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IN VITRO AND *IN SILICO* EVALUATION OF XANTHINE OXIDASE INHIBITORY ACTIVITY OF QUERCETIN CONTAINED IN *SONCHUS ARVENSIS* LEAF EXTRACT

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ABSTRACT

Objective: The aim of the present study was to examine the inhibiting effects of quercetin contained in *Sonchus arvensis* leaf extract on the activity of xanthine oxidase, an essential enzyme for uric acid synthesis.

Methods: Activity test was conducted *in vitro* by measuring the activity of xanthine oxidase using ultraviolet spectrophotometry and *in silico* by determining the interaction of quercetin and allopurinol (as comparator drug) with xanthine oxidase enzyme in terms of hydrogen bonds and binding free energy. Docking simulations were performed by AutoDock 4.2 package.

Results: The active fraction, using the solvent n-hexane, ethyl acetate, and water, tested the inhibitory activity of the xanthine oxidase enzyme *in vitro* obtained, respectively, inhibitory concentration 50% of 263.19, 16.20, and 141.80 µg/ml. Isolates with highest activity identified as quercetin. The xanthine oxidase enzyme inhibitory activity *in silico* by molecular docking showed that quercetin has free energy binding −7.71 kcal/mol, more negative than that of allopurinol −5.63 kcal/mol.

Conclusion: This shows the affinity of quercetin stronger than that of allopurinol so that it can be predicted that quercetin was more potential to inhibit xanthine oxidase enzyme activity. Thus, the extract of the *S. arvensis* leaves containing the active compound quercetin was a potential use as antihyperuricemia.

Keywords: Quercetin, *Sonchus arvensis*, Inhibitor, Xanthine oxidase, Hyperuricemia.

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INTRODUCTION

The trend of back to nature in using natural ingredients and herbs is increasing worldwide so that herbal medicine is an interesting treatment option to be developed. In Indonesia, *Sonchus arvensis* leaf has been traditionally used in as hyperuricemia remedy [1]. Hyperuricemia occurs due to excessive production of uric acid or low excretion of the acid or a combination of both [2]. Increased production of uric acid caused partly by the intake of foods that contain purine, uric acid metabolism disorder and breakdown of purines too quickly in the body also causes high levels of uric acid in the blood [3]. Decrease the excretion of uric acid is the main cause of hyperuricemia and can be caused by idiopathic primary, renal insufficiency, the interaction of drugs, and alcohol [3]. The combination of both mechanisms may occur in glucose-6-phosphatase deficiency and deficiency of fructose-1-phosphate aldolase [2]. The production of uric acid is catalyzed by xanthine oxidase in the liver [4]. Inhibition of xanthine oxidase can be one of the therapeutic avenues for the treatment of hyperuricemia. Xanthine oxidase is an essential enzyme for uric acid synthesis that converts hypoxanthine into xanthine and xanthine into uric acid, thus reducing uric acid production. Uric acid is the final product of purine metabolism in human body that exits the body through urinary excretion. This substance has very low solubility and tends to form crystals. Uric acid accumulated in the joints is commonly found in the form of monosodium uric crystals that induces inflammatory reactions [5,6]. Allopurinol is used clinically in the treatment of gout. Currently, allopurinol is one of the most widely used modern medicines to inhibit the synthesis of uric acid [2].

The results of previous studies of the structure-activity relationships and classification of flavonoids as an inhibitor of xanthine oxidase and superoxide scavengers were performed *in vitro* states that flavonoids are group of natural products that have various biological activities and pharmacological such as antibacterial, antiviral, antioxidant, antimutagenic effects and can inhibit some enzymes. Some flavonoids have been reported to inhibit the enzyme xanthine oxidase and have the ability to capture free radicals. Therefore, flavonoids can be used in the treatment of gout and ischemia in humans with lower levels of uric acid and free radicals in the body. One of them is the flavonoid quercetin, which is an inhibitor of the enzyme xanthine oxidase and has the ability to capture free radicals [7]. This is in line with the results of other studies stating that flavones and flavonols planar such as chrysin, luteolin, kaempferol, quercetin, myricetin, and isorhamnetin can inhibit xanthine oxidase enzyme strongly, while flavonoids non-planar, isoflavones, and anthocyanidins only slightly inhibit [8]. Quercetin is flavonol compounds that are widespread in plants [9]. *In silico* study was needed to analyze the xanthine oxidase enzyme inhibitory activity by predicting the affinity and interaction between quercetin and the xanthine oxidase enzyme.

Computational chemistry with *in silico* study described that in biological systems, the resulting pharmacological activity is not only dependent on the chemical structure or position of atoms that bind to receptors but also involves intermolecular force as a whole is important for proper interaction and complementarity between the two molecules. Among the various interactions between molecules, hydrogen bonds are very important. Hydrogen bonds work within a limited distance and direction, while the electrostatic interaction works in all directions and a greater distance [10]. Molecules with very different chemical structure can bind to the same site of the receptor. Many examples are known for competitive inhibition between the molecules of various structures. The shape and nature of the molecules should be complementary to the receptor sites [10]. The entire structure of drug molecules is not always necessary to bind to the receptor because not all the atoms of the inhibitor molecule

involved in the interaction with the receptor protein [10]. This article presents the evaluation of complementary in terms of *in silico* and *in vitro* pharmacological activity-related quercetin contained in *S. arvensis*.

METHODS

Plant materials

S. arvensis leaf was collected from the botanical garden of Manoko in Lembang, West Java, Indonesia. The plant materials were authenticated at Herbarium Bandungense, the School of Life Sciences and Technology, and Bandung Institute of Technology.

Plant extraction

This study used the cold maceration method for extraction, with 96% ethanol extracting solvents for the dried and ground plant materials. The macerate accumulated was concentrated using rotary evaporator under reduced pressure.

Xanthine oxidase inhibitory *in vitro* assay

Activity test for xanthine oxidase inhibition was conducted *in vitro* using ultraviolet spectrophotometry as described in previous studies [4,11-13] with the slight modification. Xanthine oxidase enzyme from bovine milk (Sigma-Aldrich, Tokyo, Japan) was prepared by dilution of the enzyme to a final concentration 2 Units/ml. 1 mM xanthine substrate solution was made by adding 5 drops of 1.0 M NaOH to increase the solubility of xanthine. Ethanolic extracts were dissolved in 1% dimethyl sulfoxide and made the test concentration at 50, 100, and 200 mg/ml. Allopurinol is used as comparator drug; total volume of the assay mixture was 3.2 ml consisting of 1 ml sample *S. arvensis* extract at various concentration, 1 ml of 0.15 M phosphate buffer (pH 7.8), and 100 µl solution of the enzyme xanthine oxidase. After pre-incubation of the test solution at 37°C for 15 minutes, the reaction was initiated by addition of 100 µl of xanthine substrate solution and incubated at 37°C for 30 minutes. The reaction was stopped by adding 1 ml of 1N HCl. Spectrophotometer absorbance at 295 nm, suggesting the formation of uric acid. Percent of inhibition of xanthine oxidase activity of the test sample was determined by measuring the absorbance of uric acid from the mixture without test extracts (blank samples) compared with the absorbance of a mixture of test extracts. Inhibitory concentration 50% (IC_{50}) values were obtained by linear regression analysis of a plot, a series of different sample concentrations against percent inhibition.

Xanthine oxidase inhibitory *in silico* assay

Xanthine oxidase inhibition assay was conducted *in silico* as described in previous studies [14-16] with the slight modification. The ligand binding domain 3D structure of bovine xanthine oxidase in complex with quercetin obtained from the Protein Data Bank (www.pdb.org) with PDB code 3NVY as well as quercetin and allopurinol. Preparation of ligands and receptors, docking process, and analysis was performed using the AutoDock Tools v.4.2 program package (http://autodock.scripps.edu).

RESULTS AND DISCUSSION

Examination compound of *S. arvensis* leaf extract by thin-layer chromatography GF254 with the mobile phase chloroform:ethyl acetate:formic acid (6:4:0.2) and comparison to quercetin standard on Rf 0.6 indicates quercetin contained in the extract. Indeed, previous studies of the evaluation of flavonoids and antioxidant activity in *S. arvensis* showed that one of the components contained in *S. arvensis* is quercetin [17]. Table 1 shows the results of inhibitory activity of the xanthine oxidase enzyme *in vitro* and obtained quercetin with IC_{50} 4.39 mg/mL, smaller than that of allopurinol 4.84 ug/ml. Indeed, previous studies on the structure-activity relationships and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers were conducted *in vitro* and showed results that quercetin was a xanthine oxidase inhibitor with an additional superoxide scavenging activity [7].

Table 1: The results of the inhibitory activity of the xanthine oxidase enzyme *in vitro* of quercetin and allopurinol

Material tested	Percent of inhibition (%)			IC_{50} (µg/ml)
	50 µg/ml	100 µg/ml	200 µg/ml	
Quercetin	58.72±2.81	75.67±2.05	95.18±1.82	4.39
Allopurinol	59.14±3.26	71.38±7.01	91.75±11.99	4.84

Previous study showed that *S. arvensis* exhibits xanthine oxidase inhibitory activity and suggests that ethyl acetate extract of *S. arvensis* leaves has the most potent activity in inhibiting xanthine oxidase activity [12,13]. *Sonchus arvensis* L. leaves extract toxicity study showed there were no toxic effects after the use of single dose administration as acute toxicity test in mice, and also no toxic effects after repeated dose administration as subchronic oral toxicity studies in rats. Lethal dose of the extract was higher than 15 g/kg bw and classified as practically non-toxic. There were no abnormalities in behavior, hematological, clinical biochemistry, and urine parameters [18].

Molecular docking simulations in this study were conducted to analyze the xanthine oxidase enzyme inhibitory activity by predicting the affinity and interaction between quercetin and the xanthine oxidase enzyme and compared with allopurinol. It was done through energy predictions bond conformation and orientation of molecules in the active site to target receptors and modeling the interactions between the receptor and the ligand. The crystal structure of bovine xanthine oxidase in complex with quercetin used as the target receptors obtained from the PDB (www.pdb.org) with PDB code 3NVY. Docking process using Genetic Algorithm Lamarckian facilities in AutoDock Tools v.4.2 program that ligands directed to various possible positions in the structure of target molecules [14]. Docking method validation was done by redocking natural ligand receptor on the active side. The validity of the docking method parameters evaluated based on the value root-mean-square deviation (RMSD) and declared valid if the value of RMSD smaller than 2.0 Å [19]. In this study, the RMSD was 0.91 Å, indicating that methods and calculation parameter settings meet the criteria of validity docking methods. Fig. 1 shown the comparison of the natural ligand conformation, that is quercetin from X-ray crystal structure of the conformation obtained from the calculation with the docking parameter grid box coordinates x; y; and z were 39.512; 21.651; and 20.246.

Analysis was performed on free energy parameter binding; inhibition constants, hydrogen bonding, and interactions occurred between the ligand (quercetin and allopurinol) with amino acid residues at the active site receptors. The value of free energy can be used to predict the binding affinity and ability of a compound to inhibit the enzyme molecule [14]. Negative free energy of binding inhibition constants indicates good affinity between ligands and receptors. As shown in Table 2, quercetin in lowest conformation energy had free energy binding −7.71 kcal/mol, more negative than that of allopurinol −5.63 kcal/mol. This suggests the affinity of quercetin stronger than that of allopurinol so that it could be predicted that quercetin was more potential to inhibit xanthine oxidase enzyme. Indeed, previous research on finding study material that has potential as the enzyme xanthine oxidase inhibitor use *in silico* docking showed that flavonoids have interaction with the active site of the enzyme xanthine oxidase [15]. Previous studies report that

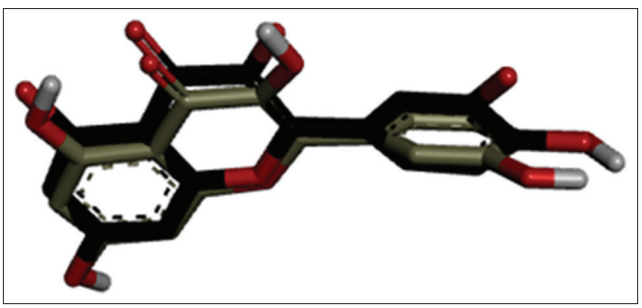

Fig. 1: The comparison between quercetin conformation of the natural ligand X-ray crystal structure (black) with the conformation obtained from docking calculations (gray)

Table 2: The results of *in silico* molecular docking of quercetin and allopurinol

Material tested	Conformation	Binding energy (kcal/mol)	Inhibition constant (μM)	Intermolecular energy (kcal/mol)
Quercetin	1	−7.71	2.24	−9.50
	2	−7.70	2.29	−9.49
	3	−7.70	2.28	−9.49
	4	−7.69	2.31	−9.48
	5	−7.69	2.29	−9.48
	6	−7.69	2.32	−9.48
	7	−7.68	2.33	−9.47
	8	−7.68	2.36	−9.47
	9	−7.68	2.34	−9.47
	10	−7.57	2.81	−9.49
Allopurinol	1	−5.63	74.96	−5.63
	2	−5.00	215.45	−5.00
	3	−4.91	249.71	−4.91
	4	−4.39	600.57	−4.39
	5	−5.63	75.00	−5.63
	6	−5.03	205.13	−5.03
	7	−4.92	245.63	−4.92
	8	−4.85	280.57	−4.85
	9	−5.26	74.96	−5.26
	10	−4.90	74.96	−4.90

the molecular modeling flavonoids could inhibit the xanthine oxidase enzyme. The apigenin, quercetin, myrisetin, isoviteksin, genistein, and naringenin were competitive inhibitor, and the kinetic analysis showed that flavonoids bind to the active site of the enzyme [16]. Likewise, another study using molecular docking showed that flavonoids provide inhibitory activity on the xanthine oxidase enzyme so as to further development for the prevention and treatment of gout and related inflammatory conditions [15].

Inhibition constants were proportional to the free energy of binding, 2.24 μM quercetin smaller than allopurinol 74.96 μM in the lowest conformation energy. It indicates quercetin easier to bind the xanthine oxidase enzyme than allopurinol. Quercetin showed intermolecular energy −9.50 kcal/mol lower than allopurinol with −5.63 kcal/mol, thus reinforcing predictions that the inhibitory activity of quercetin was greater than allopurinol.

Figs. 2 and 3 shows that quercetin had two hydrogen bonds at amino acid residue Glu802 and Thr1010 as well as allopurinol can form hydrogen bonds at amino acid residue Thr1010 and Glu802. Indeed, previous studies showed that the complement steric benefit from the structure of quercetin in the active site of the receptor by hydrogen bonds with residual catalytic at Arg880 and Glu802 as an inhibitor of flavonoids against xanthine oxidase which is useful for the treatment of hyperuricemia, gout, and diseases of inflammation [20]. In another study, it is also explained that the disposition of the substrate in the active site Arg880 and Glu802 was important for the conversion of xanthine to uric acid [21]. Results of other studies explain that the xanthine oxidase inhibitor had hydrogen bonds to Glu802, Arg880, and Glu1261 essential for enzyme reactions [22]. Thus, it appears that both quercetin and allopurinol could bind to the active side of the enzyme xanthine oxidase.

Most of the plant flavonoid binding to the sugar clusters that tend to be soluble in water is rarely found as aglycone. Flavonoid glycosides absorption in the body is very light, just flavonoid aglycones be able to pass through the intestinal wall. Hydrolysis occurs in the large intestine by intestinal microflora; hydrolytic enzymes of the intestinal microflora could convert certain flavonoid glycosides into the aglycone form then passively diffuses into the cell and provide a pharmacological activity [23]. Thus, the extract of the *S. arvensis* leaves containing the active compound quercetin was a potential use as antihyperuricemia.

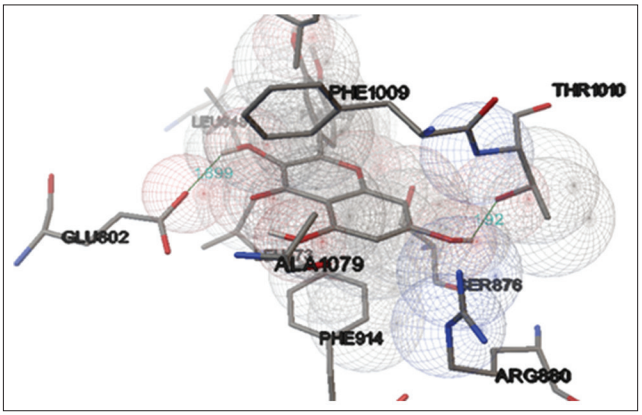

Fig. 2: The interaction of quercetin ligand on the active side xanthine oxidase receptor

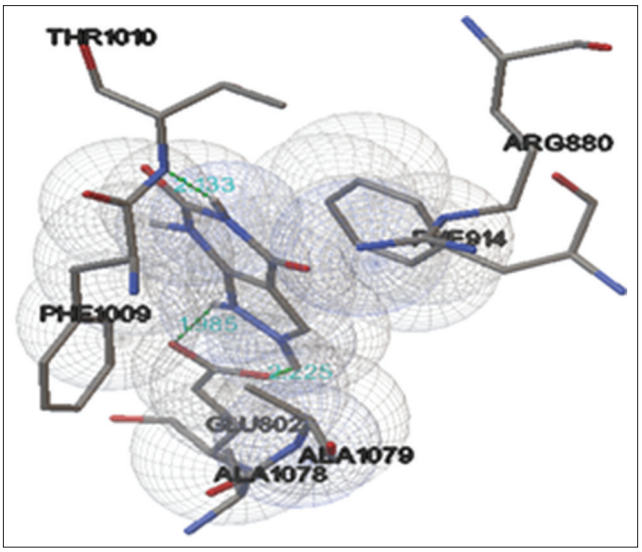

Fig. 3: The interaction of allopurinol ligand on the active side xanthine oxidase receptor

CONCLUSION

S. arvensis under investigation containing quercetin as active compound exhibits xanthine oxidase inhibitory activity. The energy bonding of quercetin was more negative compared to that of allopurinol, suggesting more affinity of quercetin to the active site of the enzyme.

Indeed, the *in vitro* result showing lower IC_{50} of quercetin than that of allopurinol. Thus, the *S. arvensis* leaf was potential to be developed as agent for treating hyperuricemia.

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