Isolation, Identification, and Inhibition of Saponin Isolates from Pineapple (*Ananas comosus* L.) and Candlenut (*Aleurites moluccanus* L.) against Xanthine Oxidase by In Vitro Assay

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Abstract. The exploration of gout treatment agent based on the natural product gains high attention in recent years. Although some current drugs effectively treat gout, fewer unbenefficial effects cannot be avoided. Thus, searching for new alternatives with fewer side effects is needed. This present work was aimed at investigating the potency of saponins in pineapple (*Ananas comosus* L.) and candlenut (*Aleurites moluccanus* L.) extract as an inhibitor of xanthine oxidase, an enzyme that responsible for gout disease, by in vitro study. The stage of this work was divided into six stages, including (1) sample preparation, (2) extraction of saponins using 70% ethanol, (3) isolation of saponins by thin-layer chromatography, (4) phytochemical test of saponins, (5) inhibitory activity testing of saponins using in vitro analysis, and (6) determination the types of saponins by UV-Vis, FT-IR and LC-MS/MS spectroscopy. The result from the preparative TLC of pineapple extract, there were three spots with Rf value of 0.95 (pi1), 0.65 (pi2), and 0.38 (pi3), while from candlenut extract there were two saponin isolates with Rf value of 0.95 (ci1) and 0.79 (ci2). The inhibition activity of each isolate against xanthine oxidase was 103.41% (pi1), 269.73% (pi2), 84.16% (pi3), 222.90% (ci1), and 157.05% (ci2) relative to Allopurinol 100 ppm. Based on spectroscopic analysis results, isolate with the greatest inhibition activity was likely a diosgenin type (C₂₇H₄₂O₃) of saponin.

1. Introduction

According to the WHO survey in 2015, Indonesia is the fourth largest country in the world with people who suffer from gout. Gout occurs every 840 per 100,000 people and represents about 5% of total arthritis [1]. Gout is caused by high levels of uric acid in the blood, due to the high intake of purines and excessive synthesis of uric acid [2].

The drug that is commonly used to treat gout is Allopurinol which reduces the activity of uric acid synthesizing enzymes, namely xanthine oxidase. However, long term use of Allopurinol can cause some side effects and even more serious diseases such as inflammation, kidney failure, and chronic heart [3].

Some traditional medicinal plants contain secondary metabolites that can inhibit the xanthine oxidase [4]. Some of them that have potential as xanthine oxidase inhibitors have been investigated for instance srikaya fruit and sendok leaf extract [5], rambutan bark extract [6], salam leaf extract [7], and mlinjo bark extract [8]. Besides that, based on testimonies from the community, pineapple and candlenut juice mixes are also effective for treating gout.

Extract pineapple and candlenut contains diosgenin saponin compounds that have the potential as xanthine oxidase inhibitors based on in silico study [9]. Therefore, in this present study, the isolation,
identification, and test of saponin isolate activity from pineapple (Ananas comosus l.) and candlenut (Aleurites moluccanus l.) on xanthine oxidase was carried out by in vitro assay.

2. Methods

2.1. Materials and Instruments
This study used pineapple (Ananas comosul L.) and candlenut (Aleurites molucannus L) flesh as samples. Some chemicals used in this research were ethanol 70%, acetone p.a, Allopurinol tablet (100 mg), phosphate buffer 0.05 M (pH of 7.5), n-hexane p.a, acetic acid p.a, iod powder, aquadest, HCl 0.1 N, gel silica plat G60F254 (MERCK), xanthine (0Sigma) and xanthine oxidase from bovine (Sigma). UV-VIS spectrophotometer (Genesys 10S UV-Vis) and LC-MS/MS (MassLynx 4.1 SCN 884 Xevo G2-S QTof/Xevo G2-S Tof) were utilized to characterize the structure of bioactive compound in both samples.

2.2. Procedures
2.2.1. Sample Preparation. The pineapple was sliced thinly followed by drying process under the sunlight. The pineapple then was dried again in the oven at the temperature of 55-65 ºC for four days until it reached the constant weight. The grinding process was carried out to obtained the powder form of dried pineapple. The next step was sifting and measuring the weight of smooth powder. Whereas the candlenut was ground directly using mortal and pastel then be weighed.

2.2.2. Sample Extraction. The powder of both samples was macerated using 600 mL of ethanol 70% p.a for 72 hours at a temperature of 30 ºC. Then the filtration was conducted using Buchner funnel. Rotatory vacuum evaporator was used to obtain the thick extract of samples. This extract was weighed to calculate the rendement.

2.2.3. Isolation of Bioactive Compound. The bioactive compound contained in the sample was isolated by qualitative and preparative thin-layer chromatography (TLC). The first step was preparing the concentrated samples that were previously dissolved in ethanol 70% p.a. The gel silica plat containing the sample was put in the chamber filled by the different eluents with varied compositions. TLC of pineapple extract was chloroform: ethanol: acetic acid (1: 2: 1) and candlenut extract n-hexane: ethyl acetate was (1:4). Iodine powder was utilized as a stain viewer to sharpen the spot formed. The spot was exposed under the UV light at a wavelength of 254 nm and 365 nm. The appropriate eluents of qualitative TLC in this step were used for the next preparative TLC. The stained spot was scraped from the plates, weighed and dissolved in methanol, homogenized using vortex apparatus and then centrifuged. The filtrate obtained was then air dried, weighed, and dissolved in ethanol. The following stages were structural analyzing using UV-Vis, FT-IR, and LC-MS / MS spectrophotometry and inhibition activity testing by in vitro analysis.

2.2.4. Phytochemical Characterization. The type of phytochemical characterization used in this study was saponin analysis. The purpose of this test was to know the saponin content in both samples. 0.1 grams of concentrated sample was added by 5 mL hot aquadest and shaked. The positive result was shown by the formation of foam. One to two drops of HCl 0.1 N was added to maintain the foam produced.

2.2.5. Inhibitory Activity Testing towards XO. Both isolated sample were examined their inhibitory activity towards XO. Allopurinol was used as a positive control of this testing. The activity of XO was examined based on the absorbance in the UV spectrophotometer at a wavelength of 284 nm. The greatest inhibition activity was used for the following structural characterization i.e. UV-Vis, FT-IR, and LC-MS/MS spectrophotometer. Table 1 presents the reagent and volume used in this analysis.
Table 1. Volume and Reagent Used in the Inhibitory Activity Testing

| Reagent                        | Volume (mL) |
|--------------------------------|-------------|
|                                | wi | Cwi | pi1 | Cpi1 | pi2 | Cpi2 | pi3 | Cpi3 | ci1 | Cci1 | ci2 | Cci2 | A   | CA  |
| Inhibitor                      | -  | -   | 1.0 | 1.0  | 1.0 | 1.0  | 1.0 | 1.0  | 1.0 | 1.0  | 1.0 | 1.0  | 1.0 | 1.0 |
| Phosphate buffer pH 7.5         | 3.9| 4.0  | 3.9 | 4.0  | 3.9 | 4.0  | 3.9 | 4.0  | 3.9 | 4.0  | 3.9 | 4.0  |     |     |
| Xanthine 0.15 mM               | 2.0| 2.0  | 2.0 | 2.0  | 2.0 | 2.0  | 2.0 | 2.0  | 2.0 | 2.0  | 2.0 | 2.0  | 2.0 | 2.0 |

**Pra incubation at temperature of 37 °C for 8 minutes**

| Xanthine oxidase 0.05 U/mL     | 0.1| -   | 0.1 | -    | 0.1 | -    | 0.1 | -    | 0.1 | -    | 0.1 | -    |     |     |
| HCl 1.0 N                      | -  | 1.0  | -   | 1.0  | -   | 1.0  | -   | 1.0  | -   | 1.0  | -   | 1.0  |     | 1.0 |

**Incubation at temperature of 37 °C for 20 minutes**

| HCl 1.0 N                      | 1.0| -   | 1.0 | -    | 1.0 | -    | 1.0 | -    | 1.0 | -    | 1.0 | -    |     |     |

Note:
wi : without inhibitor
Cwi : control without inhibitor
pi1 : pineapple isolates 1
Cpi1 : control pineapple isolates 1
pi2 : pineapple isolates 2
Cpi2 : control pineapple isolates 2
pi3 : pineapple isolates 3
Cpi3 : control pineapple isolates 3
ci1 : candlenut isolates 1
Cci1 : control candlenut isolates 1
ci2 : candlenut isolates 2
Cci2 : control candlenut isolates 2
A   : allopurinol
CA  : control allopurinol

The xanthine oxidase activity can be calculated using the following equation,

\[
\text{Activity} = \frac{\text{absorbance of sample} - \text{absorbance of control}}{\varepsilon \times \text{vol} \times \text{df}} \times 0.1
\]  
where E is absorbance of sample at 284 nm, EK is absorbance of sample control at 284 nm, vol is total volume when measuring (mL), df is dilution factor, \( \varepsilon \) is uric acid coefficient (12.2 mM), and 0.1 showed the volume of xanthine oxidase used (mL).

The amount of uric acid produced can be calculated using Lambert-Beer law using the following equation,

\[
A = \varepsilon \cdot b \cdot C
\]

where A is absorbance at 284 nm (sample absorbance - absorbance control), b is cuvette width (1 cm), and C is gout concentration (mM)

The inhibition activity towards xanthine oxidase can be calculated using this equation,

\[
\% \text{ Inhibition} = 1 - \frac{B}{A} \times 100\%
\]

where A indicates the absorbance of sample without inhibitor while B indicates the absorbance of sample with inhibitor

The percentage of inhibition activity of the sample relative to Allopurinol (% DIA) can be calculated by this formula:

\[
\% \text{DIA} = \frac{\text{inhibition activity of isolate}}{\text{inhibition activity of Allopurinol}} \times 100\%
\]

2.2.6. Structural Characterization of Saponins

2.2.6.1. UV-Vis Spectroscopy. Isolates obtained from preparative TLC were dissolved in methanol p.a and analyzed using a UV-Vis spectrophotometer at a wavelength of 200-400 nm.

2.2.6.2. FT-IR Spectroscopy. This process was carried out to analyze the functional group of bioactive compounds. The sample from preparative TLC was dissolved in ethanol 70%. The solvent was evaporated then the product was mixed with KBr until homogenous. This result was then analyzed using FT-IR spectrophotometer at a wavelength number of 400-400 cm\(^{-1}\).
2.2.6.3. LC-MS/MS Spectroscopy. This step was purposed to identify the saponins compound based on the m/z result. It was about 0.5 of both isolate volume from preparative TLC that was used to this structural characterization.

3. Results and Discussion

3.1. Pineapple and Candlenut Flesh Extract
The concentrated extract obtained from 613.6 grams of pineapple flesh was 17.45 grams while the candlenut flesh extract with the weight of 31.29 grams yielded 5.09 grams of viscous concentrated extract.

3.2. Pineapple and Candlenut Flesh Extract
Qualitative TLC is used to determine the type of eluents that are suitable for separating compounds in a sample while preparative TLC aims to isolate the bioactive compound. Based on preliminary experiments, the eluent that can separate well in TLC of pineapple extract was chloroform: ethanol: acetic acid (1: 2: 1) followed by exposing under UV254 and UV365. The stain results of qualitative and preparative TLC of pineapple extract under UV detection are shown in Figure 1. Based on those profiles, there are 3 spots found in qualitative TLC. These three spots have different Rf values, i.e 0.95, 0.65, and 0.38. These three spots indicate three isolates (pi1, pi2, and pi3) which also have different mass, i.e 1.165, 1.134, and 1.092 grams, respectively.

![Figure 1.](a) Qualitative TLC of Pineapple Extract (b) Preparative TLC of Pineapple Extract Detected by UV254 (2 isolates) and (c) UV365 (3 isolates)

Meanwhile, the TLC result of candlenut presents in Figure 2. The mixture of n-hexane: ethyl acetate (1:4) was used as a mobile phase of this TLC. The results obtained from qualitative and preparative candlenut TLC are different. Qualitative TLC showed one stain with Rf (0.95). However, for preparative TLC produced 2 stains with Rf (0.93 and 0.87). Figure 2 indicates that there are two isolates obtained from preparative TLC of candlenut extracts. Those two spots were taken and labeled as ci1 and ci2 with an isolate mass of 1.195 and 1.155 grams, respectively.

![Figure 2.](a) Qualitative TLC of Candlenut Extract (1 isolate) (b) Preparative TLC of Candlenut Extract detected by UV254 (1 isolate) (c) UV365 (1 isolate)

3.3. Phytochemical Result
Phytochemical analysis of all samples from ethanol extract, ethanol viscous extract, and all isolates in pineapple and candlenut positively contain saponins which are indicated by the formation of foam. The foam produced is due to a combination of non-polar sapogenin chains and water-soluble polar side chains. The more foam produced, the greater the concentration of saponin content in the sample.
3.4. The Inhibition Activity of Sample Isolates

Enzyme activity test was set by in vitro analysis using inhibitors and without inhibitors. Each isolate of both samples obtained from preparative TLC was tested their inhibition power toward xanthine oxidase (XO). The activity of XO was examined based on the absorbance at a wavelength of 284 nm using UV spectrophotometer. This test also used Allopurinol, a commercial drug for gout treatment, as a positive control. Based on the results of pineapple isolates, each isolate has a different inhibition activity against XO. The greatest inhibition power was shown by isolate 2 of pineapple (pi2) which more potential than Allopurinol. In addition, the candlenut isolates also show great inhibition activity. The inhibition percentage of isolate 1 of candlenut (ci1) was the greatest compared with isolate 3 and Allopurinol activity. The calculation results of this step were presented in Tables 2 and 3.

Table 2. Inhibition Activity of Pineapple Isolates and Allopurinol

| Samples          | Measurement | Absorbance | Average XO Activity (U/mL) | Average Concentration of Uric Acid (mM) | Inhibition Power (%) | Relative inhibition power to Allopurinol (%) |
|------------------|-------------|------------|---------------------------|----------------------------------------|----------------------|---------------------------------------------|
| Without Inhibitor | 1           | 0.006      | 0.086                     |                                        |                      |                                             |
|                  | 2           | 0.005      | 0.086                     |                                        |                      |                                             |
|                  | 3           | 0.005      | 0.087                     |                                        |                      |                                             |
| Allopurinol 100 ppm | 1           | 0.001      | 0.087                     |                                        |                      |                                             |
|                  | 2           | 0.001      | 0.087                     |                                        |                      |                                             |
|                  | 3           | 0.001      | 0.086                     |                                        |                      |                                             |
| Isolate 1 Pineapple (52 mg) | 1         | 0.023      | 0.081                     |                                        |                      |                                             |
|                  | 2           | 0.020      | 0.083                     |                                        |                      |                                             |
|                  | 3           | 0.019      | 0.082                     |                                        |                      |                                             |
| Isolate 2 Pineapple (71 mg) | 1         | 0.048      | 0.078                     |                                        |                      |                                             |
|                  | 2           | 0.048      | 0.079                     |                                        |                      |                                             |
|                  | 3           | 0.050      | 0.078                     |                                        |                      |                                             |
| Isolate 3 Pineapple (70 mg) | 1         | 0.024      | 0.087                     |                                        |                      |                                             |
|                  | 2           | 0.024      | 0.089                     |                                        |                      |                                             |
|                  | 3           | 0.023      | 0.090                     |                                        |                      |                                             |

3.5. UV-Vis Analysis Result

The profile of UV-Vis spectrum containing saponin in pineapple sample is shown in Figure 3. Meanwhile, the UV-Vis spectrum of candlenut isolates is shown in Figure 4. Based on the UV-Vis spectrum profile, the three saponin isolates of pineapple and the two saponin isolates of candlenut contain the n → σ* electronic transition indicating OH groups and the n → π* electronic transition, which means that there are conjugated double bonds (C=C) in the isolates. The presence of the -OH group is one of the characteristics of the saponin structure.

Figure 3. UV-Vis Spectrum of (a) the Three Pineapple Saponin Isolates and (b) Candlenut Saponin Isolates
Table 3. Inhibition Activity of Candlenut Isolates and Allopurinol

| Samples          | Measurement | Absorbance | Average XO Activity (U/mL) | Average Concentration of Uric Acid (mM) | Power Inhibition (%) | Relative inhibitory power to Allopurinol (%) |
|------------------|-------------|------------|---------------------------|----------------------------------------|----------------------|---------------------------------------------|
| Without Inhibitor| 1           | 0.011      | 0.116                     |                                        | 4.85                 | 0.0087                                      | 0%                                          | 100%                                        |
|                  | 2           | 0.01       | 0.117                     |                                        |                      |                                             |                                             |
|                  | 3           | 0.011      | 0.116                     |                                        |                      |                                             |                                             |
| Allopurinol 100 ppm| 1           | 0.001      | 0.087                     |                                        | 4.49                 | 0.0070                                      | 23.49%                                      | 100%                                        |
|                  | 2           | 0.001      | 0.087                     |                                        |                      |                                             |                                             |
|                  | 3           | 0.001      | 0.086                     |                                        |                      |                                             |                                             |
| Isolate 1 candlenut (50 mg)| 1 | 0.031      | 0.08                      |                                        | 2.81                 | 0.0041                                      | 52.36%                                      | 222.90%                                    |
|                  | 2           | 0.031      | 0.081                     |                                        |                      |                                             |                                             |
|                  | 3           | 0.03       | 0.082                     |                                        |                      |                                             |                                             |
| Isolate 2 candlenut (116 mg)| 1 | 0.056      | 0.115                     |                                        | 3.11                 | 0.0049                                      | 43.85%                                      | 157.05%                                    |
|                  | 2           | 0.055      | 0.115                     |                                        |                      |                                             |                                             |
|                  | 3           | 0.057      | 0.116                     |                                        |                      |                                             |                                             |

3.6. FT-IR Analysis Results

Based on the results of FT-IR data interpretation, it is known that pineapple second isolate and candlenut first isolate contain -OH because in the region around 3500-3200 cm⁻¹ has a strong and wide intensity peak. Absorption bands around 3300-2700 cm⁻¹ indicate the presence of aliphatic C-H groups, bands in the region of 1100-990 cm⁻¹ indicate the presence of C-O groups and reinforced with bands with wave numbers 1000-630 cm⁻¹ indicate aromatic C-H groups. The resulting clusters are characteristic of saponin compounds.

Figure 4. FT-IR Spectrum of Pineapple Second Isolates

Figure 5. FT-IR Spectrum of Candlenut First Isolates
3.7. LC-MS / MS Analysis Results

Analysis using LC-MS/MS spectrophotometer aims to determine the type of saponins found in pineapple and candlenut isolates. LC analysis of pineapple isolates 2 produces 17 chromatograms, which showed that the isolates tested were not pure (Figure 7.a). Saponin compounds from pineapple fruit isolates are found at the seventh peak at 11.56 retention times (Figure 6a). In Figure 6b there are compounds that are thought to be saponins at m/z 415.2110 with the molecular formula C_{27}H_{43}O_{3}.

Although there are many higher peaks on the LC chromatogram compared to peaks with a retention time of 11.56 (Figure 6a), it turns out that these compounds are not saponin compounds. This has been analyzed based on elemental composition that there are no saponin-containing compounds, other than those whose retention time is 11.56, because the characteristics of steroid and triterpenoid compounds are C, H, and O atoms. In addition, steroid saponin compounds contain 30 C atoms and triterpenoid saponins contain atoms C as many as 27. The peak with a retention time of 16.19 at m/z 313.2734 with the molecular formula C_{19}H_{37}O_{3} contains C, H, and O atoms, but does not contain as many as 27 or 30 C atoms so that the compounds are not classified as saponin.

That the compound with a retention time of 11.56 minutes is saponin, it can be seen in PubChem by reducing the mass of 1 sma H atom at m/z value 415.2110, because the compound is positively ionized, so the molecular formula C_{27}H_{43}O_{3} with m/z 414.2110 is obtained, which based on literature (PubChem) is a triterpenoid saponin compound, Diosgenin. The fragmentation of diosgenin compounds produced by LC-MS / MS can be seen in Figure 7.

LC analysis of candlenut 1 isolate yielded 22 chromatograms, which showed that the isolate tested was not pure (Figure 6a). Saponin compounds from isolate 1 candlenut were found at the 10th peak LC at 11.72 minutes of retention time (Figure 8a). In Figure 8b there are compounds that are thought to be saponins at m/z 415.2109 with the molecular formula C_{27}H_{43}O_{3}.

Other compounds in candlenut isolates other than those whose retention time at LC is 11.72, are not saponin compounds which can be proven based on the results of elemental composition analysis. Compounds containing saponins that contain C, H, and O atoms. Besides steroid saponin compounds it contains 30 C atoms and triterpenoid saponins contain 27 C atoms. The peak with a retention time of 11.02 at m/z 273.1479 with molecular formula contains C, H, and O atoms, but does not contain 27 or 30 C atoms.

![Image](image_url)

**Figure 6.** (a) LC Spectrum of Pineapple Isolate 2 and (b) MS after enlarged at retention time 11.56
Figure 7. a–c MS Spectrum Fragmentation of Diosgenin Compounds in Pineapple

Figure 8. (a) LC Spectrum isolates 1 of Candlenut and (b) MS after enlarged at retention time 11.72
The saponin compound at 11.72 minutes at LC with m/z 415.2109 is Diosgenin with the molecular formula C₁₇H₂₁O₃ then it can be seen in Pubchem by reducing the mass of 1 sma H atom at m/z value because the compound is positively ionized. Then the fragmentation results of LC-MS/MS analysis of compounds with 11.76 minutes retention time (Figure 9) are matched with the diosgenin fragmentation patterns (PubChem Figure 7). In Figure 8, fragments of Diosgenin compound with molecular weight of 415.2109 were fragmented at m/z 157.0828; 253.1444; and 271.1666 obtained from PubChem. The results of MS fragmentation of candlenut isolates also have a spectrum pattern with m/z which is almost the same as the results of the fragmentation spectrum in Figure 7, namely at m/z 157.0828; 271.1666; and 253.1444 which can be seen in Figure 9.

![Figure 9. a-c MS Spectrum Fragmentation of Diosgenin Compounds in Candlenut](image)

4. Conclusions
Based on this present work, it can be concluded that from the preparative TLC of ethanol extract of pineapple flesh, three isolates of saponins were obtained while from candlenut extract, there are two isolates. The greatest inhibition activity of pineapple was shown by isolate 2 while from candlenut is isolate 1. Based on spectroscopic analysis, those both isolates may be predicted as triterpenoid saponins, Diosgenin (C₂₇H₄₂O₃).

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