

Flavonoid isolation and identification of mother-in-law's tongue leaves (*Sansevieria trifasciata*) and the inhibitory activities to xanthine oxidase enzyme

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Abstract. Hyperuricemia is a disease that is characterized by high uric acid levels, in which the number of victim increase year by year in the worldwide. Flavonoid is an active compound with inhibitory activity towards Xanthine Oxidase Enzyme which is a compound that plays a role in the formation of uric acid in the body. *Sansevieria trifasciata* is an ornamental plant which is also useful as a source of antibacterial and antioxidant agent. Studies of *S. trifasciata* as Xanthine Oxidase Enzyme inhibitor have not been reported. This research isolate flavonoid compounds using open column chromatography from crude extract of *S. trifasciata* leaves that extracted by sonication method. There are six eluent used to isolate flavonoid which are methanol : ethyl acetate, chloroform : ethyl acetate, chloroform : ethyl acetate :methanol. Wilstater test is used to select the fraction that rich of flavonoid. The best result from isolation step that contains flavonoid is assessed the inhibitory activity of xanthine oxidase. It is analyzed qualitative using Liquid Chromatography Mass Spectrometry (LCMS). The inhibition percentage showed that fraction 3 was potential to inhibit XO by 85.48 %. LC-MS chromatogram can show that crude extract and positive fraction of isolation were containing flavonoid.

1 Introduction

Hyperuricemia, associated with gout, results from the overproduction or underexcretion of uric acid and it is greatly influenced by a high dietary intake of foods rich in nucleic acids. The traditional diets of Indonesia were especially high in wild meat, which could be expected to contribute to gout, meats (especially organ meats), leguminous seed, some types of seafood and food yeasts that are considered rich of nucleic acids [1]. The catalysis of xanthine by the enzyme xanthine oxidase (XO) (EC 1.2.3.2) can lead to the accumulation of uric acid and ultimately causing gout. Joint disease that occurs due to accumulation of uric acid crystals was in top five diseases with the highest prevalence in Indonesia spread in 33 provinces with prevalence reached 24.7%. Allopurinol is an XO inhibitor prescribed for chronic gout, acts as a competitive inhibitor of the enzyme. However, allopurinol has side effects for the body. The most feared side effect of Allopurinol is the so-called "allopurinol hypersensitivity syndrome". Signs and symptoms of this syndrome are wound, high fever (usually > 39°C), liver dysfunction, leukocytosis, and renal failure [2]. Many plants are used by Indigenous people for the treatment of gout, or diseases with associated symptomologies such as rheumatism or arthritis, and we postulate that these may

contain XO inhibitors. XO inhibitors have been found in a wide variety of plants used in traditional herbal medicines for the treatment of gout and rheumatism for example Chile [3] Paraguay [4] [5] [6] and Panama [7].

Flavonoids [8][9], and certain other phenols^[10], polyphenols [10] and tannins [10], as well as coumarins [11], plant growth regulators [12] and folic compounds [13] have all been reported to be potent XO inhibitors. The putative therapeutic effects of many traditional medicines have been ascribed to flavonoids in particular due to their enzyme inhibitory and antioxidant activity [14] [15].

In particular *Sansevieria trifasciata* which in South Africa and tropical America is used for the treatment of inflammatory conditions and sold as a crude drug in the market to treat victims of snake bite [16]. It is also used for the treatment of ear pain, swellings, boils and fever [17]. Previous chemical studies on *Sansevieria trifasciata* led to the isolation of flavonoid [18] steroidal saponins namely 25S-ruscogenin and sansevierigenin [19], pregnane glycosides [20], and steroidal saponins [21]. Therefore, we expect a positive correlation between XO inhibitory activity and flavonoid content of plant extracts.

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The objective of this study is to know the effect of eluent polarity index from six eluent methanol:ethyl acetate (4:1), chloroform:ethyl acetate (1:6, 9:1 and 1:2), chloroform:ethyl acetate:methanol (1:2:1 and 1:2:2) with the separation result and to determine the validity of

2 Materials and Methodology

2.1. Materials

2.1.1 Substances

The dried leaves of *Sansevieria trifasciata* were obtained from Bogor, Indonesia. methanol, chloroform, hydrochloric acid, natrium hydroxide, ethanol, ethyl acetate and mg powder (merck) were obtained from bioprocess engineering laboratory. The Xanthin (X0626) and xanthin oxidase (Sigma X4375) were obtained from Badan Pengkajian dan Penerapan Teknologi (BPPT).

2.1.2 Instruments

Ultrasonic cleaner bath, Spectrophotometer UV-VIS (BEL Engineering UV-M90), Liquid Chromatography and Mass Spectrometry (LC-MS)

2.2. Methodology

2.2.1 Preparation leaf of Mother in-law tongue simplicia

1000 g wet weight of mother in-law's tongue leaves were sorted washed, and drained by using the oven at 60°C for 8 h/day and the sample was smoothed with a blender and sieve analyzer to obtain the sample powder diameter $\pm 0.15-0.18$ mm.

2.2.2 Extraction Method

Mother-in-law's tongue leaves powder was dissolved in 70% ethanol by 1:10 ratio of mass and solvent (m/v). Then, extraction using sonicator for 60 minutes at frequency of 53 kHz and 27°C, continued by solvent evaporating using vacuum rotary evaporator.

2.2.3 Best eluent determination

The Best eluent was determined with Thin Layer chromatography (TLC) silica G 60 F₂₅₄ using different eluent that used to isolate flavonoid which are methanol:ethyl acetate (4:1), chloroform:ethyl acetate (1:6, 9:1 and 1:2), chloroform:ethyl acetate:methanol (1:2:1 and 1:2:2). The eluent that have rf number closer to standard which is quercetin and give the best separation will be used in isolation method as mobile phase.

2.2.4 Isolation Methode

plant remedies used for gout by examining their xanthine oxidase inhibitory activity and whether a relationship exists between enzyme inhibition and the flavonoid and other content.

The isolation using column chromatography method using silica Gel 60 as stationary phase, aims to separate the compounds in a crude extract into some fractions using the best eluent for mobile phase determined in the previous experiment and the same rf number of many fraction will be put together.

2.2.5 Wilstater test

In every fraction of samples were added 2-4 drops of concentrated HCl and a pinch of Mg powder. Then observe the color change occurs, it should turns into a reddish orange color [22].

2.2.6 Xanthine Oxidase Inhibition Test

The XO activities with xanthine as the substrate were measured spectrophotometrically using the procedure of Marcocci et al. (1994) [23] with the following modifications. The xanthine solution (0.15 mM) was prepared by initially dissolving xanthine (Sigma) in a minimal volume of NaOH, and adjusting pH to 7.5. The XO solution was prepared by diluting XO from bovine's milk (Sigma) to a final concentration of 0.1 U/ml in cold 50 mM potassium phosphate buffer (pH 7.5). The assay mixture consisted of 0.250 ml plant extract solution (0.4 mg/ml 50 mM potassium phosphate buffer, pH 7.5), 0.385 ml 50 mM potassium phosphate buffer (pH 7.5) and 0.330 ml xanthine solution, giving a final concentration of 100 mg plant extract per ml assay mixture. The reaction was initiated by adding 0.035 ml XO solution, and the change in absorbance recorded at 295 nm for 3 min at room temperature. Allopurinol (Sigma) was used as a standard inhibitor at a final concentration of 100 mg/ml in the assay mixture [24].

2.2.7 Quantitative test using LC-MS

Quantitative test is used to analyze the concentration of compounds in crude extract and isolate fractions by using LC-MS method.

3 Result and Discussion

3.1 Yield percentage value of Mother-in-Law's Tongue extract

The extract of leaf was done by sonication method at 37°C for 50 minutes. From this extraction results obtained viscous extract of 4.53 grams of 25 grams simplicia. Based on the calculation, the extraction yield is 18.12 percent. This method give more yield percentage if we compare to another study of *Sansevieria trifasciata* extraction using maseration and methanol as the solvent nurlaila 2011 that have 15.5

yield percentage [25]. In the sonication method, cavitation occurs when the ultrasonic wave treatment is done to break down the cell wall of the simplicia material. Cavitation is the process of forming micro bubbles (microbubbles) due to increased pressure at the time of extraction as a result of ultrasonic waves. The bubbles are so unstable that they break easily when the bubbles reach an insufficient volume to absorb energy. The breaking of the bubbles involves a large amount of energy and produces a heat effect that helps contact between the solvent and the material in the extraction so that the extraction results are maximal. The mechanical effect of the sonication method is to increase the penetration of the solvent into the material cell and increase the mass transfer so that the time required for cell splitting is not too long [26].

3.2 Best eluent determination

The Thin Layer Chromatography Method in this study was used to find the best eluent of some of the eluents present in the separation of flavonoid compounds. The concentrated extract should be diluted first with a small amount of methanol and filtered using filter paper. It aims to make the elution process work well. If the extract is too thick it will be difficult to get carried away by the solvent. The mobile phase or eluent used in the study consisted of 6 types of eluent: methanol:ethyl acetate (4:1), chloroform:ethyl acetate (1:6, 9:1 and 1:2), chloroform:ethyl acetate:methanol (1:2:1 and 1:2:2). The following is the stain sighting data from the crude extract elution using TLC, viewed using 366nm ultraviolet lamp.

Table 1. Crude extract TLC data result using some eluents

Eluent	Mark	Tailed	Well Separated
Methanol:Ethyl Acetate (4:1)	3	-	√
Chloroform:Ethyl Acetate (1:6)	1	-	-
Chloroform:Ethyl Acetate (9:1)	1	-	-
Chloroform:Ethyl Acetate (1:2)	2	-	-
Chloroform:Ethyl Acetate:Methanol (1:2:1)	1	-	-
Chloroform:Ethyl Acetate:Methanol (1:2:2)	2	-	-

In Table 1 shows the number of stains and profile of the stain formed, which is a parameter to see if the eluent provides good separation. The best eluent is the one that produces the highest number of stains and forms a clear or non-tailed spot on the plate this is due to the different distribution of the compounds present in the simplicia based on their polarity, when the eluent used is a polar eluent according to the compounds contained in the extract [27]. which is in this study methanol and ethyl acetate (4:1) was the best eluent to separate the sample.

Isolation of flavonoids is affected by the polarity of the solvent, where the polarity of a solution is related to the polarity index and if the polarity index value increases then the polarity of a solvent also increases. Based on the calculation of polarity index in every eluent, methanol and ethyl acetate (4:1) has the higher number of polarity index.

3.3 Isolation and qualitative test result

The chromatography column used is the chromatography column of silica gel. The columns are prepared by the wet method ie the method by which the silica gel incorporated into the chromatographic column has the form of a silica slurry. Wet method is chosen because silica gel is easy to mix (homogeneous). Eluent used was a mixture of methanol and ethyl acetate with a ratio of 4:1. The crude extract is placed in the column and will be absorbed into the silica gel stationary phase before eluting with methanol and ethyl acetate. When elution takes place, the color of the crude extract then gradually becomes faded. This shows that there is a reaction between the extract and the stationary phase. After that happened yellow separation and gradually the colors of the separation become lost. The column chromatographic fractions were divided into 27 fractions which were further tested using thin layer chromatography to determine similar Rf number, so it becomes 7 fractions.

Wilstater test was conducted to the 7 fractions obtained to confirm the presence of flavonoid compounds.

Table 2. Results of Wilstater test.

Number of Fraction	Flavonoid
1	-
2	-
3	+
4	+
5	-
6	-
7	-

Based on the data obtained fractions 3 and 4 contain flavonoids, which at the time of the test made a change of color from yellowish orange to reddish orange. the purpose of addition of concentrated HCl and Mg powder in this test is to reduce benzopiron contained in the flavonoid structure so that the color changes to orange or red. After the addition of Mg powder, the sample was heated and added HCl. HCl testing resulted in the result that the sample changed color to orange. This indicates a reduction oxidation reaction between Mg powder as a reducer, with a flavonoid sample. The oxidation reaction of reduction between Mg powder and flavonoids, causing the formation of complex compounds that give rise to orange in the sample.

3.4 Xanthine Oxidase Inhibition Test

Xanthine oxidase activity was expressed as percent inhibition of xanthine oxidase, calculated as $(1 - B/A) \times 100$, where A is the change in absorbance of the assay without the plant extract ($\Delta\text{abs. with enzyme} - \Delta\text{abs. without enzyme}$), and B is the change in absorbance of the assay with the plant extract ($\Delta\text{abs. with enzyme} - \Delta\text{abs. without enzyme}$) [24].

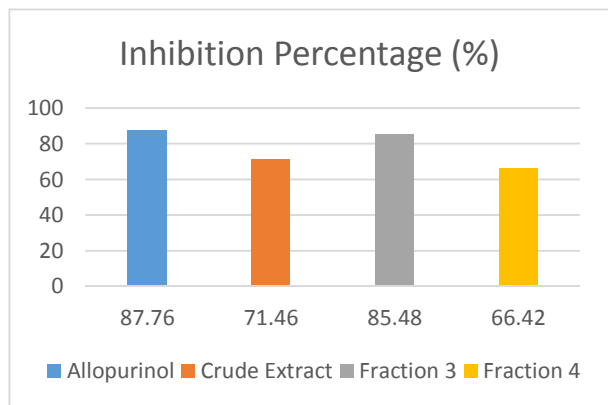


Fig 1. Results of XOI.

Figure 1 shows the results of xanthine oxidase inhibition test. Fraction 3 has the biggest inhibition

percentage compared to crude extract and fraction 4. Allopurinol is a compound that has the greatest inhibitory value in inhibiting uric acid formation, since allopurinol is a competitive reversible inhibitor, a competitive inhibitor has a structure similar to that of the substrate, causing competition between the substrate and the inhibitor in binding the active side of the enzyme [28].

Kinetical analysis that shown by *Lineweaver-Burk* [24] suggests that this type of inhibition was inhibitors with competitive inhibitory mechanisms are compounds that have structures resembling substrate structures [29] and flavonoid compounds have similar structures with xanthin substrates [27].

3.5 Quantitative test using LC-MS

The LC-MS analysis was performed on crude extract, fraction 3 and fraction 4 (Fig. 2). The results was obtained from LC-MS were chromatographic curves from LC and molecular weight graph from MS for retention time. The results of the tests performed are shown in Table 4.

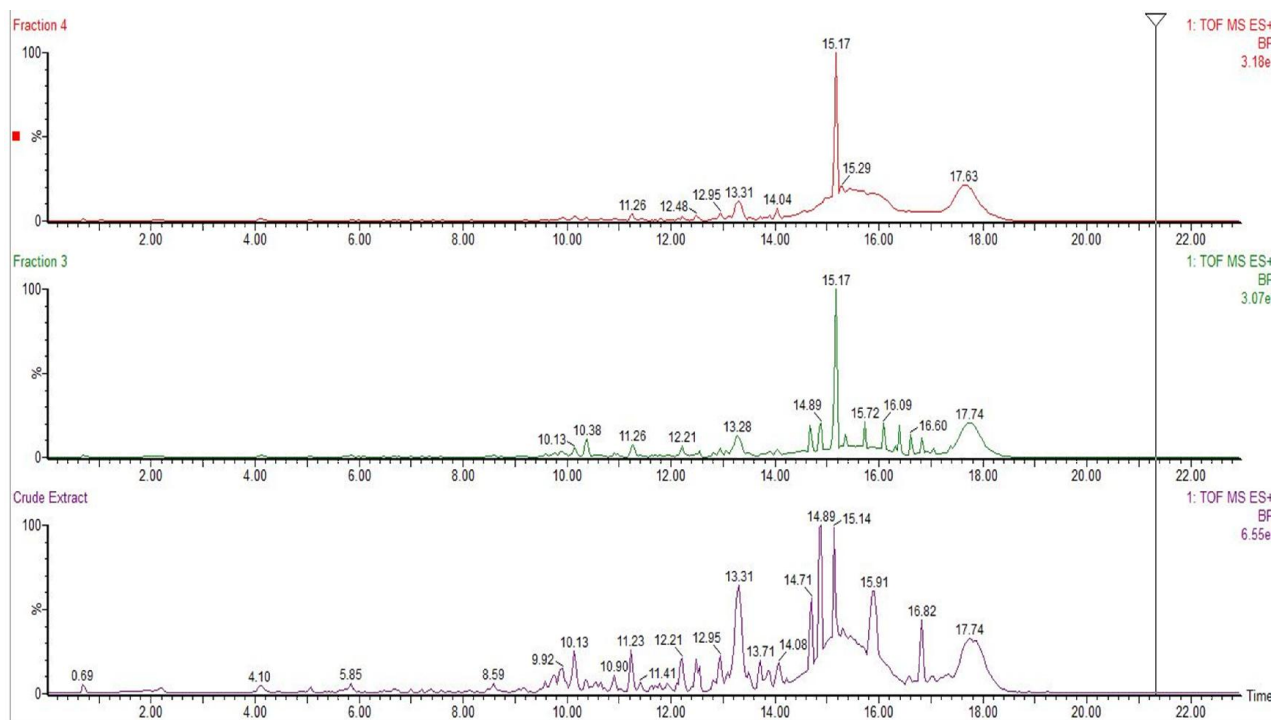


Fig. 2. LC-MS results

Table 4. LC-MS data result in every sample

Compound	Rt	Chemical Formula	Molecular Weight	Active Compound Content
Crude Extract	11.23	C ₁₆ H ₁₅ O ₅	287.28	Flavonoid (flavonones)
	12.21	C ₃₂ H ₂₇ O ₁₀	570.54	Flavonoid (isoflavone)
	14.89	C ₃₆ H ₃₃ N ₈ O ₂	68.077	Alkaloid (imidazol)
	15.14	C ₃₆ H ₃₃ N ₈ O	592.696	Pheophorbide A,
	0.69	H ₃ O ₁₄	146.143	Polyphenol (coumarins)
	12.95	C ₉ H ₇ O ₃	162.14	Polyphenol (hydrocoumarins)
	8.59	C ₄₄ H ₆₉ O ₁₇	870	Saponin
Fraction 3	11.26	C ₁₆ H ₁₅ O ₅	287.28	Flavonoid (flavonones)
	14.89	C ₃₆ H ₃₃ N ₈ O ₂	68.077	Alkaloid (imidazol)
	15.17	C ₃₆ H ₃₃ N ₈ O	592.696	Pheophorbide A,
Fraction 4	11.26	C ₁₆ H ₁₅ O ₅	287.28	Flavonoid (flavonones)
	15.17	C ₃₆ H ₃₃ N ₈ O	592.696	Pheophorbide A,

Based on these results the cooperation between flavonoid compounds, alkaloids, and pheophorbide A which are not disturbed by polyphenol compounds work better it when compared with crude extract or isolation in XOI percentage results. Although it has been said that flavonoid and polyphenol compounds may act as xanthine oxidase inhibitors ^[8] ^[9] ^[10], the results of this study show that the performance of the active compounds will be different when they separate.

4 Conclusion

The extraction result using sonication method was obtained 18,12% of yield. Methanol: Ethyl Acetate (4: 1) is a better eluent to separate the sample and from the isolation result by using column chromatography was obtained 7 main fractions and based on wilstater test fractions 3 and 4 containing flavonoid compound.

Based on the inhibition test of xanthine oxidase activity, it was found that the largest percentage of inhibition was performed by the third fraction with 85.48% followed by crude extract and the fourth fraction respectively 71.46% and 66.42%.

Based on the results of LC-MS indicated the success of the isolation process due to the survival of flavonoid compounds and the disappearance of some components in each fraction there.

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