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Inhibition Kinetic of *Apium graveolens* L. Ethanol Extract and its Fraction on the Activity of Xanthine Oxidase and its Active Compound

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Abstract: *Apium graveolens*, one of the traditional medicinal plants, has a potential as anti-gout. We have reported that flavonoid of *A. graveolens* could inhibit activity of xanthine oxidase enzyme up to 85.44%. The aim of the research was to investigate the type of inhibition kinetic of *A. graveolens* ethanol crude extract and its fraction inhibition kinetic, also to determine the active compound. The result of the research showed that the yield of *A. graveolens* ethanol crude extract was 10.40 % (LC₅₀ 1968.19 mg L⁻¹) with the inhibition activity was 6.04% until 74.01% (100-2000 mg L⁻¹). Inhibition kinetic of 1500 mg L⁻¹ crude extract caused increased K_M (0.10 mM) and unchanged V_{max}. Based on these data, the type of inhibition was competitive. Purification of crude extract resulted 7 fractions and the highest activity was achieved by fraction 6 (inhibition activity was 85.08%). The purification of crude extract caused the increasing of inhibition activity effect. Inhibition kinetic of fraction 6 (150 mg L⁻¹) caused increase K_M (0.30 mM) and unchanged of V_{max}. Based on that, the type of inhibition was competitive. Purification of fraction 6 resulted 6 fraction and the highest activity was achieved by fraction 5 (inhibition activity was 88.41%). Based on analysis of LCMS and NMR, the active compound of *A. graveolens* extract (fraction 5) were potential to inhibit the activity of xanthine oxidase, the active compound was 5, 7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one and 2, 3-dihydro-6-hidroxy-5-benzofuran carboxylic acid.

Key words: *Apium graveolens* L., ethanol extract, xanthine oxidase, inhibition kinetics, antigout

INTRODUCTION

Gout is a metabolic disorder disease caused by the deposit of uric salt crystals in joints causing acute inflammatory response or deposit of uric acid crystals in cartilage tissue not causing inflammatory response. In the last decade, gout disease is increasing both in developing and developed countries, especially in 40-50 years old male. In US, gout attacked more than 5 million people (Yu, 2007). Number of patients tended to increase from year to year, in line with the pattern of people that likes to consume more foods with high in protein. Allopurinol is used as antigout; the compound can inhibit the activity of Xanthine Oxidase (XO), while the enzyme itself can convert xanthine into uric acid in blood. However, allopurinol can cause side effects such as allergies, fever, chills and gastrointestinal disorders. Therefore, treatment with traditional medicines is important. Methanol extract of *Conyza bonariensis* could *in vitro* inhibited the activity of XO with IC₅₀ of 50.041 mM (Kong *et al.*, 2000).

Barzillian medicinal plant of *Lychnophora* (Filha *et al.*, 2006) and Indian medicinal plants such as: *Coccinia grandis* dan *Vitex negundo* (Umamaheswari *et al.*, 2007) could *in vitro* inhibited the activity of XO up to 50%.

Floral plants having potency as medicinal plants in Indonesia are about 30000 species, one of these plants known its efficacy is celery (*Apium graveolens* L.). Celery is known as vegetable, but this plant is more useful as herbal medicine to treat gout. Celery can be used as an alternative treatment to substitute synthetic drug such as allopurinol. Common known, celery has a curing effect with lower risk and side effect than other synthetic drugs. Iswantini and Darusman (2003) has studied the role of celery active compounds in inhibiting Xanthine Oxidase (XO) activity. But the XO inhibition kinetics of celery crude extract and its fractions study related to gout along to its active compounds determination have not been reported yet. Inhibition kinetics determination of natural active compound used as 'drug-to-be' is important. This is to study the apparent inhibitory mechanism, then

describe the affinity of XO enzyme as a target with the 'drug-to-be' compound, whether it is temporarily (competitive and uncompetitive inhibition) or permanently (non-competitive inhibition). Some natural compounds such as flavonoid and polyphenols were reported to be role as competitive inhibitor against XO. Among of these compounds were theaflavin, theaflavin-3-gallate, theaflavin-3-3'-digallate, (-)-epigallocatechin-3-gallate and gallic acid (Lin *et al.*, 2000) and also an apigenin derivative, apigenin-4'-O-(2''-O-p-coumaroyl)f3-D-glucopyranoside (Jiao *et al.*, 2006). Flavonoid from *Sida rhombifolia* Lamk. was a competitive inhibitor (Iswantini *et al.*, 2009). Some flavonols such as luteolin, caempferol, quercetin, myricetin and isorhamnetin had inhibitory effect through non-competitive mechanism (Nagao *et al.*, 1999). The objective of the study to investigate the type of inhibition kinetic and also determine the active compound.

MATERIALS AND METHODS

Plant materials: *Apium graveolens* L. plants were collected from its natural habitat in Semarang, Center Java, Indonesia.

Preparation of ethanol extract: Dry celery herb (stem and leaves) was extracted by ethanol with sample:solvent ratio of 1:3 (g/v) until the last filtrate showed negative result for flavonoid qualitative test. Next, the mixture was filtrated and concentrated by rotary evaporator. The obtained extract then was freeze-dried and the yield was calculated. The extract was tested for phytochemistry (Harborne, 1987), toxicity against *Artemia salina* L. (Finney, 1971), *in vitro* inhibitory activity and kinetic mechanism against xanthine oxidase, and also was fractioned with chromatography column.

The inhibitory assay to XO: The optimum condition of the assay referred to previous work (Tamta *et al.*, 2005) with modification. We tried to measure the inhibitory effect of the crude extract in various concentrations, ranging from 100 to 800 ppm, while for the purified flavonoids, the concentrations employed were based on toxicity test results into each extracts, potassium phosphate buffer 50 mM pH 7.5 was added until the volume reached 1.9 milliliter. One ml xanthine 2.1 mM and 0.1 mL⁻¹ XO 0.1 unit mL⁻¹ were added. The solutions were incubated for 45 min at 20°C and then 1 mL HCl 0.58 M were added to terminate the reaction. The absorbances of the mixed solution were measured at 262 nm.

The kinetics inhibition assay to XO: The assay was conducted only on selected ethanol crude extract, which

gave the highest inhibition effect. To determine the mode of inhibition by active compounds from the plants, Lineweaver-Burk plot analysis was performed. This kinetics study was carried out in the absence and presence of active compounds with varying concentrations of xanthine as the substrate.

Fractionation of ethanol extract: Fractionation were performed using silica gel column G-60 with the best eluent (CHCl₃: MeOH), in a gradient of polarity level. Ethanol 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 constant flow rate of 1-1.54 mL⁻¹ min. Eluates from fractionations were collected for every 5 mL and the eluate 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. The cytotoxicity values were subsequently used to determine the maximum concentration limited tolerance in the inhibitory effect assay to XO.

Compound identification of active fraction: Identification was performed on the most active fraction that showed the highest inhibition power. All fractions were identified using a Fourier transformed infrared (FTIR) spectrometer. To identify the purity of the fraction, component fragmentation pattern and molecular weight estimation, we conducted analysis using Gas chromatography-mass spectrophotometer (GC-MS).

RESULTS AND DISCUSSION

The dried sample yielded 10.40% crude extract and the phytochemistry test was positive for alkaloid, triterpenoid, steroid, quinone, tannin and flavonoid, while negative for saponnin. Cytotoxicity assay for ethanol crude extract resulted LC₅₀ value of 1969.18 mg L⁻¹ using probit analysis. Furthermore, assay of inhibitory effect of the crude extract have been performed with concentration less than its LC₅₀ value.

Figure 1 showed the inhibitory activity of ethanol crude extract. It showed the increase of inhibition activity that was not in line with increasing of extract concentration. This was suspected the existence of different compounds characteristic in sample which were extracted by ethanol-water solvent. Some groups of compounds can act as inhibitor, while the others as activator such as terpenoids and alkaloids (Harborne, 1987).

The highest percent inhibition of ethanol crude extract inhibition activity (74.01%) with concentration of

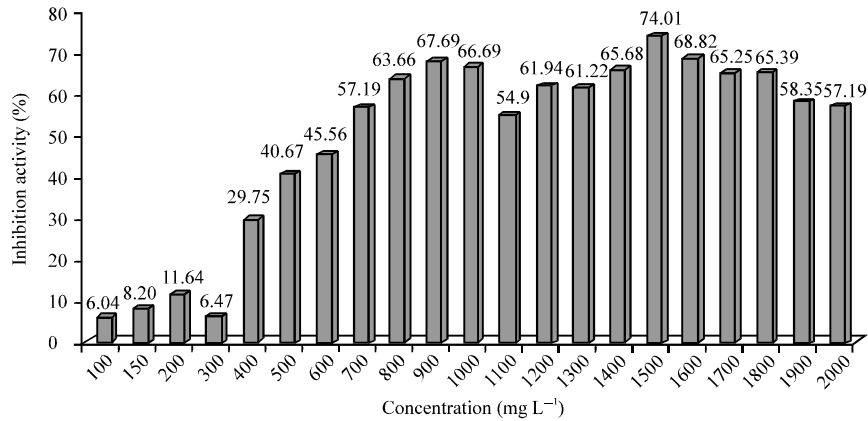


Fig. 1: Inhibition activity of ethanol crude extract against xanthine oxidase

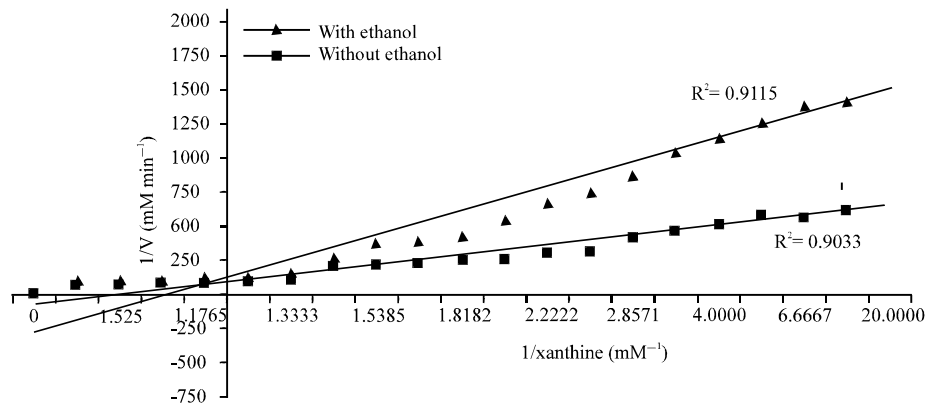


Fig. 2: Lineweaver-Burk plot of xanthine oxidase activity with ethanol crude extract 1500 mg L⁻¹ and without ethanol crude extract 1500 mg L⁻¹

1500 mg L⁻¹, showed that ethanol crude extract had a potency to be used as uric acid medicine or as a compound which was biologically active. This result is comparable with the highest percent inhibition of selected Philippine medicinal plants at 79.67% (Apaya *et al.*, 2011). Concentration of ethanol crude extract used for inhibition kinetic assay was 1500 mg L⁻¹. The selected of this concentration based on its high inhibition activity value (74.01%) and its concentration value which was lower than its LC₅₀ value. Figure 2 showed Lineweaver-Burk plot for the XO activity with and without ethanol crude extract 1500 mg L⁻¹.

Based on graphical analysis of Lineweaver-Burk plot (Fig. 2), it result significant K_M value change and very small V_{maks} change. Kinetic pattern obtained after extract addition caused K_M value increase from 0.29 mM to 0.39 mM and V_{maks} value decrease from 0.0065 to 0.0036 mM min⁻¹. The small of V_{maks} decrease was considered as none. According to Voet and Voet (2001), competitive inhibitor is an inhibition where K_M value

increases and V_{maks} remains constant. From this result, it can be concluded that the type of celery ethanol crude extract inhibition against XO was competitive inhibition. Inhibitor affinity (α) of celery ethanol crude extract was 1.4. This value was obtained by dividing the K_M value of XO activity with ethanol crude extract 1500 mg L⁻¹ addition with K_M value of XO without extract addition. α value obtained showed that competitive inhibition by ethanol crude extract was strong enough, as explained by Voet and Voet (2001) that inhibitor affinity value more than 1 shows strong inhibition activity.

Fractionation of ethanol crude extract was done in gradient mixture of eluents to obtain good separation pattern. The best eluent was CHCl₃:MeOH 9.5:0.5. Fractionation of 10 g crude extract resulted 7 fractions and gave the highest yield on the 1st fraction (22.17%), whereas fraction with the lowest yield was the 3rd fraction (1.98%) (Table 1).

Cytotoxicity assay of seven fractions gave LC₅₀ values which were lower than ethanol crude extract. The

Table 1: Yield of ethanol crude extract fractions of *Apium graveolens*

Fraction	Yield (%)
1	22.17
2	50.80
3	10.98
4	11.80
5	19.92
6	19.90
7	13.60

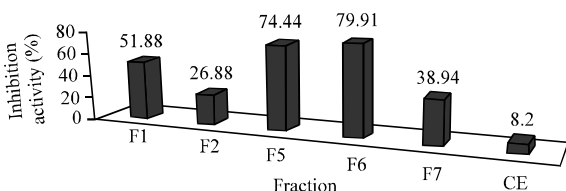


Fig. 3: Inhibition activity of ethanol crude extract (CE) and the fractions (F) against xanthine oxidase

highest LC_{50} value was fraction 7 (478.90 mg L^{-1}), while the lowest was fraction 2 (176.78 mg L^{-1}). These LC_{50} values showed that the fractions were more biologically active than ethanol crude extract. Qualitative flavonoid assay showed positive result for fraction 1, 5, 6 and 7. Furthermore, these four fractions containing flavonoid were assayed for their inhibition activity against XO.

Inhibition activity of fraction 1, 5, 6 and 7 is depicted in Fig. 3. Concentration for each fraction used was 150 mg L^{-1} . The results showed that fraction 6 had the strongest inhibition activity against xanthine oxidase with this concentration, next was fraction 5 and the last was fraction 1 (Fig. 3).

Compared with allopurinol in concentration of 300 mg L^{-1} with inhibition activity of 68.07% (Iswantini *et al.*, 2005), fraction 6 inhibition activity was better (79.91%) or equal to 39.96% when allopurinol concentration was 150 mg L^{-1} . While compared with the crude extract, fraction 6 inhibition activity was 9.75 fold stronger. It is suspected to be caused by the existence of active compound in fraction 6 which was potent antixanthine oxidase. Figure 3 showed the comparison of crude extract and fractions inhibition activity. The fractionations could increase the inhibition activity. In general, inhibition activity of the fractions were stronger than crude extract in concentration 150 mg L^{-1} , this showed that fractionations had effect in increasing the inhibition activity.

Fraction 6, having the highest inhibition activity was assayed for inhibition kinetic (Fig. 3). Figure 4 showed the Lineweaver-Burk plot for XO activity with and without the addition of fraction 6 150 mg L^{-1} .

According to Lineweaver-Burk plot analysis, K_M values for XO activity without and with the addition of

Table 2: FTIR absorbance patterns and functional group estimation of fraction 6

Wavelength No.	Estimated functional group	Wavelength No.
Absorbance peak (cm^{-1})		No. Range (cm^{-1})
3407.90	Stretching -OH phenol	3650-3300*
2927.54	Stretching C-H aromatic	2926±10*
1765.14	Stretching C = O	1900-1550 [†]
1464.15	Stretching C = C aromatic	1496-1466 and 1650-1600
1378.58	Stretching C-O phenol	1425-1350 [‡]
1028.18	Stretching C-O cycle ether	1200-1150
726.33	Fold C-H aromatic	900-675

*Pavia *et al.* (1996); [†]Colthup *et al.* (1975); [‡]Sudjadi

Table 3: The inhibition activity of the fractions from fraction 6

Fraction	Yield (%)	Inhibition activity (%)
1	30.50	40.60
2	11.67	50.76
3	90.55	40.90
4	16.49	80.05
5	80.83	88.41
6	36.97	44.69

fraction 6 were 0.29 and 0.59 mM, respectively while the V_{max} values were 0.0065 and 0.0029 mM min^{-1} for XO activity without and with the fraction 6 addition respectively. K_M and α values of fraction 6 were higher than crude extract. It showed the high competition between substrate and inhibitor for XO active site. Besides, it was caused by the inhibitor having more pure component. Based on these data, it can be concluded that the type of fraction 6 inhibition against XO was competitive inhibitor.

To identify the active compounds as XO inhibitor, we did FTIR, LC-MS and NMR analysis. FTIR analysis for fraction 6 gave absorbance as showed in Table 2.

Advanced purification was done of fraction 6 to identify the more pure compound as XO inhibitor. Fraction 6 was fractionated again using HPLC and gave 6 fractions. Each fraction then was tested for its inhibition activity against XO and fraction 5 was the highest one (Table 3). After that fraction 5 was analyzed using LC-MS and NMR.

LC-MS analysis for fraction 5 showed that this fraction contained two compounds with $[M+H]^+$ 270.88 and 195.01 g mol^{-1} . The value from LC-MS analysis gave two compounds: 5, 7-dihydroxy-2-4-(hydroxyphenyl)-4H-1-benzopyran-4-one and 2, 3-dihydro-6-hydroxy-5-benzofurane carboxylic acid, with structure shown in Fig. 5 and 6, respectively from NMR database.

Proton NMR analysis for fraction 5 gave chemical shifts (i, ppm) as below:

- **Compound 1:** 168, 8 (C, t), 156, 6 (C, t), 125, 6 (C, t), 115, 6 (C, t), 105, 0 (C, q), 97, 6 (C, t), 94, 9 (C, t)
- **Compound 2:** 152, 0 (C, t), 132, 3 (C, q), 121, 5 (C, q)

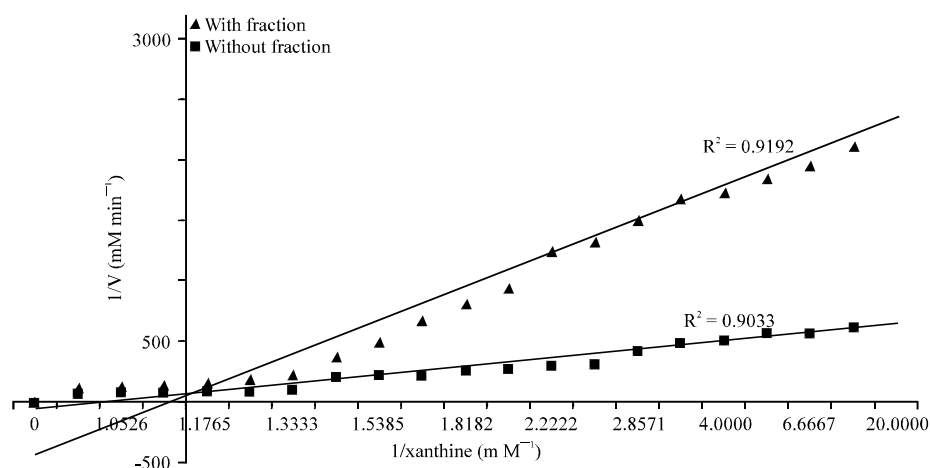


Fig. 4: Lineweaver-burk plot of xanthine oxidase activity with fraction 6150 mg L⁻¹ and without fraction 6150 mg L⁻¹

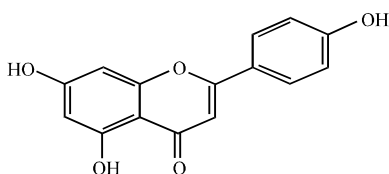


Fig. 5: Compound 1 : 5, 7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one

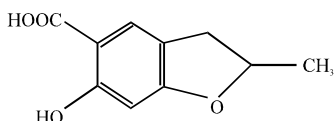


Fig. 6: 2, 3-dihydro-6-hydroxy-5-benzofurane carboxylic acid

NMR database informed that compound 1 was a flavonoid apigenin derivative while compound 2 was unknown. These two compounds were considered as XO inhibitor as reported by Jiao *et al.* (2006) that active compound from *Palhinhae cernua* which could inhibit XO activity was a flavonoid group compound.

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