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Research Article

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The Effectiveness of Indonesian Tradional Therapy from Blimbing Wuluh Leaf Extract in Reducing Uric Acid in Mus Musculus

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ABSTRACT

Uric acid includes degenerative diseases caused by the oxidation of purine compounds by xanthin oxidase to uric acid. In traditional medicine in Indonesia, in general a stew made of the existing plant of Blimbing Wuluh Leaf (Averrhoa bilimbi L) is drunk. The content of Blimbing Wuluh Leaf is flavonoids, where most of the flavonoids are quercetin. Quercetin including antioxidant compounds, which can inhibit xanthin oxidase, does not produce uric acid. Each test group consisted of seven white male mice (Mus musculus) aged 2-3 months. To make high mice uric acid, potassium oxonate with a dose of 14mg/20g body weight was used. Negative control (CMC-Na), Positive control (allopurinol, and three doses of Blimbing Wuluh Leaf extract based on quercetin content (0.13, 0.39 and 0.65 mg/20 g body weight) were included in this study. The treatment was done for 21 days, and the determination of uric acid level was done every three days. For data analysis, ANOVA software (one way) was used with a confidence degree of 5%. Toxicity test of Blimbing Wuluh Leaf extract was performed for 24 hours using calculated doses based on quercetin content (200 ; 600 ; 800 and 5400 mg/kg body weight). The effectiveness of Blimbing Wuluh Leaf extract in decreasing uric acid was obtained at the dose of 0.65 mg/20 g weight body of white mice after 13 days of administration ($p<0.05$), and it was proved to be not toxic because no dead mice were seen.

Keywords: *Blimbing Wuluh Leaf Extract, Effectiveness and Toxicity, Uric Acid, Quercetin, White Mice (Mus Musculus), UV Spectrophotometry Method*

INTRODUCTION

Hyperuricemia is a degenerative disease characterized by increased uric acid levels above the normal range of the body, resulting from uric acid crystal deposition in bone and kidney with uric acid incidence occurring 21.2% in men and 21.6% in women in the USA, and 42.1% in men and 27.4% in women in Taiwan [1, 2]. Uric acid is a soluble compound and a major end product of purine metabolism. Xanthine oxidase enzyme will catalyze hypoxanthin to xanthin. The formed xanthin will be re-catalyzed by the xanthine oxidase to uric acid [3-5].

The synthetic drug commonly used to inhibit the enzyme xanthine oxidase (XO) is Allopurinol with a dose of 0.52 mg/20 g BW for mice. Allopurinol works by inhibiting xanthine oxidase, but the drug provides side effects such as liver inflammation and allergies [6]. Therefore, it is a necessary alternative medicine that has better treatment activity and lower side effects. The use of herbs and natural ingredients is now a major alternative in lowering uric acid levels.

Belimbing wuluh (Averrhoa bilimbi L) which is the name of a traditional plant in Indonesia, included in the family of Oxadilaceae, is one of medicinal plants that were potentially exploited for gout disease [6, 7]. Almost all parts of the belimbing wuluh plants can be utilized, one of which is the leaf. Starfruit leaves contain flavonoids, saponins, triterpenoids, amino acids, citric acid, phenolic, and sugar [8, 9].

Flavonoids are the largest polyphenol compounds found in nature. One example of the flavonoid group is quercetin which has been reported to have antioxidant activity by inhibiting xanthine oxidase which is an enzyme that can

convert purine into uric acid, and therefore decreasing uric acid levels in the body [9, 10]. The level of quercetin in belimbing wuluh leaf extract which has been done by previous researchers was reported to be 1.67 mg/gram and 341.2 µg/g dry weight [11, 12]. Preliminary study with a dose of 150 mg of quercetin in humans, and 50-100 mg/kg in rats can reduce uric acid levels [13, 14].

The effectiveness and toxicity tests as the development of traditional medicine were performed because they were needed for herbal medicine requirements in addition to providing security guarantees for consumers.

MATERIALS AND METHODS

Instruments : Single-beam UV spectrophotometer (Hewllet Pakard), analytical balance (O-Haus Adventurer), commonly used glass tools for chemical analysis, Easy Touch Blood Uric Acid Meter, Easy Touch gout strip, weight scales, sounde spuite injection, and cage mice.

Chemicals : Leaves of belimbing wuluh (Averrhoa bilimbi L) were collected from Purwodadi Botanical Garden, and determined by Biology Laboratory of Airlangga University's Science and Technology Faculty, quersetin (Pharmaceutical Grade ; Sigma Aldrich), acetone p.a. (Merck), ethanol p.a. 96% (Merck), aquadest, potassium oxonate p.a. (Sigma Aldrich), 0.9% NaCl, CMC Na, allopurinol tablets.

Animal Ethics : As the experimental animals used as research subjects, healthy male mice weighing 20-30 g, aged three to four months, were used. Each group consisited of 7 mice, which were adapted for a week before treatment. For using the experimental animals, the Certificate of Veterinary Ethics from the Research Ethics Commission, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia with Number : 671-KE, was obtained.

Dosage : In the activity test, the mice were induced with potassium oxonate with a dose of 7 mg/20 g BW of mice intraperitonial [15, 16]. The treatment was administered for 21 days on an oral basis with daily body weight measurements, and the uric acid levels were measured every three days using the Uric Acid Meter (Easy Touch). In a preliminary study that was standard, quercetin with a dose of 150 mg in humans can reduce uric acid levels [13, 14]. The dose was converted to the dose of mice, and 0.39 mg/20 g BW mice was obtained. Therefore, the activity test was conducted on 3 different doses of blimbing wuluh extract of 0.13 mg/20 g BW mice, 0.39 mg/20 g BW mice, and 0.65 mg/20 g BW mice, along with negative control CMC-Na 0.3%, and positive control I (allopurinol dose 0.52 mg/20 g BB mice), and positive control II (quercetin 0.39 mg/20 g BW mice). Four levels of quercetin dose of 200 mg/kg BW, 600 mg/kg BW, 1800 mg/kg BW, 5400 mg/kg BW of blimbing wuluh leaf extract standardized with quercetin, and negative control including CMC-Na 0, 3%, were used in the toxicity test, and the observation was done in 24 hours.

Water Content : The moisture content of simplicia and extracts was determined by gravimetric method [17].

Making Extract : Leaf belimbing wuluh extraction was done by a maceration method on acetone solvent pa (1 : 6) for 24 hours.

Standardization : Standardization of leaf belimbing wuluh leaf extract was based on quercetin content in samples performed by validated UV-Spectrophotometric method (selectivity, linierity, precision and accuracy) [18, 19].

RESULTS AND DISCUSSION

Belimbing wuluh leaf extraction was done by a maceration method on acetone solvent pa (1 : 6) for 24 hours, and evaporated with rotary vapor. The extract was dried by oven at 400^0 C. The standardization of belimbing wuluh leaf extract was based on quercetin content in samples performed by validated UV-Spectrophotometric method. Previously, the moisture content of simplicia and extracts was obtained to be12.16±0.51% (w/w) and 5.91±9.22% (w/w), respectively by gravimetric method. Selectivity test with standard quercetin solution indicated 372 nm to be the maximum wavelength. The maximum wavelength for quercetin was 258 nm and 375 nm ; 257 and 372 nm and 380 nm [20-23]. Linearity test with a standard solution of quercetin concentration of 4-12.8 ppm obtained regression equation of y = 0.0658x-0.0084 with a coefficient correlation of (r) = 0.9995 and the coefficient value of function variation (Vxo) = 4.29%. Precision test with standard solution of 6.4 ppm of quercetin (C.V.) resulted in 0.85%. The obtained accuracy of the percent of recovery was 99.78 ± 1.22% (w/w) which met the percent requirement of recovery for biological sample (bioassay) which is 80-120% (C.V.) ≤ 15% [24, 25]. The quercetin content in the extract of blimbing wuluh leaf was determined to be 5.42 ± 1.91% (w/w). While the results of research indicated 1.67 mg/g [11, 12]. The differences of these levels can be caused due to the differences in environmental conditions of leaf belimbing wuluh leaf such as climate and soil structure.

Researches on the reported decrease in uric acid have included a mixture of blimbing wuluh leaves' extract with bay leaves or other plants, and they were not even standardized to the active ingredient compounds (quercetin). So, the resulted effect was the effect of synergism, which can hardly be compared with this study examining a single extract of blimbing wuluh leaf. Data on uric acid level and body weight of mice were obtained (Table 1 and 2), and the normality was tested by using Kolmogorov Smirnov test, and it was found that each data of uric acid level and body weight were normally distributed with a significance value greater than 0.05; p>0.05). Then, a homogeneity test was conducted on each treatment group using Levene Test test. From the analysis, it was found that each treatment group on uric acid data and body weight was homogeneous with p>0.05.

Table 1. Uric Acid Assay

Group	Number mice	Uric acid assay, day to- (mg/dl)								
		0	1	4	7	10	13	16	19	21
Negative control	7	3.5	7.4	7.8	8.0	7.7	7.4	7.0	6.7	6.6
Allopurinol	6	3.5	7.4	6.2	5.3	4.6	3.9	3.3	3.2	3.2
Quersetin	7	3.6	7.5	6.7	5.5	4.9	4.6	4.1	3.9	3.6
Extract dose 1	7	3.7	7.4	7.0	6.3	5.7	5.0	4.4	3.8	3.9
Extract dose 2	7	3.5	7.5	6.7	6.0	5.3	4.7	4.2	3.7	3.5
Extract dose 3	7	3.7	7.5	6.4	5.4	4.5	3.9	3.5	3.4	3.2

To determine the different levels of uric acid level among treatment groups, One Way Anova analysis was used, p value = 0.000 (p <0.01) meant that the treatment group had a significant difference (Table 2).

Table 2. Statistical Analysis of One Way Anova Among Uric Acid Groups

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	62.327	5	12.465	15.960	0.000*
Within Groups	27.337	35	0.761		
Total	89.664	40			

*) significant if p< 0.01

Then the LSD analysis was proceeded to find which pair of groups had significant differences. From table 3, it was found that the positive controls compared with the negative control groups had p = 0.000 (p <0.01), so it can be said that there was a significant difference between negative and positive controls. All treatments with negative control had p = 0.000 (p <0.01), meaning that there was a significant difference in all treatments with negative controls. In the positive control group (allopurinol) and the Blimbing wuluh leaf extract group of dose 1, p = 0.027 (p <0.05) meaning that there was a significant difference between allopurinol treatment and the Blimbing wuluh leaf extract group of dose 1. The treatment of dose 1-3 of Blimbing wuluh leaf extract with the negative control resulted in p = 0.000 (p<0.01), which can be induced that all the dose treatment of the Blimbing wuluh leaf extract can decrease uric acid.

Table 3. Statistical Analysis of LSD Test

Group	Significantly					
	Negative control	Allopurinol	Quercetin	Extract Dose 1	Extract Dose 2	Extract Dose 3
Negative control		0.000*	0.000*	0.000*	0.000*	0.000*
Allopurinol	0.000*		0.197	0.027**	0.124	0.939
Quercetin	0.000*	0.197		0.312	0.787	0.156
Extract dose 1	0.000*	0.027**	0.312		0.456	0.018**
Extract dose 2	0.000*	0.124	0.787	0.456		0.94
Extract dose 3	0.000*	0.939	0.156	0.018**	0.094	

*) significant if p<0.001; **) significant if p<0.005

For statistical analysis, the paired t test was used to find the differences between groups in lowering uric acid levels. Positive control 1 (Allopurinol) and positive control 2 (quercetin) showed decreased uric acid levels from day one to day 16. In the extract group 1, the decrease was significant on the first day until the 19th day, in the extract group 2 it was significant on the first day until the 16th day, and in the extract group 3, it was significant from the first day until the 13th day. The differences between the results could be due to the differences of doses among the groups. The lowered levels of uric acid were also different (Table 4 & 5, Fig 1).

Table 4. Statistical Analysis Paired t Test

Day to-	difference significantly (p)					
	Negative control	Allopu-rinol	Quercetin	Extract Dose 1	Extract Dose 2	Extract Dose 3
1 - 4	0.670	0.000*	0.003*	0.008*	0.000*	0.000*
4 - 7	0.504	0.000*	0.000*	0.000*	0.025**	0.000*
7 - 10	0.108	0.000*	0.001*	0.000*	0.002*	0.000*
10 - 13	0.025**	0.000*	0.005*	0.000*	0.000*	0.006*
13 - 16	0.046**	0.000*	0.001*	0.000*	0.015**	0.081
16 - 19	0.076	0.411	0.128	0.001*	0.418	0.322
19 - 21	0.272	0.793	0.104	0.289	0.339	0.407

*) significant if p<0.01; **) significant if p< 0.05

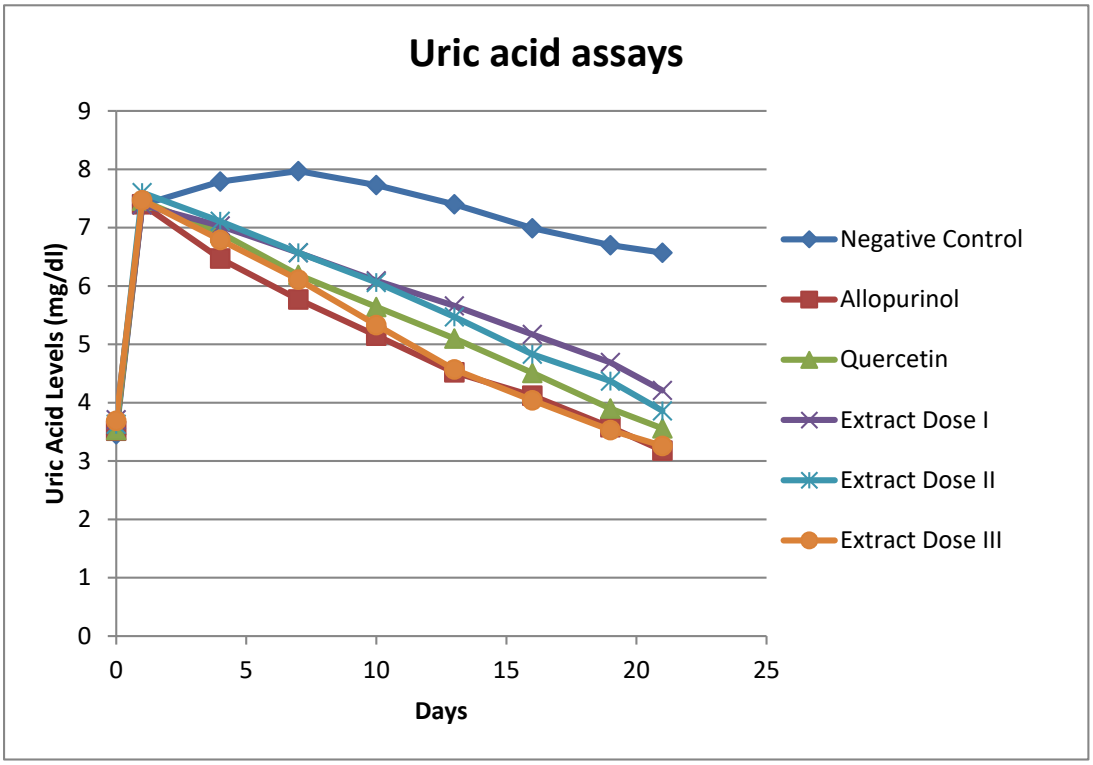

Figure 1. Uric Acid Assays

The sensitivity of decreased uric acid levels in each group can be observed by looking at the value of b or tangents α. The results indicated that 0.65 mg dose of Blimbing wuluh leaf extract was the most effective dosage, because it had the greatest b value or the sharpest decrease in uric acid compared with other treatment groups. In a study, blimbing wuluh leaf extract gave the largest activity at doses of 100 and 200 mg/kgBW, with the induction of potassium oxonate with a dose of 5 mg/20 gBW mice by the treatment of one hour after induction, and the measurement of uric acid levels performed one hour after the treatment, and allopurinol dose of 10 mg/kgBW as a positive control [8, 9].

Table 5. Values of Tangent α (b)

Group	Regression Equation	Tangent α (b)
Positive control 1	y = -0.2178x + 7.4230; R^2 = 0.9857	0.2178
Positive control 2	y = -0.1974x + 7.6465; R^2 = 0.9983	0.1974
Extract dose 1	y = -0.1515x + 7.6012; R^2 = 0.9987	0.1515
Extract dose 2	y = -0.1836x + 7.8341; R^2 = 0.9978	0.1836
Extract dose 3	y = -0.2420x + 7.7480; R^2 = 0.9989	0.2420

Based on the research, belimbing wuluh leaf extract (Averrhoa bilimbi L) with standardized quantity of doses of 0.13 mg ; 0.39 mg ; and 0.65 mg/20 gBW mice can lower uric acid levels in mice. Whereas, for human use, they are needed to be converted by correction factor of 387.9 to obtain doses (50.43-252.14) mg/70 kgBW human [23].

In the toxicity test of belimbing wuluh leaf extract for 24 and 48 hours, no mice died [7, 26]. The data obtained were analyzed using three methods namely Weil, Probit and line equation method. Observations for 24 and 48 hours found no dead mice (Table 6).

Table 6. Toxicity of Blimbing Wuluh Leaf Extract

Group	Quercetin dose (mg/kg BW)	Log Dose	Number Mice	Mortality	
				24 Hour Observation	48 Hour Observation
Negative control	-	-	7	0	0
Extract dose 1	200	2.301	7	0	0
Extract dose 2	600	2.778	7	0	0
Extract dose 3	1800	3.255	7	0	0
Extract dose 4	5400	3.732	7	0	0

The results were obtained by the three methods of analysis. The LD50 value could not be determined because no deaths found in experimental animals. So, it can be concluded that leaf belimbing wuluh leaf extract of quercetin is not toxic and has LD50 more than 5400 mg/kgBW. It meets the requirements of the dosage test for more than 2000mg/kgBW there is no animal mortality then the test preparation can be declared to be nontoxic [7, 26]. Based on the previous studies, large doses of quercetin in a given period do not cause carcinogenicity in rodents [26, 27]. Epidemiologically, it has been indicated that high quercetin administration will correlate with the given benefit. According to The International Agency for Research on Cancer (IARC, 1999) in states, the quercetin has been classified as a non-toxic compound to humans. Research on humans showed that quercetin was tolerable at a dose of 1000 mg/day for several months of use, and had no side effects on liver parameters, renal function, hematology and electrolyte [28]. This study was consistent with [28], in which quercetin in leaf starch extract was non-toxic.

CONCLUSION

Blimbing wuluh leaf extract of standardized quercetin can be a traditional therapy to decrease uric acid levels significantly in male mice with dose of 0,65 mg/20 g BW mice within 13 days with an interval of giving extract every day, and it was also proved to be not toxic.

Suggestion

Blimbing wuluh leaves can be used as a safe traditional therapy to decrease uric acid levels.

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