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Research Article

XANTHINE OXIDASE INHIBITORY ACTIVITY OF METHANOL EXTRACT FRACTIONS OF VARIOUS INDONESIAN ETHNOPHARMACOLOGICAL PLANTS

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ABSTRACT

Objective: Hyperuricemia involves an increase in serum uric acid levels, resulting in kidney damage, increased mortality, and reduced quality of life. Inhibitors of xanthine oxidase, which catalyzes the last step in uric acid synthesis, are targets for therapeutic intervention.

Methods: An ethnopharmacological approach, screening four native Indonesian herbal medicinal plants with reported activity against hyperuricemia, was used for preliminary studies, fractionating methanolic extracts by solvent partitioning. Fractions were then tested *in vitro* for xanthine oxidase inhibitory activity, and the most active fraction was then subjected to preliminary phytochemical screening.

Results: The target tissue of the four herbal medicinal plants investigated was Indian bay leaf (*Syzygium polyanthum* Wight.), God's crown fruit (*Phaleria macrocarpa* Boerl.), snake fruit peel (*Salacca edulis* Reinw.), and Job's tears tuber (*Cyperus rotundus* Linn.). Each sample was extracted by maceration with 80% methanol. The concentrated extract was then fractionated by the liquid-liquid partition method (1:1 v/v) using n-hexane, ethyl acetate, butanol, and methanol sequentially as solvents. The results revealed that the ethyl acetate fraction was the most active fraction. *S. polyanthum* leaf and *C. rotundus* tuber showed the greatest potential in inhibiting xanthine oxidase, with half-maximal inhibitory concentrations of 18.43 and 10.50 µg/ml, respectively. Enzyme kinetics analysis shows that each plant fraction works as a competitive inhibitor of xanthine oxidase.

Conclusion: Preliminary screening identified the ethyl acetate fractions of two native Indonesian herbal medicinal plants as showing potential for anti-hyperuricemia activity.

Keywords: Ethnopharmacological, Hyperuricemia, Phytochemical screening, Xanthine oxidase.

INTRODUCTION

Hyperuricemia is a pathological condition where uric acid levels in the blood are elevated above the normal range [1]. This condition can persist for a long time without any symptoms. During the asymptomatic period, the deposition of uric acid crystals may lead to chronic pain, causing joint damage in some patients [2]. In young patients, hyperuricemia stimulates oxidative stress, inflammation, and inflammatory response as feedback from the oxidative stress triggered by the high uric acid concentration itself [3]. Acute and chronic inflammation due to crystal deposition in joints and soft tissues is a consequence that occurs when hyperuricemia is not immediately treated [4].

Uric acid is the final product of the catabolism of purine nucleotides. There are two main sources of purines. The first one is endogenous, originating from the synthesis of purines *de novo* and their subsequent breakdown as part of nucleic acid turnover. Purines can also come from exogenous sources, such as food [5]. Many enzymes are involved in the purine catabolism pathway, where eventually adenine will be converted into hypoxanthine and guanine will be converted to xanthine. Hypoxanthine will be oxidized to xanthine, and finally, xanthine is oxidized again by xanthine oxidase to form uric acid [1]. Xanthine oxidase inhibitors, such as purine analogs, are used to treat hyperuricemia and gout, and there is pharmaceutical interest in the exploitation of natural plant xanthine oxidase inhibitors such as inositols and flavonoids.

An ethnopharmacological approach investigates the basis of the traditional use by native people of natural materials, such as plants, animals, fungi, microorganisms, and minerals, in treating specific medical conditions [6]. Many modern pharmaceuticals originated from traditional medicine and ethnopharmacology [7], either directly, using natural compounds as drugs, or as lead compounds in drug development.

Tubers of rumput teki or Job's tears (*Cyperus rotundus* Linn.) have been widely used in Indonesia as a traditional medicine to treat dysentery and painful joints, and as a diuretic [8], whereas leaves of the Indian bay leaf (*Syzygium polyanthum* Wight.) are used as a traditional medicine to treat muscle pain and uric acid accumulation [9]; a water extract of *S. polyanthum* has also been reported to significantly reduce blood plasma uric acid concentrations [10]. God's crown (*Phaleria macrocarpa* Berl.) is a native plant from Papua, Indonesia, the fruit of which has been used traditionally for a number of medicinal uses, including the treatment of kidney disease [11]. Snake fruit (*Salacca edulis* Reinw.) peel is traditionally used to reduce uric acid levels and has been shown to reduce uric acid levels in the Wistar rat model [12]. The aim of the current study was to investigate the potential of these ethnopharmacological plants to reduce uric acid levels through inhibition of the xanthine oxidase pathway.

MATERIALS AND METHODS

Plant material

Indian bay leaf (*S. polyanthum*) and God's crown fruit (*P. macrocarpa*) were obtained from a local market in West Java, Indonesia. Fruit of snake fruit (*S. edulis*) was obtained from Magelang, Indonesia and tubers of Job's tears (*C. rotundus*) were obtained from Bogor, Indonesia. Each of the samples was authenticated by Herbarium Bogoriense. The samples were cleaned, impurities were removed, and each sample was then dried at 60°C. The dried samples were powdered and stored in desiccator cabinets at room temperature until further analysis.

Preparation of extract

The preparation of extract was performed based on Yanti *et al.* with a number of modifications [13]. Each sample powder (1 kg) was extracted using the maceration method with 80% (v/v) methanol as the solvent. The powder was allowed to extract in the methanol for approximately 24 h, before being filtered. The solid was re-extracted 3 more times, and the filtrates obtained were combined and the pooled filtrate was then evaporated to dryness using a rotary vacuum evaporator at 40°C. This crude extract was stored at −20°C before use for further analysis.

Procedure of fractionation

The extract obtained was dissolved in 200 ml dH_2O, and then fractionated by liquid-liquid partitioning (1:1 v/v), using a sequence of solvents, from non-polar to polar, namely, n-hexane, ethyl acetate, butanol, and methanol. Partitioning was carried out 3 times in a separating funnel, each for 1 h. The fractions from one extract were combined and dried with a rotary vacuum evaporator to produce a concentrated extract fraction [14].

Determination of xanthine oxidase inhibitory activity

The determination of xanthine oxidase inhibitory activity was performed based on Ahmad *et al.* with a number of modifications [15]. A sub-sample (10 mg) of an extract or extract fraction was dissolved in 4 gtt of dimethyl sulfoxide (DMSO), before adding CO_2 free dH_2O to obtain a 1.0 µg/ml stock solution. The stock solution was then diluted to a 0.1 µg/mL working solution. One milliliter of working solution was added to 2.9 ml 0.05 M phosphate buffer pH 7.5, 2 ml 0.2 mM xanthine, and 1 ml of 1N HCl, the reaction mixture then being shaken vigorously until homogeneous. The mixture was incubated for 10 min at 25°C, before adding 0.1 ml 0.1 unit/ml xanthine oxidase and 1 ml 2N HCl. The final mixture was incubated at 25°C for 30 min and absorbance (A_{290}) was measured after the incubation was terminated. The control used DMSO without the extract or fraction sub-sample. The percentage inhibition was calculated from the equation below:

$$\text{Inhibition}(\%) = 1 - \frac{\text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

Extracts or fractions that achieved an inhibitory effect of more than 50% were tested again using the same method at concentrations of 5, 10, 20, 40, or 80 µg/mL to determine the half-maximal inhibitory concentration (IC_{50}) value by regression.

Preliminary phytochemical screening

In this study, preliminary phytochemical screening was carried out to determine the presence/absence of phytochemicals of a particular group in the extract fractions that had the greatest inhibitory activity against xanthine oxidase. Phytochemical screening was performed for a number of chemical groups, namely, alkaloids, glycosides, saponins, flavonoids, tannins, and terpenes.

Identification of alkaloids

A sample (500 mg) of extract fraction was dissolved in 10 ml of 0.2 N aqueous HCl, then heated for 2 min. The mixture was filtered, and the filtrate was used as the test solution (TS). Each 1 ml aliquot of TS was reacted with 2 ml of various test reagents. Bouchardat's reagent resulted in a dark-brown precipitate in the presence of alkaloids, whereas Mayer's reagent resulted in a white precipitate for a positive reaction, and Dragendorff's test showed a reddish-orange precipitate as a positive result [16].

Identification of glycosides

A sample (300 mg) was dissolved in 15 ml of 10% HCl, then filtered. The filtrate obtained was washed with ether 3 times, and the resulting filtrate was evaporated to dryness at 40°C. To the filtrate were then added 2 ml methanol, this solution being used as the TS. An aliquot (1 ml) of TS was evaporated to dryness and dissolved in 20 ml concentrated acetic acid and 1 ml concentrated sulfuric acid. A green or blue color indicated the presence of glycosides. Identification with Molisch's test was performed by dissolving the evaporated TS in 2 ml dH_2O and 5 ml Molisch's reagent. To the solution, 2 ml sulfuric acid was added carefully. The formation of a reddish-purple colored ring at the junction between the two layers indicated the presence of glycosides [17].

Identification of saponins

The identification of saponin compounds was carried out by the simple froth test method. A sample (500 mg) of the extract fraction was put in a test tube, to which was added 10 ml of hot distilled water, and the tube was shaken vigorously for 10 s. The formation of foam that lasted for at least 10 min and that did not disappear with the addition of 1 ml of 2N HCl, indicated the presence of saponin compounds [17].

Identification of flavonoids

Flavonoid identification was performed using Shinoda test methods. A sample (500 mg) of the fraction was dissolved in 2 ml of ethanol, to which was added 500 mg of Zn powder and 2 ml 2N HCl, following which the reaction mixture was allowed to stand for 1 min. Following the addition of 10 ml concentrated HCl, the appearance of a red color after 2–5 min incubation indicated the presence of flavonoids. The same method was used with 100 mg of Mg powder replacing the Zn powder to identify the presence of flavones, chalcones, and aurones. The appearance of a yellow to orange color indicated the presence of compounds of flavones, chalcones, or aurones [16].

Identification of tannins

A sample (200 mg) of the fraction was dissolved in 5 ml of hot distilled water, to which was added 10% NaCl, and the solution was filtered. The filtrate (TS) was then tested using the gelatin test and $FeCl_3$ test methods. To 1 ml of TS was added 3 ml 10% gelatin solution, with the formation of a white precipitate, indicating the presence of tannins. The ferric chloride test was performed by added 2 ml 3% $FeCl_3$ to 1 ml of TS, with a change in coloration to violet-green indicating that the sample contained tannins [17].

Identification of terpenes

A sample (200 mg) of the fraction was dissolved in a solution of concentrated acetic acid:concentrated sulfuric acid (2:1 v/v), at which point the formation of a greenish-red or violet-blue color indicated the presence of terpenes. To confirm the result, the procedure continued by spraying a sample with a solution of *p*-anisaldehyde in concentrated sulfuric acid, when dark-blue, green, red, or brown fluorescence at 366 nm ultraviolet light indicated the presence of terpenes [17].

RESULTS AND DISCUSSION

Xanthine oxidase inhibitory activity

The data obtained showed that the ethyl acetate fraction from each sample showed the highest inhibitory activity against xanthine

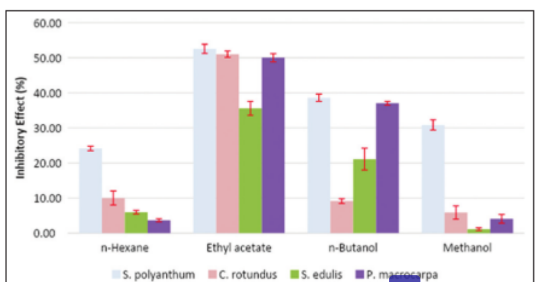

Fig. 1: Xanthine oxidase inhibitory activity from solvent partitioning fractions of a methanolic extract of *S. polyanthum*, *C. rotundus*, *S. edulis*, and *P. macrocarpa*. Each sample is expressed as the mean±standard error of the mean of two independent experiments

Table 1: Natural deep eutectic solvents combinations and mole ratios used in this study

Samples	Phytochemical presence					
	Alkaloid	Glycoside	Saponin	Flavonoid	Tannin	Terpene
Syzygium polyanthum	+	+	+	+	+	+
Cyperus rotundus	−	+	−	+	−	−
Salacca edulis	−	+	−	+	+	−
Phaleria macrocarpa	−	+	+	+	+	+

(+) present, (−) not detected

oxidase, in a comparison with the other extract fractions (Fig. 1). The ethyl acetate fraction of all four plant crude extracts had the highest mean±standard error of the mean inhibitory activity, namely, *S. polyanthum* (52.54 ± 1.29%), *C. rotundus* (51.01 ± 0.95%), *P. macrocarpa* (50.00±1.1[illegible]%), and *S. edulis* (35.58±2.00%). Interestingly, *S. polyanthum* exhibited the highest xanthine oxidase inhibitory activity in each of the solvent fractions (Fig. 1). Fractions that exhibited a xanthine oxidase inhibitory effect of greater than 50% could have their IC_{50} value determined; this was not possible for *S. edulis*. Analysis showed that *S. polyanthum* had a IC_{50} of 18.43 µg/ml, *C. rotundus* 10.50 µg/ml, and *P. macrocarpa* 19.23 µg/ml, whereas allopurinol, a well-known inhibitor of xanthine oxidase activity exhibited a IC_{50} of 0.067 µg/ml, making it more than 100 times more active than the most active plant extract, *C. rotundus*.

To investigate the kinetics of the xanthine oxidase inhibition by the plant fractions, we also performed the Lineweaver–Burk plot. The results showed the intersection of the straight line from the test with and without the inhibitor at the Y-axis. This is interpreted as all four plant fractions operating a competitive inhibition mechanism against xanthine oxidase. In such a system, there is no change in the V_{max} value, but there is a change in the value of K_m that needs a higher substrate concentration to reach the K_m value [18]. The difference between reversible and irreversible inhibition is that reversible inhibitors are easier to dissociate, thus causing shorter inhibition time. Competitive inhibition allows both the inhibitors and the substrates to bind to the same active site of the enzyme, but they cannot occupy the same active site of the enzyme at the same time. Therefore, the inhibitors will compete with the substrate to occupy the active site of the enzyme [19]. Basically, xanthine oxidase is an enzyme with low specificity. Because xanthine oxidase participates in the catabolism of xenobiotics, such as antiblastic and antimetabolic drugs, a xanthine oxidase inhibitor should be used carefully when these drugs are also being taken [20].

Phytochemical screening

The most active fraction (the ethyl acetate fraction) of each sample extract in terms of xanthine oxidase inhibitory activity was subjected to phytochemical screening to reveal which groups of phytochemical compounds were present. As shown in Table 1, all of the ethyl acetate fractions from the original crude methanolic extract of the four medicinal plants studied contained glycosides and flavonoid compounds.

The *S. polyanthum* ethyl acetate fraction of the leaf extract contained all of the compounds tested for, namely flavonoids, terpenes, alkaloids, saponins, tannins, and glycosides (Table 1). Recent work showed a number of bioactive compounds in the ethyl acetate fraction of the *S. polyanthum* leaf methanolic extract, including α-pinene, linalool, nerolidol, caryophyllene oxide, farnesol, phytol, squalene, β-tocopherol, α-tocopherol, β-sitosterol, α-humulene, neophytadiene, hentriacontane, and octanal [21].

The Abd Rahim *et al.* study [21] also reported that the ethyl acetate fraction of the *C. rotundus* tuber methanolic extract contained glycoside compounds, which were suspected to be the terpenoid iridoid glycoside groups such as 1α-meth-oxy-3β-hydroxy-4α-(3′,4′-dihydroxyphenyl)-1,2,3,4,-tetra-hydronaphthalin and 1α,3β-dihydroxy-4α-(3′,4′-dihydrox-yphenyl)-1,2,3,4,-tetrahydronaphthalin or other rotundus side compounds and their isomers that had been reported by another research team [22], although this tentative identification requires confirmation. The presence of flavonoid compounds in the *C. rotundus* fraction, which we identified, was also reported by Kilani *et al.* [23]. They described that the ethyl acetate fraction of *C. rotundus* contained flavonoid compounds such as afzelechin, catechin, quercetin, and luteolin.

We reported that the ethyl acetate fraction of *S. edulis* contained a number of phytochemical constituents such as glycosides, flavonoids, and tannins (Table 1). Afrianti *et al.* discovered that the ethyl acetate fraction of *S. edulis* contained a number of phytochemical compounds such as 3-hydroxystigmastan-5(6)-en and pyrolle-2,4-dicarboxylic acid, methyl ester [24], whereas another phytochemical screening study also reported that *S. edulis* contained flavonoids and tannins [24,25]. The positive results for glycosides detected in this current research are expected to be associated with the high sugar content in *S. edulis*.

Based on Table 1, the phytochemical compounds present in the ethyl acetate fraction of *P. macrocarpa* include glycosides, saponins, flavonoids, tannins, and terpenes. These data are similar to those in an article reviewing data reported by Alara *et al.* The positive results for glycosides, saponins, and terpenes could have been due to the presence of fevicordin in *P. macrocarpa*, which is classified as terpenoid. In the form of sugar conjugates, fevicordin would give positive results for glycoside as well as saponin. It was also reported that *P. macrocarpa* contains flavonoids, tannins, and gallic acid [26].

CONCLUSION

Various plants used in ethnomedicine have been scientifically proven to have beneficial effects in treating hyperuricemia through a competitive inhibitory mechanism against xanthine oxidase. In the current study, *C. rotundus* tuber was the herbal medicinal plant with the greatest potential for development as a hyperuricemia treatment by inhibiting xanthine oxidase (IC_{50}=1 0.50 µg/ml).

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