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Research Article

In Vitro Xanthine Oxidase Inhibitor Activity of Ethanol Extract and Fraction Roselle Calyx (*Hibiscus sabdariffa* L.)

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ABSTRACT

Objective: The objective of this study is to evaluate xanthine oxidase inhibitor activity of ethanol extract and fraction of roselle calyx (Hibiscus sabdariffa L) in vitro. Methods: The principle of measurement of xanthine oxidase activity inhibition is measuring the reduce of uric acid amount formed from the reaction catalyzed by xanthine oxidase, The uric acid is formed from the reaction between hypoxanthine and xanthine with xanthine oxidase enzyme. In this study allopurinol is use as reference drug. Result: The results showed that ethanol extract (100 µg/ml) can inhibit the activity of xanthine oxidase enzyme 25.13 %, water fraction inhibit 25.81% and the ethyl acetate fraction inhibit 32.25 %. with IC_{50} value of extract is 1294.37, IC_{50} water fraction 830.71 and IC_{50} value of ethyl acetat fraction is 290.62 µg/ml . Conclusion: Ethanol extract and fractions Roselle calyx have xanthine oxidase inhibitor activity with IC_{50} value of extract is 1294.37, IC_{50} water fraction 830.71 and IC_{50} value of ethyl acetat fraction is 290.62 µg/ml

Keywords: *Hibiscus sabdariffa* L, xanthine oxidase, extracts, fractions, antihyperuricemia.

INTRODUCTION

The prevalence of patients suffered from gout or hyperuricemia in Indonesia is around 6-7%, and it will increases every year[1-3]. Hyperuricemia is a state of high uric acid levels, which achieve blood level of ≥7 mg/dl in man and ≥6 mg/dl in women. High levels of uric acid in the blood lead to precipitation of uric acid in joint and cause spam and several pathologic condition namely gout[1,4,5]. Lowering of elevated uric acid level in the blood could be achieved by xanthin oxidase inhibitors and inhibitor of renal urate reabsorption[6]. Uric acid is the end product of purine catabolism in the body to be excreted through the kidneys. In the metabolism of purines, xanthine and hypoxanthine dioxidase will be converted into uric acid by the enzyme xanthine oxidase. Excessive production of uric acid in the body can affect the excretion of uric acid by the kidneys; it can decrease the excretion of uric acid and causes hyperuricemia[5]. Hyperuricemia may occur as a result of an increase in uric acid metabolism, decreased excretion of uric acid or a combination of both. Gout will lead to kidney disorders disease, hypertension, diabetes, cancer. Chronic hyperuricemia may stimulating rennin-angiotensin system and inhibit the release of endothelial nitric oxidase, causing renal vasoconstriction and increase blood pressure. Hyperuricemia can also lead to metabolic syndrome through endothelial dysfunction, inflammation, as well as kidney disease, and prognosis existing kidney disease[5,6]. Up to know, xanthine oxidase inhibitor known as medicine is only allopurinol which has several side effect including neuritis peripher, nephrolithiasis, allergic reaction and increase the toxicity of 6- mercaptopurine[13] therefore it is necessary to find plant materials that could be used to lower uric acid levels. One of the plants that can be used to lower uric acid is Roselle calyx that traditionally has been used for treating hypertension, diabetes, lowering cholesterol, as a diuretic, protects infection, blood circulation, prevent heart disease and can lower uric acid levels[7,8]. Because of the potential of Roselle calyx, it is necessary to do further research to develop the ethanol extract of Roselle calyx as antihyperuricemia. Preliminary studies have been conducted in *in vivo* assay on male Wistar rats by using allopurinol as comparator. Allopurinol is antihyperuricemic agent with the mechanism is to inhibit the action of the xanthine oxidase enzyme that converts hypoxanthine into xanthine and xanthine into uric acid, therefore can reduce uric acid production. This research aims are to test the activity of extract and its fraction of Roselle calyx as xanthine oxidase.

MATERIALS AND METHODS

Plant Material

Roselle (*Hibiscus sabdariffa* L) calyx obtained from the Botany Garden of Manoko in Lembang, West Java, Indonesia. The plant materials were authenticated at Herbarium Bandungence, Institut Teknologi Bandung.

Materials

Xanthine oxidase enzyme derived from bovine (Sigma Aldrich, USA), Xanthine (Sigma Aldrich, USA) and Phosphate buffer Ph 7.5, HCl 1 N

Preparation of the extracts and its fractions

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Table1: The result of phytochemical screening tests botanicals, extracts and fractions Roselle (*Hibiscus sabdariffa* Linn) calyx.

	Crude drugs	EER	WFR	EAF	HF
Flavonoids	+	+	+	+	-
Saponins	+	+	+	+	-
Alkaloids	-	-	-	-	-
Polyphenols	+	+	+	+	-
Tannin	-	-	-	-	-
Steroids	-	-	-	-	-
Triterpenoids	-	-	-	-	-
Quinone	+	+	+	+	+
Mono &sesquiterpen	+	+	+	+	-

EER: ethanol extract of Roselle, WFR: Fraction water Roselle, EAFR: Fraction ethyl acetate Roselle, HFR: n-hexane fraction Roselle

Table 2: Xanthine oxidase inhibitory activity of allopurinol.

Concentration (µg/ml)	Percentage inhibition (%)
5	35.31±3.74
10	49.53±13.25
20	65.57±8.01
50	78.65±3.32
100	94.57±5.35

Extraction was done by continuous extraction using a Soxhlet with 96 % ethanol. Filtrate was concentrated by rotary evaporator until viscous extract, was obtained. extract yield was 35.8 %.

Then concentrated ethanol extract was fractionated by liquid-liquid extraction. Fractionation is done in stages using several solvent, including n- hexane, ethyl acetate and water, with the aim to separate the soluble components with different polarity[9].

Xanthine oxidase inhibitory activity assay

The principle of measurement of the activity of xanthine oxidase inhibition is quantification of uric acid which formed in the reaction catalyzed by xanthine oxidase using UV Spectrophotometry[10].

The procedure based on the procedure of Tamta[11] who has been modified. The following procedures were 1.9 ml of 50 mM phosphate buffer pH 7.5 was added with1 ml 0.15 mM xanthine, also 100 µl of the test substance was added and then incubated at 37°C for 10 minutes. After incubation, then 100 µl 0.1 U enzyme xanthine oxidase was added, and then incubated for 30 min at 37°C. The reaction was stopped immediately by addition 1 ml 1 N HCl, and then absorbance was read at λ 290 nm. Percent of inhibition by measuring the absorbance of uric acid from the mixture without test extract (blank samples) compared with the absorbance of mixture of test extract[8]. Controls were conducted in the same manner as the sample group without addition of the enzymes.

Inhibition of xanthine oxidase calculation:

% Inhibition= (1-B / A) x 100%..... (I)

Description

A: absorbance without sample (absorbance with enzyme – absorbance without enzyme)

B: absorbance changes by the sample test solution [absorbance Samples with enzyme – absorbance sample without enzyme]

The assay was done using several concentrations of the extracts and fractions at 100, 200, 300, 400, 500 ppm. Allopurinol with concentrations of 5.10, 20 , 50 and 100 µg/ml were used as reference drug. Xanthine oxidase inhibition test performed on extracts and results of fractionation using various variants of concentration aims to look at the effect of the concentration of the test substance on the increase in inhibition. Concentration of sample is 100 - 500 µg/ml. It also made observations of enzyme activity without the addition extract (blank) to see the effect of the inhibition of the extract as well as the results of fractionation.

RESULTS AND DISCUSSION

Ethanol Extract Roselle calyx yield was 35.8 % Water fraction yield was 46.27%, Ethyl acetate fraction: 15.28% and n-hexane fraction 8.67 % Extract and fraction then conducted phytochemical screening test to see the secondary metabolic. The result of phytochemical screening was shown in table 1. Phytochemical screening is important step in identification a new source of therapeutic agents and to test the existence of groups of secondary metabolites in a sample. Phytochemical screening showed that the ethanol extract, fraction of water and ethyl acetate fraction contained flavonoids, saponins, polyphenols and quinones, whereas n-hexane fractions contained only the quinone. Flavonoid is a phenolic group which have been proved to inhibit xanthine oxidase activity[14]. Based on the results of phytochemical screening extracts and fractions that contain flavonoids then tested the enzyme xanthine oxidase inhibitory activity *in vitro*. Besides test *in vitro* is done to see how much the ability of extracts and fractions in inhibiting the enzyme xanthine oxidase, as in previous studies *in vivo* extracts or fractions shown to have activity antihyperuricemia which extracts

Table 3: Xanthine oxidase inhibitory activity of ethanol extract and its fraction.

Concentration (µg/ml)	Percentage inhibition (%)		
	Ethanol extract Roselle	Water fraction	Ethyl acetate fraction
100	25.13± 4.90	25.81±3.63	32.25± 0.32
200	27.12±2.45	22.82±4.87	24.23±12.12
300	26.74±1.11	12.46±2.83	22.54±2.11
400	25.03±2.74	12.03±3.25	26.06±2.63
500	18.03±3.29	16.25±7.06	28.41±10.71

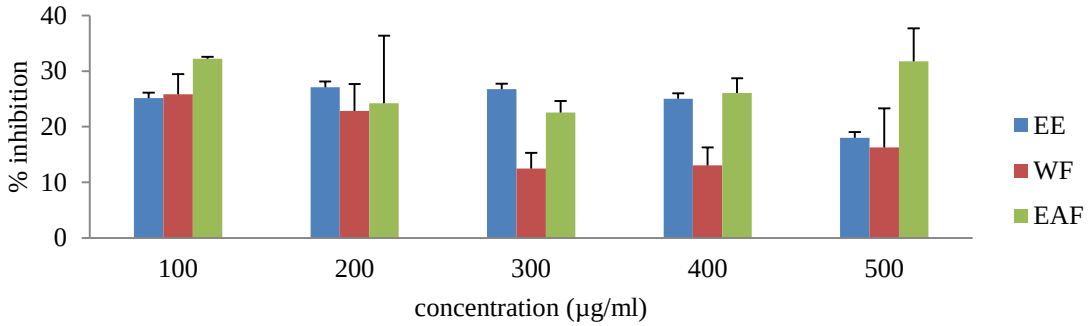

Figure 1: XO inhibitory activity of the ethanol extract and its fraction. EE: ethanol Extract, WF : water fraction, EAF : ethyl acetate fraction

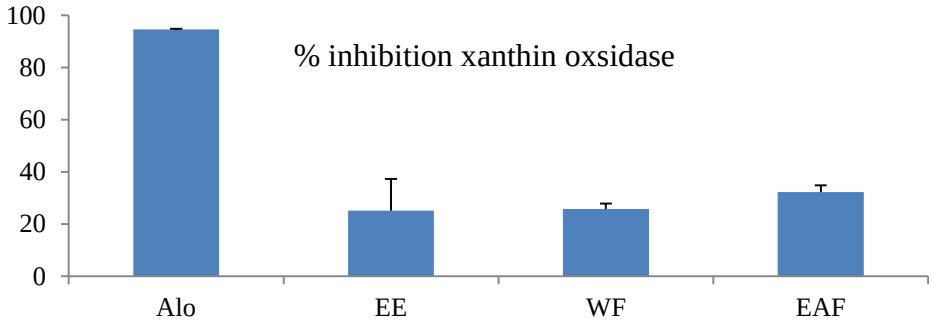

Figure 2: XO inhibitory activity of allopurinol, ethanol extract and its fraction. EE: ethanol Extract, WF: water fraction, EAF: ethyl acetate fraction

and fractions Roselle calyx can lower uric acid levels in the blood of rats wistar male. Ability inhibition of xanthine oxidase is important in one of the working mechanism of antihyperuricemia. Fractionation performed to purify the crude extract and for possible inhibitory compounds that have better power than the extract. Allopurinol is a drug that is uricostatic antihyperuricemia, where the mechanism of action of allopurinol is to inhibit the activity of the enzyme xanthine oxidase to convert hypoxanthine into xanthine and uric acid[6]. The xanthine oxidase is an enzyme flavoproteins containing molybdenum and iron oxidizes hypoxanthine and further into human urate. Uric acid is the end product of purine breakdown and excreted through the urine[5,6]. Principle of the assay of inhibition of xanthine oxidase activity is measurement of the amount of uric acid formed in the reaction catalyzed by xanthine oxidase. This test is an *in vitro* assay were done Spectrophotometric at maximum wavelength (λ 290)[12]. Xanthine oxidase inhibition assay is performed on extracts and its fraction using various variants of concentration, which the aims are to look inhibition effect in increasing concentration. Concentrations of the extract and its fraction were 100 - 500 µg/ml. It also made observations of enzyme activity without the addition extract (blank) to see the effect of the inhibition of the extract as well as the results of fractionation[11]. The xanthine oxidase inhibitor activity of allopurinol as reference drug and extract and its fraction is presented in table 2 and 3. The results of the extracts and its fractions showed that extracts and fractions can inhibit the xanthine oxidase enzyme activity and the reference drug (allopurinol) also showed similiar effect. Although *in vivo* extracts and its fractions showed activitity to reduce uric acid levels, but it was had lower activity compared to allopurinol. There is an interesting thing here is ethanol extract and water fraction in their dose escalation seen a decline in the ability to inhibit the enzyme xanthine oxidase which ethanol extract peak inhibition of the best is at a concentration of 200 µg/ml as well as in the water fraction, but on improving decreased inhibition of the enzyme xanthine osidase. The possible of there is content of other in the extract a long with the rise of concentration also improve metabolit other possible to disturb against the process of inhibition enzyme xanthine oxidase. *In vivo* ethyl acetate give antihyperuricemia effect better than extract, but stil very low when compared with allopurinol. It is shown also with the IC_{50} allopurinol 10.25 ppm while ethanol extract is 1294.37 µg/ml water fraction 830.71 µg/ml and ethyl acetate fraction is 290.62 µg/ml, but IC_{50} ethyl acetate fraction better compared with ethanol extract and water fraction. The activity of inhibitory xanthine oxidase extract and the fraction showed in fig 1 and fig 2. The results also showed that the ethanol extract of Roselle calyx may inhibit xanthine oxidase enzyme at 100 µg/ml by 25.13%, while the water fraction at 100 µg/ml at 25.81% and ethyl acetate fraction can inhibit the enzyme xanthine oxidase by 32.25%, while the reference drug allopurinol at 20 µg/ml already provide inhibit by 65.57%. This indicates that the activity of inhibitory xanthine

oxidase of extract and the fraction were still low when compared with allopurinol, it is possible that extracts and fractions of Roselle calyx has other mechanism as antihyperuricemia which is uricosuric.

CONCLUSION

The mechanism of action *in vitro* test showed that the inhibition of xanthine oxidase activity at 100 µg/ml of ethanol extract can inhibit the action of the xanthine oxidase by 25.12%, water fraction by 25.81 and ethyl acetate fraction by 32.25%. It is possible that extracts and fractions of Roselle calyx has other mechanism as antihyperuricemia which is uricosuric.

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