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Original Article

THE EFFECT OF ETHYL ACETATE FRACTION OF *VERNONIA AMYGDALINA* DEL. LEAVES ON URIC ACID LEVELS OF MALE WHITE MICE (*MUS MUSCULUS*) HYPERURICEMIA

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ABSTRACT

Hyperuricemia is a condition where uric acid levels in the blood are increased above normal. High levels of uric acid in the blood can cause a buildup of uric acid crystals in the joints and other organs of the body. One of the plants used as traditional medicine to reduce uric acid levels in the blood is *Vernonia amygdalina* Del. plant. This study aims to determine the effect of ethyl acetate fraction of *V. amygdalina* on uric acid levels of hyperuricemic male white mice. The experimental animals used were 54 male white mice which were divided into 6 groups, namely the negative control group, the positive control group, the comparison group, the 12.5 mg/kgBW dose treatment group, the 25 mg/kgBW treatment group, and the 50 mg/kgBW doses treatment group with a treatment duration of 5 days, 10 days and 15 days. Data analysis used two-way analysis of variance (ANOVA) followed by Duncan's Post Hoc test with 95% confidence intervals ($P<0.05$). The results obtained showed that uric acid levels decreased along with the length of time the ethyl acetate fraction was administered with the optimal reduction effect at a dose of 50 mg/kgBW. This study shows that the ethyl acetate fraction of *V. amygdalina* exhibits antihyperuricemic activity and can be used as an alternative medicine in the community.

KEYWORDS: Ethyl Acetate Fraction, *Vernonia amygdalina* Del. Leaves, Uric Acid, Hyperuricemia.

1. Introduction

Joint pain is one of the complaints that many humans suffer from. This complaint can attack all ages, from young to old age [1]. The risk factors for this complaint are important as age and gender increase [2]. Often this complaint is associated with increased levels of uric acid in a person's blood [3]. Increased uric acid levels can be triggered by food, lifestyle such as doing too heavy exercise, or daily activities. When uric acid increases, symptoms or signs will appear in the body that can cause abnormalities [4].

Hyperuricemia is a condition where there is an increase in blood uric acid levels above normal [5]. A person is said to be hyperuricemic if the uric acid level is >7 mg/dL in men and >6 mg/dL in women [6]. Uric acid is the end result of purine metabolism in the body [7]. Under normal circumstances, there is a balance between the formation and degradation of purine nucleotides and the kidney's ability to excrete uric acid [8]. The condition of hyperuricemia can be caused by two main factors, namely high production of uric acid levels in the body due to excessive uric acid synthesis and decreased uric acid excretion in the distal tubules of the kidney [9].

The Basic Health Research Results Report stated that as many as 1.23% of the 15-24 year age group had suffered from joint disease. Moreover, gout attacks more men than women. If this disease attacks women, generally the women who suffer from it have reached menopause [10]. In Indonesia, gout is in second place after osteoarthritis. The results of basic health research show that the prevalence of joint disease in Indonesia based on the diagnosis of health workers in the population aged ≥ 15 years according to characteristics reached 7.30%. The highest prevalence of joint disease occurred at age ≥75 years based on the diagnosis of health workers in residents aged ≥ 15 years according to characteristics of 18.95%. The prevalence of joint disease in West Sumatra based on the diagnosis of health workers

in residents aged ≥ 15 years according to characteristics is 7.21% [11].

One of the treatments used for hyperuricemia is allopurinol. This drug can be used with the aim of reducing high uric acid levels in the blood. However, if consumed, this drug can cause side effects such as skin rashes or allergies, intestinal-stomach disorders, headaches, dizziness, hair loss thrombocytopenia, and aplastic anemia in patients with impaired kidney function [12]. One of the plants used as traditional medicine to reduce uric acid levels in the blood is the *Vernonia amygdalina* Del. plant [13].

Vernonia amygdalina Del. plants are believed to reduce uric acid levels in the blood. The results of Ijeh's research in 2011 [14] showed that *V. amygdalina* contains many nutrients, for example carbohydrates, protein, fiber, fat, ascorbic acid, carotenoids, calcium and iron. The chemical compounds contained in *V. amygdalina* are saponins, coumarins, lignin, terpenes, flavonoids, phenolic acids, and luteolin [15].

The results of Jumain's research in 2018 [16] showed that *V. amygdalina* contains a flavonoid compound, namely luteolin, which can inhibit the activity of the xanthine oxidase enzyme on purine bases so that it can reduce uric acid levels in the blood. Flavonoids are antioxidant compounds that can prevent the oxidation of hypoxanthine and xanthine to uric acid by xanthine oxidase. Inhibition of xanthine oxidase can reduce uric acid production in the blood [17,18]. Based on the description above, researchers are interested in conducting further research on the effects of fractionation of *V. amygdalina* with various variations in dose and duration of administration on uric acid levels in experimental animals. It is hoped that the results of this research will provide information and knowledge about the benefits of fractionated *V. amygdalina* leaf extract as an antihyperuricemia drug and can be used as an alternative treatment among the public.

2. Materials and Methods

This research was carried out from December 2020 to April 2021 at the Pharmacology Laboratory, Quantitative Chemistry Laboratory, Pharmacognosy Laboratory and Central Laboratory of the Faculty of Pharmacy, Andalas University, Padang. The samples used were *V. amygdalina* leaves taken in Kasik Lolo Gunung Sarik, Gunung Sarik Village, Kuranji District, Padang City, West Sumatra. The stages of making simplicia *V. amygdalina* leaf are plant collection, wet sorting, washing, drying, dry sorting, and storage.

2.1. Tools and Materials

2.1.1. Tools

The tools used in this research include an animal scale (Triple Beam Balance, Ohause®), an analytical balance (Denver® Instrument), animal cages, food and water containers for mice, glassware (measuring cylinders (Pyrex®), dropper pipettes, vials, funnels, beakers (Pyrex®), 500 ml infusion bottles), mortar, pestle, scissors, grinder, filter paper, cotton, gavage needle, syringe, centrifuge (Hettich EBA 20), micropipette (Eppendorf®), rotary evaporator (Buchi®), photometer 5010 V5+ (Riele®), gloves, lab coat, mask, stationery, and calculator.

2.1.2. Materials

Vernonia amygdalina Del. leaves, 70% ethanol, distilled water, n-hexane, ethyl acetate, 0.5% Na-CMC, Allopurinol (Promedrahardjo Pharmaceutical Industry), potassium oxonate (Medika Laborta), uric acid analysis reagent (ReiGed®), chicken liver juice, and standard feed for mice.

2.2. Sampling

The samples used were *V. amygdalina* leaves taken at Kasik Lolo Gunung Sarik, Gunung Sarik Village, Kuranji District, Padang City, West Sumatra. The part taken as a sample was 7 kg of *V. amygdalina* taken on the morning of November 29, 2020.

2.3. Preparation of *V. amygdalina* Leaf Extract

Extracts from dry powdered simplisia were made by maceration using 70% ethanol. The simplisia powder was put into a macerator, one part of the simplisia plus 10 parts of solvent, then soaked while occasionally stirring for the first 6 hours. Then it was let stand for approximately 18 hours. Then the extract was filtered to get the macerate. The process was repeated at least twice with the same type and amount of solvent. All the macerates were collected, then evaporated with a rotary evaporator to obtain a thick extract. After that, the calculation of the yield obtained is the percentage of weight (b / b) between the yield and the weight of the simplisia powder by weighing [19].

2.4. Fractionation of *V. amygdalina* Leaf Extract

The thick ethanol extract of *V. amygdalina* leaves was fractionated successively using the solvents n-hexane, ethyl acetate and distilled water. The thick ethanol extract of *V. amygdalina* leaves was first diluted using 300 mL of distilled water, then put into a separating funnel, then 600 mL of n-hexane solvent was added, shaken until homogeneous and let stand until a separation boundary was visible between the two solvents: the bottom layer (water fraction) and the top layer (n-hexane fraction). After separation, the n-hexane fraction was removed from the separating funnel. The 600 mL of ethyl acetate solution was added to the remaining fraction of distilled water, then shaken until homogeneous and let stand until a separation boundary was visible between the two solvents, then the ethyl acetate fraction was removed from the separating funnel.

Extraction of each fraction was carried out three times using 600 mL of solvent for each extraction. The first, second and third juices are collected. The fractionated extract was then concentrated using a rotary evaporator and the percent yield was calculated.

2.4.1. Fractions Characterization

2.4.1.1. Phytochemical Test of Ethyl Acetate Fraction of *V. amygdalina* Leaves

Phytochemical testing aims to determine the class of compounds contained in the ethyl acetate fraction of *V. amygdalina* [20]. This phytochemical test is a simple, fast, and selective method that can be used to identify groups of compounds and active compounds contained in plant tissues [21]. Secondary metabolite testing is an examination of the presence of alkaloid, saponin,

phenolic, and flavonoid content in the ethyl acetate fraction [22,23].

2.4.1.2. Determination of Chromatogram Pattern of Fraction *V. amygdalina* Leaves

Chromatogram pattern testing with Thin Layer Chromatography (TLC) analysis using several eluents with different levels of polarity to produce solvents that are able to provide good separation and good stains. Stains on the plate will be observed under UV lamp 254 nm [24].

2.5. Experimental Animals

The animals used were 54 2-3 month old male white mice with a body weight of 20-30 grams. Before the research was carried out, the mice were left for 7 days to adapt to their environment. Mice were also given enough food and drink while in the laboratory environment. The inclusion criteria for mice were healthy, not hyperuricemic, not showing significant changes in body weight (maximum deviation of 10%) and showing normal behavior visually.

2.5.1. Treatment Procedurs for Test Animals

Experimental animals were given hyperuricemia induction, namely chicken liver juice orally and potassium oxonate intraperitoneally. Chicken liver juice was given on days 1 to 14, while potassium oxonate was given on days 5, 10, and 15. On days 5 to 15, treatment groups IV, V, VI were given suspension with results of fractionation of *V. amygdalina* leaf extract with doses of 12.5 mg/kgbb, 25 mg/kgbb and 50 mg/kgbb, the positive control group was given 0.5% Na-CMC suspension, and the comparison group was given allopurinol suspension, while the negative control group was not given any treatment, it was given only standard food and drink [25].

On days 5, 10, and 15, the fractionated suspension was given 1 hour after administration of potassium oxonate. Then, after 1 hour of administration of the fractionated suspension, blood was drawn to measure uric acid levels in experimental animals [16].

2.6. Measurement of Uric Acid Levels

Determination of uric acid levels in male white mice was done on days 5, 10, and 15 after administering the suspension resulting from *V. amygdalina* leaf fractionation. The examination was carried out using a 5010 V5+ photometer. The samples used were blood taken from the neck veins of male white mice. The blood was collected in a gel activator tube, left to stand for 15 minutes, then centrifuged at 4000 rpm for 20 minutes to obtain serum [26]. Serum was separated with a micropipette and transferred into a microtube to determine uric acid levels in experimental animals.

Determination of uric acid levels in male white mice was carried out by pipetting 25 µL of serum with a micropipette into a test tube, then adding 1000 µL of uric acid kit reagent and vortexing, then incubating for 10 minutes at 37°C. Next, levels will be read at a wavelength of 546 nm [27].

2.7. Data Analysis

The research data were analyzed statistically using the two-way Analysis of Variance (ANOVA) statistical method to see the differences in the average uric acid levels of the treatment groups and the length of observation in experimental animals, then continued with the Duncan test to determine the effect of giving African leaf fractions on days 5, 10, and 15. The confidence level is 95% and the results are said to be significant if $p<0.05$. Duncan's further test is conducted if the ANOVA test results show positive results (there is a significant difference between the variables used). Duncan's further test is conducted to find out in more detail what treatments show different results.

3. Results

This study is different from previous studies because in this study the fractionation results, namely the ethyl acetate fraction of *V. amygdalina* leaves, were used with hyperuricemia inductors using chicken liver juice and potassium oxonate, while the similarity of previous studies is a decrease in uric acid levels in hyperuricemic male white mice.

3.1. *V. amygdalina* Leaf Extract Yield

The results of the maceration were collected after repeating three times using ethanol solvent. The macerate is then evaporated with rotary evaporator with the aim of reducing air pressure on the surface so as to reduce the vapor pressure of the solvent and further reduce the boiling point of the solvent, then the macerate will concentrate and a thick extract is obtained [28]. The thick extract of *V. amygdalina* leaves was calculated as the % yield of the extract, then the obtained yield of *V. amygdalina* leaf extract was as much as 20.55%.

The calculation of extract yield aims to determine the percentage of extract produced from 2 grams of *V. amygdalina* leaf simplisia powder [29].

3.2. Fraction of *V. amygdalina* Leaf Extract

The extract that has been obtained was then continued with the fractionation process. The viscous extract was fractionated to obtain polar, semipolar, and nonpolar active compounds carried out with three kinds of solvents with different polarity properties, namelywater, ethyl acetate and n-hexane [28].

From 150 grams of *V. amygdalina* leaf extract used, the yield of water fraction, ethyl acetate fraction and n-hexane fraction were 41.5377 g (27.69%); 8.0043 (5.33%); and 0.2125 g (0.14%), respectively. Organoleptically, the results of the *V. amygdalina* leaf fraction are in the form of a thick fraction resembling caramel, smelling distinctive, and blackish brown in color.

3.2.1. Phytochemical Testing of Ethyl Acetate Fraction

Secondary metabolite testing is an examination of the presence of alkaloid, saponin, phenolic, and flavonoid content in the ethyl acetate fraction. Based on tests that have been carried out using several specific reagents, the ethyl acetate fraction of *V. amygdalina* leaves contains saponins, phenolics, and flavonoids. Alkaloid examination in the ethyl acetate fraction obtained negative results as evidenced by the formation of no white precipitate for Mayer reagent, and no brown precipitate for Dragendroft and Wagner reagents.

Table 1. Secondary metabolite testing results of ethyl acetate fraction of *V. amygdalina* leaf

	Reagents	Observations	Results
Alkaloid	Mayer	No white precipitate formed	-
	Dragendroft	No brown precipitate formed	-
	Wagner	No brown precipitate formed	-
Saponin	Hot water and HCl 2N	Froth formed	+
Phenolic	$FeCl_3$ 5%	Blue green solution	+
Flavonoid	Metal Mg, concentrated HCl	Orange solution	+

Description:
(+): Contains secondary metabolite compounds
(-): Does not contain secondary metabolite compounds

3.2.2. Determination of Chromatogram Pattern of Ethyl Acetate Fraction

Thin Layer Chromatography (TLC) is a separation method that relies on absorption, partition, or a combination of both. A thin layer of absorbent or supporting granules is applied to a glass plate, metal, or other material. To achieve saturation conditions in the chromatography vessel, a sheet of filter paper is placed along the vessel wall. The mobile phase used in this study was n-hexane : ethyl acetate with a ratio of 2 : 3 [30].

The mobile phase was poured into the vessel, making sure the filter paper was completely wet. The vessel was filled with the mobile phase, reaching a height of about 5-10 mm. The vessel was sealed for one hour and a temperature range of 20-25°Celcius was maintained [30]. After that, it was seen under UV light with a wavelength of 254 nm and then the Rf of the ethyl acetate fraction of *V. amygdalina* leaves of 0.63 was obtained.

Fig 1. Thin Layer Chromatography (TLC) analysis results of ethyl acetate fraction
Description:
K : Quercetin
Et: Ethyl acetate fraction of *V. amygdalina* leaf

The results of Thin Layer Chromatography (TLC) of the ethyl acetate fraction of *V. amygdalina* leaves were a stain that was almost parallel to the quercetin stain, namely with the Rf of the ethyl acetate fraction 0.63 while the Rf of quercetin used as a comparison was 0.61. These results indicate that in the ethyl acetate fraction of *V. amygdalina* leaves there are flavonoid group compounds that have activity as xanthine oxidase inhibitors so that they can cause the effect of reducing uric acid levels in the blood [28].

3.3. Uric Acid Levels Testing

Measurement of uric acid levels was carried out by the enzymatic method. In this method uric acid is converted by uricase into allantoin and hydrogen peroxide. Hydrogen peroxide will bind to 4-aminoantipyrine to 3,5 dichloro-2-hydroxybenzene sulfonic acid (DHBS) to form a chromogen that will be measured on a 5010 v5+ photometer with a wavelength of 546 nm. The selection of this method is based on its simple workmanship, which can directly read serum uric acid levels through the measured absorbance, is sensitive, and is a method commonly used in clinical laboratories [28].

From the results of measuring uric acid levels, it can be seen that the lowest average decrease in uric acid levels was in the 50 mg/kgbb dose group, followed by a dose of 25 mg/kgbb and then the third with a dose of 12.5 mg/kgbb. These results show that there was a decrease in uric acid levels in mice after administration of the ethyl acetate fraction of *V. amygdalina* leaves. This decrease shows that the greater the dose of *V. amygdalina* leaf fraction given, the better the reduction in the average uric acid levels.

Table 2. Results of measuring uric acid levels in male white mice that had been induced by potassium oxonate and chicken liver juice when given *V. amygdalina* leaf ethyl acetate fraction for 15 days

Doses (mg/kgbw)	Average Uric Acid Levels (mg/dL) ± SE			Average (mg/dL)
	5 days	10 days	15 days	
Negative control	1.33 ± 0.594	1.33 ± 0.594	1.33 ± 0.594	1.33 ± 0.343^{a,b}
Positive control Na CMC 0.5%	5.36 ± 0.594	4.06 ± 0.594	0.87 ± 0.594	3.43 ± 0.343^{d}
Allopurinol 13	0.73 ± 0.594	0.70 ± 0.594	0.57 ± 0.594	0.67 ± 0.343^{a}
Ethyl acetate fraction 12.5	5.03 ± 0.594	2.86 ± 0.594	0.73 ± 0.594	2.87 ± 0.343^{c,d}
Ethyl acetate fraction 25	3.30 ± 0.594	2.80 ± 0.594	0.63 ± 0.594	2.24 ± 0.343^{b,c}
Ethyl acetate fraction 50	2.00 ± 0.594	1.87 ± 0.594	0.80 ± 0.594	1.55 ± 0.343^{a,b}
Average ± SE	2.96 ± 0.243^{p}	2.27 ± 0.243^{q}	0.82 ± 0.243^{q}	

Note:
a, b, c, d are different superscripts in the same column
p,q are different superscripts on the same line

The decrease in uric acid levels shows that there is a pharmacological effect of the ethyl acetate fraction in reducing uric acid levels. In Jumain's (2018) research, it was also stated that the ability of *V. amygdalina* leaf extract to reduce uric acid levels is thought to have a similar action to the uricostatic antihyperuricemia drug, namely allopurinol, which works by inhibiting the xanthine oxidase enzyme, thereby influencing the conversion of

hypoxanthine and xanthine into uric acid, because of the presence of flavonoid compounds in the *V. amygdalina* leaf extract with a mechanism that inhibits xanthine oxidase activity on purine bases [16].

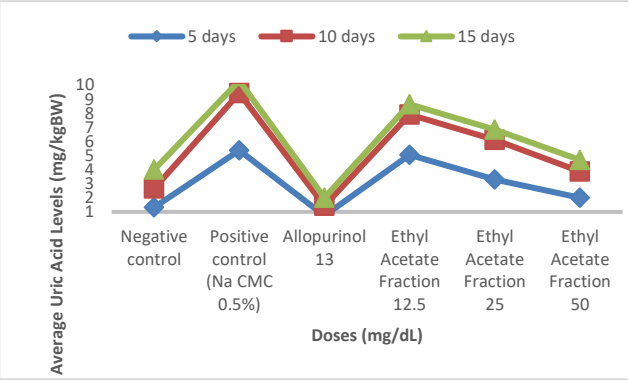

Fig 2. Line diagram of the results of measuring uric acid levels in male white mice that had been induced by potassium oxonate and chicken liver juice when given *V. amygdalina* leaf ethyl acetate fraction for 15 days.

Based on data processing using two-way Analysis of Variance (ANOVA) on the research results, it was found that the ethyl acetate fraction of *V. amygdalina* leaves showed a real influence on dose variations with a significance value of 0.000 ($p<0.05$). Meanwhile, the duration of administration also showed a significant effect with a significance value of 0.000 ($p<0.05$) on reducing uric acid levels in hyperuricemic male white mice. From these results, testing can be continued with the Duncan test on the variables of dose variation and duration of administration to determine the influence of each of these factors.

The results obtained from the three variations in doses of the ethyl acetate fraction of *V. amygdalina* leaves are that the dose of 50 mg/kgbb had the lowest average uric acid levels compared to the doses of 12.5 mg/kgbb and 25 mg/kgbb and the group The 15 day treatment resulted in the lowest uric acid levels compared to the 10 and 5 day treatment groups.

The ethyl acetate fraction of *V. amygdalina* leaves can reduce uric acid levels because the fraction contains flavonoid compounds which can inhibit the activity of the xanthine oxidase enzyme, thereby influencing the change of hypoxanthine to xanthine and xanthine to uric acid. Inhibition of xanthine oxidase can reduce uric acid production in the blood. Kharimah's research (2016) states that *V. amygdalina* leaves contain many chemical compounds, including saponins, coumarins, flavonoids, phenolic acids, lignans, terpenes and luteolin. The most important uses are for the treatment of gout, diabetes, hypertension, and cancer [31].

Based on these results, it can be concluded that the ethyl acetate fraction of *V. amygdalina* leaves can reduce uric acid levels in hyperuricemic male white mice. This is in accordance with previous research, which concluded that *V. amygdalina* leaves contain flavonoid chemical compounds which have the ability to inhibit the action of xanthine oxidase so that they can inhibit the formation of uric acid in the body [16].

4. Conclusion

Based on the tests that have been carried out and data analysis using ANOVA on the administration of varying doses and duration of administration of varying doses of the ethyl acetate fraction of *V. amygdalina* leaves, it was stated that there was a significant effect on reducing uric acid levels in hyperuricemic male white rats. This is because the ethyl acetate fraction of *V. amygdalina* leaves contains flavonoid chemical compounds that have the ability to inhibit the work of xanthine oxidase so that it can inhibit the formation of uric acid in the body.

From the results of the ANOVA test, the test can be continued with the Duncan test on the dose variation variables and duration of administration to determine the effect of each of these factors. The results of the Duncan test showed that the dose that had the best effect of reducing uric acid levels to normal was a dose of 50 mg/kgbb.

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