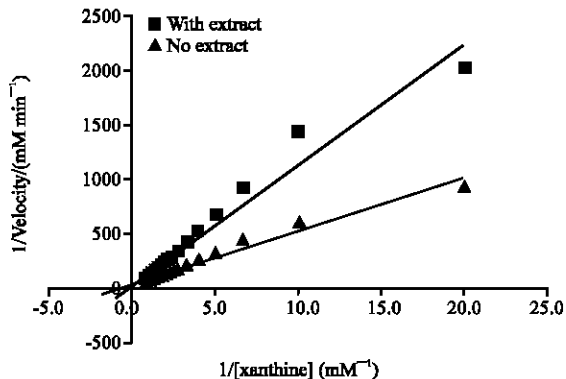


Fig. 1: Lineweaver-Burk plot of XO inhibition with various concentrations of xanthine, addition of 200 ppm of flavonoids extract of *Sida rhombifolia* L., no extract and Lineweaver-Burk transformed data were plotted and followed by linear regression of the points

extractable by methanol-water mixtures are carbohydrates, terpenoids and to a small extent alkaloids (Harborne, 1987), including NADH which acts as a XO activator (Millar *et al.*, 2002). Noro *et al.* (1983) reported that an extract can be considered as useful for gout treatment if its inhibitory effect on XO is more than 50%. Present findings indicate that concentrations above 200 ppm have inhibition of more than 50%, suggesting that the flavonoids crude extract has potential as a treatment against gout.

The inhibition kinetic assay was conducted only for 200 mg L<sup>-1</sup> of the flavonoid crude extract. At higher concentrations, various compounds in the extract may interfere with the enzymatic reaction. To understand the mode of the enzyme inhibition, the Lineweaver-Burk plot was established (Fig. 1).

The extract caused a change of kinetic parameters. Michaelis-Menten constant of K<sub>M</sub> increased from 0.87 to 2.02 mM, V<sub>max</sub> decreased from 0.01892 mM min<sup>-1</sup> to 0.01893 mM min<sup>-1</sup>. ANOVA analysis on V<sub>max</sub> values showed a non-significant value (p<0.01). Based on this data, the inhibition of XO was in a competitive mode. The inhibitor affinity value (α) on XO was calculated by ratio of the K<sub>M</sub> value of the reaction containing extract/inhibitor with the K<sub>M</sub> value of the reaction without extract/inhibitor. The resulting α was 2.32, which is better than pyrazolopyrimidin (α = 2.10; Tamta *et al.*, 2005), but less than quercetin from *Allium cepa* (α = 2.92; Hanaee *et al.*, 2004). This data showed that the competitive inhibition is strong (α>2). The competitive inhibition kinetic pattern from this research supports former research (Lin *et al.*, 2000, 2002),

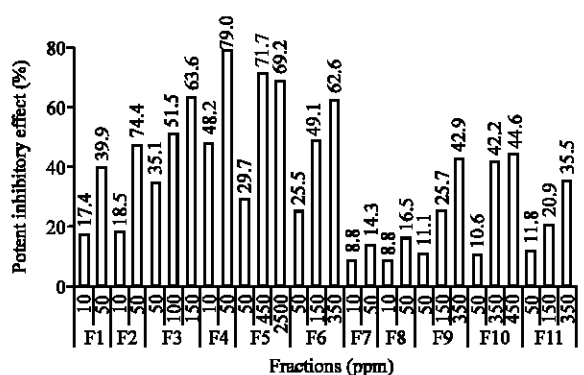


Fig. 2: Potent inhibitory effect of several purified fractions on the activity of xanthine oxidase

Table 2: Profile of chromatography column and yield of fractions

Fraction*	Color/fraction form	Yield (%)
1	Dark green/semi-solid	0.93
2	Green/semi-solid	1.64
3	Pale green/semi-solid	0.54
4	Pale green/semi-solid	0.41
5	Yellow-brown/solid	1.60
6	Dark green/solid	1.41
7	Brown-red/solid	3.36
8	Dark brown/solid	17.14
9	Brown-red/solid	4.25
10	Brown/solid	13.29
11	Dark brown/solid	30.18

suggesting that flavonoids may be competitive inhibitors of XO. An active compound from wheat leaf (4-aminopyrazolo[3,4-d]pyrimidine) also exhibited the competitive inhibition of XO (Hsieh *et al.*, 2007).

Fractionation of flavonoids crude extract was done in gradient mixture of eluents to obtain good separation pattern. The best eluent was CHCl<sub>3</sub>:MeOH 9:1. Fractionation of 4.6086 g crude extract gave the highest yield on the 11th fraction (30.18%), whereas the fraction with the lowest yield was the 4th fraction (0.41%) (Table 2).

Each fraction from the purification step has a different inhibitory effect (Fig. 2). Among the 11 fractions, fraction 4 showed the strongest inhibitory effect at concentration assay 50 mg L<sup>-1</sup>. And the inhibitory effect of fraction 4 at 50 mg L<sup>-1</sup> was stronger than the highest tested concentrations of the six other fractions that were tested at concentrations higher than 50 mg L<sup>-1</sup>. Based on this, we assume that the compounds in fraction 4 have potential as gout medicine. If we compare with allopurinol at 300 mg L<sup>-1</sup> (inhibitory effect 68.07%, data not shown), fraction 4 has better inhibitory effect. However, compared with the crude extract in the same concentration, allopurinol inhibitory effect is 1.23 times stronger (Table 1). Based on these facts, fraction 4 and the crude

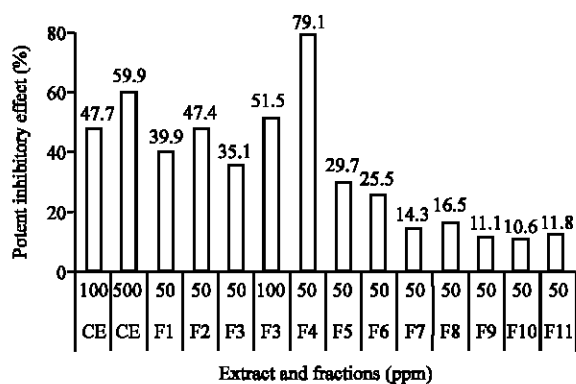


Fig. 3: The inhibitory effect of crude extract and its fractions on the activity of xanthine oxidase. CE: Crude extract; F: Fraction

extract are potential as antigout/gout medicine. The increase of concentration did not always show with a linear increase in the inhibitory effect. This is indicated by fraction 5. When the concentration reached 450 mg L<sup>-1</sup>, the inhibitory effect increased to 71.7% but when the concentration was increased to 2500 mg L<sup>-1</sup> the inhibitory effect was only 69.2%.

Figure 3 shows the potent inhibitory effect of flavonoid crude extract compared with fractions at the concentrations 50 and 100 mg L<sup>-1</sup>. It shows that fractionation generally had little effect on increasing the inhibition power. The purified fraction 3 at the concentration 100 mg L<sup>-1</sup> only showed a slight increase of 8.11% in the inhibitory effect (51.58%) as compared with the crude extract at the same concentration (47.71%). Generally, the potent inhibitory effect of the purified fraction at 50 mg L<sup>-1</sup> was similar or much lower than the potent inhibitory effect of crude extract of 100 mg L<sup>-1</sup> (Fig. 3). Fraction 4 was the exception, showing a stronger potent inhibitory effect (79.1%) compared to that of the flavonoids crude extract (47.7%).

Since, fraction 4 was the most active fraction in term of inhibitory effect we conducted further analysis to characterize its compounds. We used FTIR to reveal the functional groups contained in fraction 4. To estimate the degree of purification of this fraction, we analyzed this fraction with GC-MS.

The FTIR analysis showed some absorbencies with peaks at 3437.50, 2928.28, 2361.30, 1654.82, 1461.79, 1377.42, 1172.50 and 722.19 cm<sup>-1</sup> (Table 3). Wide peak at 3437.50 cm<sup>-1</sup> showed an absorbance caused by -OH stretching from bounded phenol.

GC-MS analysis from fraction 4 showed that there were 39 organic compounds and flavonoid fragments with retention times of 4.14, 6.53 and 6.74 that have resemble

Table 3: The IR absorbance patterns and functional group estimation of fraction 4

Absorbance peak (cm <sup>-1</sup> )	Estimated functional groups	Absorbance range (cm <sup>-1</sup> )
3437.50	Stretching-OH phenol	3650-3300*
2928.28	Stretching C-H aromatic	2926±10*
2361.30	Stretching R-CN aromatic	2400-2350*
1654.82	stretching C = O	1900-1550
1461.79	Stretching C = C aromatic	1496-1466; 1650-1600
1377.42	Stretching C-O phenol	1425-1350 <sup>o</sup>
1172.50	Stretching C-O ether cyclic	1200-1150
722.190	Fold C-H aromatic	900-675

\*Pavia *et al.* (1996)

fragmentation of benzoic acid compounds based on instrument database (chromatograms not shown). These results indicated that fraction 4 was not completely pure. The phytochemical assay was determined on fraction 4. Result indicated that flavonoids are the class of compound identified in this particular fraction. This result is also supported by earlier studies that several flavonoids such as apigenin and isovitexin, quercetin, myricetin and genistein can inhibit XO and exhibit competitive inhibition (Lin *et al.*, 2002). Other research also reported that the size and geometry of the flavonoids are important factors in the positioning of the molecule in order to block the access of the physiologic substrate (hypoxanthine) to the catalytic center of XO. Flavonoids will be good as XO inhibitor when the size is small and the hydrophobic character high (Silva *et al.*, 1996). We did not find research related to identification of flavonoids from *S. rhombifolia* or the genus *Sida*. But some flavonoids have been found in some plants belong to the family of Malvaceae, such as hibiscin, hibiscitrin, hibiscetin, gossypitrin, sabdaritrin, gossypetin and quercetin from *Hibiscus sabdariffa*, luteolin and quercetin from *Kitaibelia vitifolia*, herbacetin, gossypetin and isoscutellarein from *Malope trifida*. These or other flavonoids may also be found in *S. rhombifolia*.

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