

ANTIHYPERURICEMIC ACTIVITY OF FOUR PLANTS ANNONACEAE USING HYPERURICEMIC RATS MODEL AND ENZYME ASSAY**TITIK SUNARNI^{1*}, FRANSISKA LEVIANA¹, IRDA FIDRIANNY², MARIA IMMACULATA IWO³,
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ABSTRACT

Objective: The aim of the research is to study the antihyperuricemic activity of *Annona muricata* L, *Annona squamosa* L, *Annona reticulata* L, and *Stelechocarpus burahol* (Bl.) Hook. F. & Th. (Annonaceae).

Methods: The dried powdered leaves were extracted using ethanol by maceration method. The ethanolic extracts of four plants were administered orally to potassium oxonate-induced rats. Serum samples were collected from rats at the 1st to 3rd hr after drug administration. Enzyme assay was done by xanthine oxidase (XO). The XO inhibitory activity was measured by UV-Vis spectrophotometry, and the degree of enzyme inhibition was determined by measuring the absorbance of uric acid formation.

Results: The result of this study revealed that all of the ethanolic extracts had a significant effect on hyperuricemic rats, and *S. burahol* extract decreased uric acid level was equal to level normal control. However, the antihyperuricemic activity was not parallel with XO inhibitor activity which showed weak activity with IC₅₀ of *A. reticulata* was 171.73 µg/ml, while IC₅₀ of *A. muricata*, *A. squamosa*, and *S. burahol* more than 200 µg/ml.

Conclusion: The ethanolic extract of *S. burahol* was more potential than three other extracts in decreasing in uric acid levels. The results scientifically confirmed that *S. burahol* can be developed as potential antihyperuricemic agents.

Keywords: Antihyperuricemia, *Annona muricata*, *Annona squamosa*, *Annona reticulata*, *Stelechocarpus burahol*, Potassium oxonate, Xanthine oxidase.

INTRODUCTION

Hyperuricemia is a common metabolic disorder in human. It may be an asymptomatic condition, associated with uric acid blood level greater than 6.8 mg/dl [1]. Increasing in uric acid level could happen either on man or woman. Epidemiology study showed an increasing prevalence of gout, especially in the older group [2]. Increasing in uric acid level also leads to form urate crystals in the joints, causing gout, and in the kidney, predisposing to urate nephrolithiasis [3]. Recently, hyperuricemia linked to cardiovascular diseases [4]. Uric acid is the end product of nucleic acid metabolism in human with the loss of uricase that is formed from nucleic acid either endogenously from cell breakdown or exogenously from metabolism of food [5]. The amount of urate in the blood depends on the dietary intake of purines, urate biosynthesis, and the rate of urate excretion. Overproduction or under excretion of uric acid leads to hyperuricemia [6].

Xanthine oxidase (XO), a molybdenum-containing enzyme, catalyzes the oxidation of hypoxanthine to xanthine and ultimately to uric acid [7]. The target of drugs against hyperuricemia and gout is XO. Synthetic XO inhibitor from purine analog group, allopurinol, is widely used in the therapeutic and clinical management of gout [8]. However, the use of allopurinol can be related to a number of side effects, indicating allergic reactions, such as skin rash. In some cases more severe hypersensitivity reactions may be seen, such as Steven-Johnson Syndrome [9].

A potential source of such compounds can be obtained from medicinal plants. Therefore, the attention in recent times has been focused on the isolation, characterization, and utilization of antihyperuricemia natural. Many Indonesian medicinal plants have been used for the preventing and gout treatment and related with an inflammatory disorder, but they still lack of sufficient scientific evidence.

Stelechocarpus burahol, *Annona muricata*, *Annona squamosa*, and *Annona reticulata* belong to the Annonaceae, which are traditionally used by the local people in Indonesian for gout treatment, rheumatic, and cancer. It has been reported in previous studies that *A. muricata* leaves had antinociceptive, anti-inflammatory [10], antipyretic [11], antidiabetic, antihyperlipidemia, antioxidant [12], and cytotoxic [13] effect. Recently, butanol leaves extract of *A. muricata* was confirmed to have antihyperuricemia in rats [14]. Several reports showed that the leaf extract of *A. squamosa* had antioxidant, anti-lipidemic activity [15,16], and antidiabetic activity [17]. The leaves of *A. reticulata* had been investigated for antimicrobial [18] and NO inhibitor activity [19]. *S. burahol* leaves demonstrated that had antioxidant [20], antihyperuricemic activity on rooters [21], and rats [22]. There was no study regarding antihyperuricemic activity of four plants Annonaceae using hyperuricemic rats model and enzyme assay. Therefore, this study aimed to study XO inhibitor and antihyperuricemic activities in oxonate-induced rats of four Annonaceae (*A. muricata*, *A. squamosa*, *A. reticulata*, and *S. burahol*).

METHODS**Analytical materials**

Xanthine, XO, potassium dihydrogen phosphate, dimethyl sulfoxide (DMSO), potassium oxonate were purchased from Sigma Chemicals Co. Allopurinol was obtained from Ifars Pharmaceutical Laboratories. The uric acid kit was purchased from Sclavo Diagnostic.

Plant materials

The plant materials were collected from Central of Java, Indonesia and determined in School of Life Science and Technology, Bandung Institute of Technology. The materials were washed, wet sortation, dried, and grinded into powder.

Preparation of extracts

The dried leaves powder of each plant 500 g was macerated with 2.5 l ethanol 96% for 5 days at room temperature. After 5 days, the mixture was filtered and the residue washed by using ethanol and treated for 5 days of the same treatment as before. Solvent was evaporated by rotary evaporator yielding extract of *A. muricata* 39.7 g, *A. squamosa* 69.7 g, *A. reticulata* 45.4 g, and *S. burahol* 51.1 g, respectively.

Animal

Male Wistar rats weight 170-200 g (age, 7-8 weeks) were obtained from the animal laboratory (School of Pharmacy, Bandung Institute of Technology). The rats were allowed to adapt to their environment at a constant temperature of 25°C for a week before being used. They were given free access to feed standard pellets and water during the study. All the procedures were approved by Animal Ethics Committee, Bandung Institute of Technology.

Animal model of hyperuricemia in rats and drug administration

Hyperuricemic rat model was prepared by induced using potassium oxonate which act as uricase inhibitor. Experiments were performed using the Liu's method [23] with minor modification in potassium oxonate dose. All of the rats were divided at random into normal control, hyperuricemic control, allopurinol control, and sample administration groups (n=6 each). Allopurinol and ethanolic extract of *A. muricata*, *A. squamosa*, *A. reticulata*, and *S. burahol* were suspended in 0.3% carboxymethylcellulose sodium (CMC-Na). Ethanolic extract of plants at a dose of 75 mg/kg body weight (bw) were given by orally to rats. Allopurinol suspension was used as reference drug. It was also administered in the same manner at a dose of 10 mg/kg bw. The volume of the suspension which was administered based on bw. The rats were fasted 1 day before being used in the experiment. Water was withdrawn from the animals 1 hr prior to the drug administration. The animals were transferred to the laboratory at least 1 hr before the potassium oxonate-induced hyperuricemia experiment. Before inducing by potassium oxonate, blood was collected for uric acid level on 0th hr. Briefly, rats were injected intraperitoneally with potassium oxonate (250 mg/kg bw suspended in CMC-Na 0.3%) to increase the serum uric acid level, except normal control group, 1 hr before the drug administration. Blood samples were collected from rats by tail vein bleeding in duration 1st hr to 3rd hr for obtaining uric acid level on 1st, 2nd, and 3rd hr after drug administration. The blood was allowed to clot for 30 minutes at room temperature and then centrifuged at 10.000 × g for 7 minutes to obtain the serum. The serum was stored at -20°C. Serum uric acid level was determined by enzymatic-colorimetric method, using a standard diagnostic kit, according to manufacturer's instructions.

In vitro XO inhibitor activity

All of the ethanolic leaves extracts *A. muricata*, *A. squamosa*, *A. reticulata*, and *S. burahol* were tested for their *in vitro* XO inhibitory activity. The XO inhibitor activity was determined by spectrophotometrically under aerobic conditions using xanthine as the substrate. Experiment was conducted according to methods which were reported by Abdullahi [24] with minor modification in phosphate buffer volume. The mixture which consisted of 1 ml extract (25-200 µg/ml), 0.9 ml phosphate buffer 50 mM (pH 7.5 at 25°C), and 0.1 ml XO enzyme solution (0.1 unit/ml in phosphate buffer, pH 7.5) was prepared immediately before using. After preincubation at 25°C for 15 minutes, the reaction was initiated by adding 2 ml of substrate solution (0.15 mM xanthine solution, prepare fresh). Then the mixture was incubated at 25°C for 30 minutes. The reaction was then stopped by adding 1 ml 1N hydrochloric acid, and the absorbance was measured at λ 287 nm using UV-Vis spectrophotometer. Different concentrations of the extract (25-200 µg/ml) were dissolved in DMSO. Extracts were added in appropriate volume so that the final concentration of DMSO in the assay did not exceed 4% of the total volume. Allopurinol (0.2-1.6 µg/ml), a known inhibitor of XO, was used as the positive control. All of the experiments were conducted in triplicate. The inhibitory activity of XO was assessed as the inhibitory percentage (%):

$$\text{Inhibition (\%)} = \left\{ \frac{(A - B) - (C - D)}{(A - B)} \right\} \times 100$$

Where, A is the activity of the enzyme without the extract, B is the control of A without the extract and enzyme; C and D are the activities of the extract with and without XO, respectively. The assay was done in triplicate, and IC₅₀ values were calculated from the percentage of inhibition.

Statistical analysis

Results showed and represent the mean ± standard deviation. The statistical significant of difference was calculated by the Analysis of Variance followed by Tukey *post hoc* test. Statistical significant was set at p<0.05.

Phytochemical screening

Preliminary phytochemical screening of the plant extract was performed for determining the presence of alkaloids, phenolics, tannins, and flavonoids.

RESULTS AND DISCUSSION

Antihyperuricemic activity

Hyperuricemia is the most important risk factor for the development of gout. It has been involved in many diseases such as renal dysfunction, cardiovascular diseases, hypertension, hyperlipidemia, diabetes, and metabolic syndrome [25]. Hyperuricemia occurs, as a result, increasing in uric acid production; impair renal uric acid excretion, or a combination of these mechanisms [3]. The development of gout requires three distinct steps: Prolonged hyperuricemia, the formation of monosodium urate monohydrate (MSU) crystals and interaction between MSU crystals, and the inflammatory system [26]. Control of hyperuricemia is most often achieved by reducing uric acid production with an inhibitor of XO, the enzyme catalyzing the two terminal reactions in uric acid synthesis (the oxidation of hypoxanthine to xanthine and of xanthine to uric acid) or, less frequently, by employing uricosuric agents to increase renal clearance of uric acid. Accordingly, the use of the XO inhibitor that blocks the synthesis of uric acid in the body should be one of the therapeutic approaches for hyperuricemic treatment. Until recently, allopurinol was the antihyperuricemic drug with worldwide availability [27].

A potential source of novel antihyperuricemic agents may be derived from the natural products. Flavonoids are groups of natural products with varied biological and pharmacological activities. Some studies on the structure - activity relationship of different chemical classes of flavonoids as potential inhibitors of XO *in vitro*. Thus, various flavonoids were evaluated *in vitro* effect on XO, which showed that the planar flavones and flavonol with the 7-hydroxyl group had a highly inhibitory effect on XO [28]. A natural product, such as polyphenol alkyl caffeate [7] and dicaffeoyl quinic acid methyl ester [29], was demonstrated that effective in inhibiting uric acid formation *in vitro*. Oral administration of quercetin, morin, myricetin, kempferol, apigenin, and puerarin with a dose of 50 and 100 mg/kg bw for 3 days was able to elicit hypouricemic actions in hyperuricemic mice induced by potassium oxonate. In addition, quercetin, morin, myricetin, kempferol, and puerarin exhibited significant inhibition on the liver XO activities [30]. The extract of *S. burahol* and *A. muricata* leaves were being frequently used in traditional for gout treatment [14,31], and so *A. squamosa*, *A. muricata*, and *A. reticulata* for rheumatic treatment [10,32,33].

Uricase inhibitor potassium oxonate treatment caused hyperuricemia in rats, which was indicated by drastic increasing in serum uric acid levels. As shown in Fig. 1, before potassium oxonate treatment at 0 hr, uric acid level in all of treatment groups were not significantly different from each other as well as from the normal control rats. The normal control group which was given only by CMC-Na showed no significant different in serum uric acid level. In contrast, after potassium oxonate and oral

drug administration, at 1 and 2 hrs, uric acid level of the hyperuricemic control group was significantly increased compared to normal control group. The extracts treatment groups, 1 hr after oral administration, *S. burahol* extract at a dose of 75 mg/kg bw was significantly reduced uric acid level compared to the hyperuricemic control group and at the same dose, *A. squamosa*, *A. muricata*, and *A. reticulata* extract gave significantly reduced in uric acid level. The activity of *S. burahol* extract on the uric acid level was higher than three other extracts. The profile uric acid level at 2 and 3 hrs after drug administration orally still increased in the hyperuricemic control group. Furthermore, the serum uric acid level of an oxonate-induced rat in extract group at 3 hrs was significantly different with normal control ($p < 0.05$). These data indicated that the all of extracts had potential activity, but they did not complete the recovery, except *S. burahol* extract. It demonstrated that *S. burahol* extract which was administered orally could reduce uric acid level significantly compared to hyperuricemic control ($p < 0.05$). The allopurinol and *S. burahol* treatment group could reduce uric acid level until normal level.

The oral pre-treatment of allopurinol at 10 mg/kg bw elicited significant reduction of serum urate level in the hyperuricemic rats to the normal value at 1 and 2 hrs. Among the four plants extracts of Annonaceae that have been examined, *S. burahol* seemed to be more effective in reducing the uric acid level than *A. muricata*, *A. squamosa*, and *A. reticulata* extracts after oral administration. Comparison study showed that potency *S. burahol* extract appeared to be a not significantly difference with allopurinol group at 1 hr. These results suggested that *S. burahol* extract was capable in reducing the accumulation of purine metabolites in blood following oxonate-induction. Previously, *S. burahol* was disclosed to contain flavonoids in an ethanolic fraction of the aqueous extract [20].

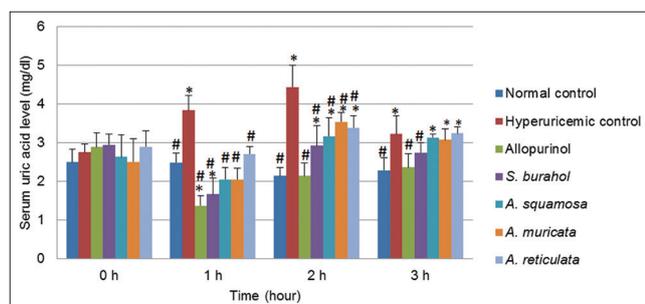


Fig. 1: The effect of *Stelechocarpus burahol*, *Annona squamosa*, *Annona muricata*, *Annona reticulata* leaves extracts and allopurinol on serum uric acid level in hyperuricemic rats. Note: Blood was collected before the potassium oxonate treatment and after drug administration (0, 1, 2, and 3 hrs). Data represent mean value (\pm standard deviation) of serum uric acid level (mg/dl). *significant different compared to normal control ($p < 0.05$), #significant different compared to the hyperuricemic control ($p < 0.05$)

XO inhibitor

In this study, the extracts of four different plants that belong to Annonaceae families were investigated their potential as XO inhibitors *in vitro*. The XO inhibitory activity of extracts was evaluated by the xanthine-XO enzymatic system. The degree of XO inhibition activity was evaluated for all of the extracts at a concentration of 25-200 $\mu\text{g/ml}$. The influence of the extracts in XO inhibitor activity was evaluated by decreasing in the production of uric acid, which was measured by UV-Vis spectrophotometry. The result of XO inhibition activity of four plants was summarized in Table 1. The results indicated that ethanolic extract of *S. burahol*, *A. squamosa*, and *A. muricata* inhibited the XO with the IC_{50} value > 200 $\mu\text{g/ml}$, except *A. reticulata* extract was more potent than three other extracts with IC_{50} 171.73 $\mu\text{g/ml}$. The IC_{50} value of XO inhibitor > 100 $\mu\text{g/ml}$ was a less inhibitory effect [28]. The IC_{50} of *S. burahol*, *A. squamosa*, and *A. muricata* extracts were not identified at 200 $\mu\text{g/ml}$. The extract could not be tested at a higher concentration more than 200 $\mu\text{g/ml}$ due to their poor solubility and the final concentration of DMSO exceeds 5% [24]. In contrast, allopurinol, a known inhibitor of XO, was used as positive control in the studies showed IC_{50} 1.21 $\mu\text{g/ml}$.

Phytochemical screening of four plants showed the presence of alkaloid, phenolic, tannin, and flavonoid compounds. Moreover, many studies also indicated that flavonoid compounds had XO inhibitor activity [28,34] and antihyperuricemia in an animal model [30]. The previous research by Chen *et al.* [35] exposed that 6,8,4'-trihydroxyflavanone-7-C- β -D-glucopyranoside, and epiphyllocoumarin-3-O- β -D-allopyranoside had significantly inhibited XO activity *in vitro* and reduced serum uric acid levels *in vivo* [35]. The present study revealed that leaves extract of *A. muricata*, *A. squamosa*, *A. reticulata*, and *S. burahol* did not show strong XO inhibitor activity *in vitro*, but had significant effect on antihyperuricemia *in vivo*. Inconsistency between the *in vitro* and *in vivo* data may be due to the differences in bioavailability of the extract constituents and their metabolism in rats. In general, bioactivities of drugs *in vivo* can be influenced by many factors which was involved in absorption, metabolism, and distribution. When administered orally, drugs are susceptible to metabolize by intestinal bacteria flora and various enzymes, in some cases the metabolites are responsible to have pharmacological activity. Allopurinol is converted by XO to its main metabolite, oxipurinol, which is also a potent XO inhibitor and contributes to the hypouricemic action of allopurinol [29]. Previous study [36], regarding antihyperuricemia *in vitro* and *in vivo* of flavonoid demonstrated that genistein, apigenin, quercetin, rutin, and astilbin did not show any significant effect on XO activity *in vitro*, but had significant effect on XO activities *in vivo*.

CONCLUSION

The ethanolic leaves extract of *A. muricata*, *A. squamosa*, *A. reticulata*, and *S. burahol* reduced serum uric acid level in oxonate- induce rats, but less inhibitor effect on XO activity *in vitro* assay.

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Table 1: Percentage of XO inhibitor of extracts

Extract	Percentage of XO inhibition				IC_{50} ($\mu\text{g/ml}$)
	25 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$	200 $\mu\text{g/ml}$	
<i>S. burahol</i>	2.40 \pm 2.03	5.29 \pm 0.29	8.54 \pm 1.72	12.03 \pm 1.44	> 200
<i>A. squamosa</i>	2.33 \pm 1.18	2.98 \pm 0.66	6.37 \pm 0.53	-20.74 \pm 5.99	> 200
<i>A. muricata</i>	8.53 \pm 1.41	11.27 \pm 0.78	14.18 \pm 4.29	20.46 \pm 1.09	> 200
<i>A. reticulata</i>	12.1 \pm 1.09	28.34 \pm 4.74	47.38 \pm 4.31	50.38 \pm 2.96	171.73 \pm 17.74
Standard	Percentage of XO inhibition				IC_{50} ($\mu\text{g/ml}$)
	0.2 $\mu\text{g/ml}$	0.4 $\mu\text{g/ml}$	0.8 $\mu\text{g/ml}$	1.6 $\mu\text{g/ml}$	
Allopurinol	3.61 \pm 2.54	9.85 \pm 1.29	35.65 \pm 4.39	69.02 \pm 2.28	1.21 \pm 0.05

XO: Xanthine oxidase, *A. muricata*: *Annona muricata*, *A. squamosa*: *Annona squamosa*, *A. reticulata*: *Annona reticulata*, *S. burahol*: *Stelechocarpus burahol*

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