Bioactivity of Flavonoid in Ethanol Extract of *Annona squamosa* L. Fruit as Xanthine Oxidase Inhibitor

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Abstract. The global gout prevalence was increased rapidly in recent decades, especially in some developing countries. One of the most commonly used to treat gout is allopurinol, that act as xanthine oxidase inhibitor. However, consuming allopurinol in the long term can cause some unwanted side effects. On the other hand, many kinds of flavonoid compounds identified in some Indonesian grown plants have bioactivity as xanthine oxidase inhibitor. One of that is sugar apple (*Annona squamosa* L. fruit). Therefore, the aims of this study were to isolate the flavonoid compounds in ethanol extracts of sugar apple and to test the activity as xanthine oxidase inhibitor relative to allopurinol. The study has been carried out in some phases: (1) flavonoid extraction using 70% ethanol, (2) purification of the flavonoid isolate using n-hexane, chloroform and ethyl acetate as solvent, (3) flavonoids identification and (4) inhibitory activity test against xanthine oxidase, relative to allopurinol using spectroscopy methods. The results have shown that: from 275 grams of sugar apple fruit can be isolated 6 grams of flavonoid with the purity of 95.26% (according to HPLC peak) and has xanthine oxidase inhibitory activity was higher (83%) than allopurinol (48%) at the same concentration (100 ppm). Moreover, based on in silico study, there was also confirmed that a flavonoid compound found in *Annona squamosa* L., has higher binding affinity to the xanthine oxidase than allopurinol. This result thus indicated the flavonoid isolated from *Annona squamosa* L. might be a promising herbal drug for gout treatment.

Keywords: *Annona squamosa* L., xanthine oxidase inhibitor, flavonoids, anti gout fruit

1. Introduction

Gout prevalence has increased in recent years, especially in some developing countries, such as in Indonesia. A survey result from WHO, Indonesia occupies fourth place as a country with high prevalence of gout[1]. Gout is one of serious disease generally caused by abnormally production of uric acid [2-3]. The accumulation of uric acid in the blood will initiate production of urate crystal or monosodium urate-monohydrate crystals which trigger some pains in joints, tendons, and tissues [4].

The uric acid production occurs during purine catabolism catalyzed by the enzyme of xanthine oxidase (XO) [5]. This oxidoreductase group of enzyme catalyzes the oxidation reaction of hypoxanthine and xanthine to uric acid. Thus, the potency of next therapeutic agent to treat gout are examined based on their capability to inhibit xanthine oxidase activity [6]. A well common drugs that have this inhibition activity toward xanthine oxidase is allopurinol. However, consumption this drug
for long period can cause some side effects, such as hypersensitivity reaction, nephrolitiasis, Stevens-Johnson syndrome, renal toxicity, allergic reaction, and liver necrosis [7-8].

The attentions for searching new antigout from natural resources have increased recently due to their lower side effects. Indonesia is a country with highly diversified distribution of many grown plant species that have been explored and utilized to treat many diseases [9]. Some plants in Indonesia are well known to possess potential bioactive compound such as flavonoid that can inhibits xanthine oxidase [10-11]. One of natural source to isolate flavonoid is sugar apple or *Annona squamosa* L. This plant not only have antigout property but also antidiabetic, antimicrobial, antifertility potential, etc [12].

The previous research showed that flavonoids have inhibition activity toward xanthine oxidase based on in vitro and in vivo study [13-14]. Another research using in silico analysis also proved these flavonoid activity [15]. However, based on those studies and literatures, the different flavonoid derivatives show the different inhibition activity. Thus, searching for bioactive compound which is more potential as antigout agent is necessary to carried out. Therefore, in this present study, inhibitory activity of flavonoid isolated from *A. squamosa* L. was evaluated using in vitro study. Further analysis using in silico was also performed to identify the potential binding site of enzyme which is the target of flavonoid.

2. Experimental.

2.1. Materials and Instruments.
Sugar apple (*Annona squamosa* L.) was collected from local region of Kediri district, East Java, Indonesia. Xanthine substrate and xanthine oxidase (XO) were purchased from Sigma Aldrich, whereas pH universal, phosphate buffer 0.05 M with pH of 7.5, aquadest, ethanol 70%, HCl 37%, NaOH 1 N, acetone, ethyl acetate, chloroform, acetic acid, n-butanol, n-hexane, gel silica plat G60F254 were purchased from MERCK. Allopurinol generic tablet (100 mg) was purchased from Indofarma. High Performance-Liguid Chromatography (HPLC) was used to analyze bioactive compound in the extract. Some free softwares were also used for in silico analysis to determine the inhibitory activity, i.e PyMol (*Python Molecular Viewer*), PyRx 0.8, and discovery studio software.

2.2. Procedures

2.2.1. Preparation of ethanol extract. A 275 grams flesh of sugar apple fruit (*A. squamosa* L.) were blended then followed by filtration. The simplicia were weighed and extracted for 3 x 24 hours using 70% ethanol to obtain ethanol extract.

2.2.2. Purification of ethanol extract. This step was carried out by solvent-solvent partition using three different solvents which were n-hexane, chloroform, and ethyl acetate respectively. Five milliliters of crude ethanol extract was dissolved in 10 mL n-hexane then shaked before being allowed to settle down for 24 hours in room temperature. This process was conducted twice and resulted the first ethanol fraction. First ethanol fraction then was extracted by 10 mL chloroform to obtain second ethanol fraction which was followed by next extraction using ethyl acetate to produce third ethanol fraction. These last fraction was considered as purified ethanol extract.

2.2.3 Purity testing of the extract. The purity of ethanol extract was determined using high performance liquid chromatography (HPLC). Then the purified ethanol extract was separated by thin layer chromatography (TLC) using G60F254 silica gel as stationary phase and mixture of 70% ethanol: ethyl acetate (3:1) ; (1:2), 70% ethanol : chloroform (1:4) ; (4:1), and n-butanol : acetic acid : water (4:1:5) ; (6:2:9) as mobile phase.
2.2.4 Flavonoid testing. The ethanol extract of sugar apple was dissolved in 3 mL of 70% ethanol. One milliliter of those solution was added by 37% HCl then boiled for 5 minutes. The positive result of this test was shown by color change of solution become reddish color. The same procedure was also carried out to check flavonoid content in purified ethanol extract.

2.2.5. Inhibitory activity analysis using in vitro study. The XO activity, uric acid concentration and inhibition percentage were determined to analyze the inhibition activity of the test sample. The XO activity and uric acid concentration were calculated by [9],

\[
\text{XO activity} = \frac{(A_E - A_{EC}) \times V \times df}{\varepsilon \times 0.1}
\]

\[
C \text{ (concentration of uric acid)} = \frac{A}{\varepsilon \times b}
\]

Where, \(AE\) is absorbance of sample, \(AEC\) is absorbance of control of sample, \(V\) is volume total when measurement (mL), \(df\) is dilution factor, \(\varepsilon\) is uric acid concentration (12.2 mM) and 0.1 show the XO volume used (u/mL). \(C\) is uric acid concentration, \(A\) is absorbance at 284 nm, \(\varepsilon\) is molar absorptivity (mM\(^{-1}\)cm\(^{-1}\)) and \(b\) is length of cuvette (1 cm).

The inhibition percentage of ethanol extract and purified ethanol extract was examined. The mixture of 1 mL of the test sample (ethanol extract and purified ethanol extract), A 2.9 mL phosphate buffer (pH 7.5), and 0.1 mL of xanthine oxidase enzyme (0.05 units/mL). The mixture was incubated at 30°C for 10 minutes followed by addition of 2 mL xanthine substrate (0.15 mM). The mixture was then incubated again at 30°C for 30 minutes. The reaction was stopped by adding 1 mL of 1 N HCl then the absorbance was measured at 284 nm using UV spectrophotometer. This step was proceeded triplicate. The positive control used is allopurinol (100 µg/mL). The percentage inhibition was determined by the following formula [9],

\[
\% \text{ inhibition} = \left[1 - \frac{B}{A}\right] \times 100\%
\]

Where, \(B\) is the absorbance change of the test sample with inhibitor while \(A\) is absorbance change of test sample without enzyme.

2.2.6. Inhibitory activity analysis using in silico study. The 3D structure of xanthine oxidase P80457 (code: 1FIQ) receptor was obtained from Protein Data Bank at uniprot website while the 3D structure of ligand which were xanthine, allopurinol, and quercetin-3-glucoside was obtained from Pubchem database. The flavonoid structure used in this study was based on the structural characterization result of \textit{A. squamosa} leaves from previous research conducted by Panda, et. al (2007) [12]. The 3D structure of receptor and ligand was visualized using Pymol software. Each ligand interaction with receptor (XO) was determined using molecular docking. At first, the enzyme was sterilized using Pymol to eliminate water molecule and all the ligand then followed by identification enzyme interaction with ligand using PyRx 0.8 to obtain binding affinity value. The binding position of each ligand toward XO then was visualized using Pymol. The bond type, bond distance, and amino acids involving in the formation of ligand-enzyme complex were identified using Discovery Studio software.

3. Results and Discussion

3.1. The mass comparison of sugar apple and the products of extraction results
The maceration result of 275 grams of sugar apple fruit were obtained in dark brown extract (Figure 1a). While the purified ethanol extract was in more light brown color (Figure 1b). The color depends on the amount of secondary metabolites contained in the extract. This data indicates the separation of several secondary metabolites due to the partitioning process, resulting in purified extracts.
After ethanol extraction process, the mass of sugar apple obtained was 31 grams that equal to 11.27% (w/w) of the total initial mass of fruit. Six grams of purified ethanol extract was obtained from three times partition process using n-hexane, chloroform, and ethyl acetate respectively. The mass of purified ethanol extract was equal to 2.18% (w/w) of total initial mass of fruit.

3.2. Qualitative analysis of flavonoid
In this study, identification of flavonoids was carried out with the addition of concentrated hydrochloric acid, which will show positive results if there is a reddish color. Figure 2 shows the positive result of both ethanol extract and purified ethanol extract of sugar apple. Thus, the two results show the presence of flavonoids in both extracts.

![Figure 1. Maceration of sugar apple in (a) ethanol extract (b) purified ethanol extract](image)

3.3. In vitro XO inhibitory activity
This test was conducted to determine how much enzyme activity in forming uric acid with and without the addition of inhibitors. The uric acid formed was shown in Table 1. The addition of both ethanol extract and purified ethanol extract of sugar apple caused the greater decrease of XO activity compared to addition of allopurinol. This proved that both extracts have the greater inhibitory activity than allopurinol at the same concentration of 100 ppm (v/v).

In order to find out the mass of the extract to have the same inhibitory power as one tablet of allopurinol, equalization was carried out. The mass of ethanol extract and purified ethanol extract needed to be equivalent to one tablet of allopurinol were 157.43 mg and 120.65 mg respectively. Whereas to make ethanol extract and purified ethanol extract required fruit as much as 1396.55 mg and 2320.19 mg respectively. In purified ethanol extract, fewer extracts are needed. This is due to the
gradual treatment of the partitioning process to separate secondary metabolites based on their polarity. The more specific the secondary metabolite group contained in the extract, the more specific the interaction with xanthine oxidase. This interaction may increase the inhibition activity toward enzyme.

**Table 1. Inhibitory Activity of *Annona squamosa* L. Fruit Extract Compared to Allopurinol.**

| Sample                  | Measurement Number | Absorbance | Average of XO activity (U/mL) | Average of Uric Acid Concentration (mM) | Inhibitory Activity (%) |
|-------------------------|--------------------|------------|-------------------------------|----------------------------------------|--------------------------|
| Without inhibitor       | 1                  | 0.0178     | 0.0752                        | 0.0048                                 | 0%                       |
|                         | 2                  | 0.0204     | 0.0835                        | 3.36                                   |                          |
|                         | 3                  | 0.0272     | 0.0837                        |                                        |                          |
| Allopurinol             | 1                  | 0.0524     | 0.0824                        | 0.0024                                 | 48.44%                   |
| 100 ppm                 | 2                  | 0.0545     | 0.0842                        | 1.72                                   |                          |
|                         | 3                  | 0.0564     | 0.0870                        |                                        |                          |
| Ethanol extract         | 1                  | 0.0456     | 0.0679                        | 0.0017                                 | 63.52%                   |
| 100 ppm                 | 2                  | 0.0551     | 0.0767                        | 1.22                                   |                          |
|                         | 3                  | 0.0529     | 0.0730                        |                                        |                          |
| Purified ethanol extract| 1                  | 0.0482     | 0.0580                        | 0.0008                                 | 82.88%                   |
| 100 ppm                 | 2                  | 0.0521     | 0.0565                        | 0.56                                   |                          |
|                         | 3                  | 0.0500     | 0.0651                        |                                        |                          |

Compare to the previous studies, the sample used in this study, both in ethanol or purified ethanol extract, have the greater inhibitory activity than the extract from other plants, as can be seen in table 2. Thus the extract of *Annona squamosa* fruit has a great potency as xanthine oxidase inhibitor.

**Table 2. The XO inhibition activity of *Annona squamosa* fruit extract relative to some medical plants extract at 100 µg/mL**

| Scientific Name         | Local Name | Part       | Solvent Used | XO inhibition (%) |
|-------------------------|------------|------------|--------------|-------------------|
| *Alpinia galanga* [16]  | Lengkuas   | Rhizome    | Ethanol      | 57.99             |
| *Annona muricata* [2]   | Sirsak     | Leaves     | Ethanol      | 14.18             |
| *Curcuma longa* [17]    | Kunyit     | Whole plant| Methanol     | 28.31             |
| *Punica granatum* [7]   | Buah delima| Seed       | Methanol     | 15.53             |
| *Cucurbita pepo* [17]   | Labu       | Seed       | Methanol     | 27.33             |
| *Annona squamosa*       | Srikaya    | Fruit      | Ethanol      | 63.52             |
| (In this study)         |            |            |              |                   |
| *Annona squamosa*       | Srikaya    | Fruit      | ethanol (and then be purified) | 82.88 |
| (In this study)         |            |            |              |                   |

3.4. *Identification the purity of bioactive compound in the purified ethanol extract*

High Performance Liquid Chromatography (HPLC) was used in order to analyze the purity of bioactive compounds contained in the ethanol extract. The chromatogram of ethanol extract at wavelength of 275 nm was depicted in Figure 3 that showed three signals with one highest peak (95.26%) and two small peaks (4.17% and 0.55%). The signals appear indicated that there were three compounds contained in ethanol extract with the presence of dominant compound shown from the presence of highest peak, that based on phytochemical data analysis (Fig. 2) is flavonoid.
Figure 3. HPLC Chromatogram of *A. squamosa* L. Purified Ethanol Extract

The TLC result of purified ethanol extract of sugar apple, in some variations of eluen was depicted in Table 3. The data also showed that there was a single spot under UV detection although the retention factor (Rf) was different. This result means that the purified ethanol extract contains one compound. This result was also supported by HPLC result on Figure 3 that confirms one dominant compound in the extract. Further structural characterization was necessarily performed to analyze the type of compound in the extract, such as Liquid Chromatography-Mass Spectrometry.

| Eluens                  | Ratio (v/v) | Eluent Distance (cm) | Compound Distance (cm) | Retention Factor (Rf) | Spot Number |
|-------------------------|-------------|----------------------|------------------------|-----------------------|-------------|
| Ethanol 70% : ethyl acetate | 3 : 1       | 6.5                  | 2                      | 0.31                  | 1           |
|                         | 1 : 2       | 6.5                  | 0.5                    | 0.08                  | 1           |
| Ethanol 70% : chloroform | 1 : 4       | 6.5                  | 1.5                    | 0.23                  | 1           |
|                         | 4 : 1       | 6.5                  | 1                      | 0.15                  | 1           |
| N-butanol : acetic acid : water | 4 : 1 : 5 | 6.5                  | 1                      | 0.15                  | 1           |

Table 3. Data of TLC Result using Different Eluen Systems.

3.5. Preliminary in silico analysis

This method was used to develop the potency of the flavonoid obtained from this study as XO inhibitor compared to allopurinol as a well known drug for anti gout treatment. The previous study
showed that *A. squamosa* L. extract contains flavonoid type. The other study also performed the completely structural analysis on leaf extract of *A. squamosa* L. which confirmed that the extract contains quercetin-3-glucoside, one of flavonoid group [12]. To predict the ability of this flavonoid compound to inhibit the xanthine oxidase enzyme, relative to allopurinol, this study also carried out in silico analysis.

The interaction between ligand and enzyme (XO) was determined based on their binding affinity. The binding affinity of three ligand (xanthine, allopurinol and flavonoid) toward XO was shown in Table 4. The binding affinity energy exhibited the minimum energy used to form the interaction of ligand-enzyme complex. As shown in Table 4, docking result of all of ligands have different and negative value of binding affinity in each mode. However, the more negative value was shown by the predicted flavonoid (quercetin-3-glucoside). The more negative value indicated the greater binding affinity of ligand and XO. It also showed the lower energy needed to form a complex of ligand and enzyme thus the binding affinity will increase. This result confirmed that quercetin-3-glucoside have the greater affinity toward XO compared to xanthine and allopurinol. So this results proved that those flavonoid type compound was more potential as xanthine oxidase inhibitor compared to standard allopurinol.

| Ligand              | Mode 0 | Mode 1 | Mode 2 | Mode 3 | Mode 4 | Mode 5 | Mode 6 | Mode 7 | Mode 8 |
|---------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Xanthine            | -6.4   | -6.3   | -6.2   | -6.0   | -5.9   | -5.9   | -5.8   | -5.7   |
| Allopurinol         | -6.3   | -5.9   | -5.8   | -5.8   | -5.6   | -5.6   | -5.5   | -5.5   |
| Quercetin-3-glucoside| -9.6   | -9.4   | -9.3   | -9.3   | -9.2   | -9.2   | -9.1   | -8.9   |

Furthermore, the binding position between each ligand to XO was also analysed and visualized using PyMol Software, as can be seen in Figure 4, while the specific distance, type of chemical bond and amino acid residue that contributing in the interaction, can be further analysis using Discovery studio software as can be seen in Table 4. The letter code and number of amino acid name indicate chain and residual number that involved in the intermolecular interaction.
Figure 4. Binding Position of Xanthine (a), Allopurinol (b), and Quercetin-3-glucoside (c) on Xanthine Oxidase (XO).

Table 5. The Intermolecular Interaction on Ligand-XO Complexes

| Ligand          | Amino acid | Distance (Å) | Intermolecular Interaction   |
|-----------------|------------|--------------|------------------------------|
| Xanthine        | ASP B: 429 | 3.14         | Pi-Anion                     |
|                 | HIS C: 1220| 2.50         | Conventional Hydrogen Bond   |
|                 | ARG C: 1222| 2.41         | Unfavorable Donor-Donor      |
|                 | ARG C: 1222| 4.78         | Pi-Alkyl                     |
|                 | THR C: 1226| 2.75         | Conventional Hydrogen Bond   |
|                 | SER C: 1225| 2.91         | Conventional Hydrogen Bond   |
|                 | SER C: 1225| 2.46         | Conventional Hydrogen Bond   |
|                 | GLU A: 45  | 2.60         | Conventional Hydrogen Bond   |
| Allopurinol     | SER C: 1234| 2.34         | Conventional Hydrogen Bond   |
|                 | SER C: 1234| 2.72         | Conventional Hydrogen Bond   |
|                 | ILE C: 1235| 2.14         | Conventional Hydrogen Bond   |
|                 | ASN C: 1175| 2.42         | Conventional Hydrogen Bond   |
|                 | THR C: 1237| 2.49         | Conventional Hydrogen Bond   |
| Quercetin-3-glucoside | LEU B: 404 | 2.85         | Conventional Hydrogen Bond   |
|                 | LEU B: 404 | 2.34         | Conventional Hydrogen Bond   |
|                 | GLY B: 349 | 3.55         | Carbon Hydrogen Bond         |
|                 | SER B: 347 | 3.29         | Conventional Hydrogen Bond   |
|                 | SER B: 347 | 2.82         | Conventional Hydrogen Bond   |
|                 | ILE B: 264 | 5.38         | Pi-Alkyl                     |
|                 | ASN B: 261 | 2.80         | Conventional Hydrogen Bond   |
|                 | LYS B:256  | 4.29         | Pi-Cation                    |
|                 | LYS B:256  | 5.38         | Pi-Alkyl                     |
|                 | LYS B:256  | 3.57         | Pi-Sigma                     |
As shown in the Table 5, the binding site of xanthine and allopurinol was relatively the same (that is around 1200 residues), but was different to quercetine-3-glucoside (around 200, 300 and 400 residues). This data then may exhibits the different inhibition mechanism of the flavonoid (quercetin-3-glucoside) compared to allopurinol. The study from Umamaheswari et al. also clarified that allopurinol possess competitive inhibition mode toward xanthine oxidase [18]. Thus, the quercetin-3-glucoside may have non-competitive inhibition mode, because of the different binding site compared to xanthine substrate. However further investigation such as using kinetic assay, was need to be performed in order to characterize the specific mechanism.

Both in vitro and in silico analysis of flavonoid isolates from sugar apple showed a higher inhibitory activity against xanthin oxidase, compared to the antigout drug: allopurinol, so that the flavonoid isolate has a high potential as an antigout drug.

4. Conclusion
Based on in vitro and in silico analysis, flavonoid isolated from Annona squamosa L. exhibited the greater inhibition activity toward xanthine oxidase compared to allopurinol. Because of their inhibitory activity, the flavonoid from sugar apple could be developed as a potential antigout agent.

Acknowledgement
This work was supported by PNBP Research Grant from Universitas Negeri Malang.

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