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Original Article

TOXICITY TEST FROM GLORIOSA SUPERBA L LEAVES EXTRACT IN RATS (RATTUS NOVEGICUS)

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ABSTRACT

Objective: Plants *Gloriosa Superba L* family of *Liliaceae*, containing one type of alkaloid that is colchicines. Colchicine is used in the treatment of gout and rheumatism. The aims of this study were to make standardized herbal preparations by determining the effects of decreasing of uric acid level and conducting toxicity test.

Methods: The determination of the effect of *Gloriosa Superba L*. leaf extracts, toward decrease uric acid level using a white male Wistar rats were induced by potassium oxonic dose of 250 mg/kg BW, and acute toxicity testing was conducted using a chemical substance once or multiple times within 24 hour period. Toxicity tests are designed to determine the LD_{50} of the drug. And acute toxicity test has been carried out in the colchicine of standardized *Gloriosa Superba L* with a dose of 121.24 mg/kg, 363.73 mg/kg, 1091.196 mg/kg and 3273.6 mg/kg, and pure colchicine at a dose of 0.045 mg/kg, 0.45 mg/kg, 4.5 mg/kg, 45 mg/kg and 450 mg/kg using rats. Toxicity test used rats as experimental objects.

Results: Data were analyzed with SPSS version 20, the real difference between treatments was tested with Two Way ANOVA and gave a significant difference (significant) between the treatment of hyperuricemia with the negative control group, positive control, and the test preparation group with a significant price of each group was 0.000 ($\alpha < 0.05$). The results showed that the extract of *Gloriosa Superba L* decreased uric acid level in the blood of male Wistar strain rats which were induced with potassium oxonic.

Conclusion: Results LD_{50} for *Gloriosa Superba* L leaf extract falls into the category of practical non-toxic (>1500 mg/kg BW). Obtained LD_{50} of colchicine was 7.92 mg/kg.

Keywords: Uric acid, *Gloriosa Superba* L leaf, Hyperuricemia, LD_{50}.

INTRODUCTION

Gloriosa Superba L (*Liliceae*) has been also known as Malabar glory lily or kembang telang (Java, Indonesia), it is called as "Mauve beauty", "Purple prince", "Modest", "Orange gem", "Salman Glow", and "Orange Glow" [1], is a semi-woody herbaceous branched climber reaching approximately 5 meters height, with brilliant wavy edged yellow and red flowers [2]. Some researchers suggest that *Gloriosa Superba* L can be used as a commercial source of colchicine [3,4]. It has been reported that *colchicum* of 0.62% of colchicine and 0.39% of *colchicoside.* Whereas *Gloriosa Superba* L contains 0.9% of colchicines *colchicine* and 0.82% of colchicoside [5]. *Gloriosa Superba* L is an imperative medicinal plant where all part are used in the medicine and contains two important alkaloid, *colchicine* and *colchicoside* [6]. Which are used in the treatment of gout and rheumatism [7,8].

Colchicine is a chemical substance that has been used as a remedy against gout, a disease caused by deposits of uric acid in the joints [9]. Colchicine is widely used in the treatment of acute gouty arthritis, joint pain and swelling do to uric acid. It also helps to prevent gout and gout attacks. Moreover colchicine relieves pain and reduces inflammation of gout arthritis [10]. Anti-inflammatory activity of colchicine is specific for gout. If relieves pain in acute gout within 12 hours [11]. Hyperuricemia is a situation where there is an increase of uric acid levels above normal in the blood [12]. This resulted in the deposition of sodium urate crystals in the tissues, especially the kidneys and joints. Hyperuricemia does not always lead to gout, but gout is always preceded by hyperuricemia [11]. Gout is a metabolic disorder of uric acid and purine, a type of inflammatory arthritis caused by the deposition of monosodium uric crystals in synovial fluid and other tissues, and is often associated with hyperuricemia [13,14,15]. Toxic dose of colchicine in humans approximately 10 mg and it could be fatal if consumed more than 40 mg within 3 days [16]. Plants *Gloriosa Superba* L effectively overcome gout rheumatic diseases, by lowering the levels of uric acid in the blood. The use of this plant is limited to empirical experience, mean while scientific data that ensures the efficacy and safety is still very limited.

Toxicity test is required to ensure the safety in the use of *Gloriosa Superba* L as standardized herbal preparations. Acute toxicity test was designed to determine the lethal dose of a substance (LD_{50}). LD_{50} is defined as a single dose of the drug substance that is statistically expected to kill 50 experimental animals [17].

MATERIALS AND METHODS

Samples: Gloriosa Superba L leaf, potassium oxonic p.a (Sigma-Aldrich Chemical Company), colchicine p.a (Xi'an Olin Biological Technology. Co. Ltd), 0.9% saline (Widatra Bhakti), pro water injection (Otsuka), distilled water.

Instruments: glass (Pyrex), UASure uric acid meters (Apex Biotechnology Corp., Hsinchu, Taiwan), strip UAsure, 1.0 ml injection syringe (Terumo), gastric sonde, cage, gloves, where drinking water and animal feed container (Pellet, BR 594), tools, glass (Pyrex), scales test animals (Berkel, Type EH No. 106.601).

Sample collection: Samples used in this study are Gloriosa Superba L leaf which grows wild in the area of Purwodadi-Pasuruan and taken in November 2012 and December 2012. Determination of plant was performed to ensure the validity of the plant identity. This plant has been the determined in UPT Purwodadi Botanical Gardens Plant Conservation.

Preparation of Test Samples

Gloriosa Superba L leaf were collected, washed with water, drained and weighed, dried in the open air for two weeks. After obtaining dried simplicia, size reduction was done to obtain powder simplicia. From 100 gram of this powder, an extract with concentration of 10% and total volume of 2500 ml was prepared. Then the extract was freeze dried to obtain frozen extract [18].

Animals: Male Wistar rats (2 -3 months old, 200-250 g, n = 20) were maintained on a 12-h light/dark cycle. They were fed with a standard laboratory diet and allowed to cunsume food and water ad libitum for an acclimatization period of 7 days prior to the experiment. The temperature and humadity were kept at 25^0 C and 45 -65%, respectively. Animals have passed Conduct Feasibility Teston by Research Ethics Commitee of the Faculty of Veterinary Medicine, Airlangga University, 2013.

Preparation of Experimental Animals: Animals were acclimatized for 1 week before being used for experiments, placed in standard cages and feed indefinitely during the study. Then the test animals were fasted approximately 18 hours prior to the experiment, while still be allowed to consume. The animals used in this study were healthy rats with characteristics of clear red eyes, white hair, weight gain and active.

Assay of Test preparation Dose: Dose of test preparation used was 121.24 mg/ kg freeze dried extract of G*loriosa Superba* L leaf which is equivalent to colchicine dose for rats 0.23 mg/kg orally [19].

Animal model of hyperuricemia in rat: An in vivo induction of potassium oxonic to make the test animals experience the effects of hyperuricemia. A 250 mg of potassium oxonic was dissolved in 0.9% NaCl solution and intraperitonially given for each test animals 1 hour prior to oral administration of another test preparation. The blood samples were taken from each rat after two hours of drug administration [20, 21, 22]. The preparations made by dissolving potassium oxonic in saline solution and heating it until completely dissolved on a hot plate. A new potassium oxonic solution was always made before being intraperitoneally injected into rat.

Assay of colchicine dose: The dose of colchicine for rats 0.23 mg/ kg orally [19].

In vivo activity of hyperuricemia: in this experiment, tested animals were randomly divided into 4 treatment groups. Where each group consisted of 5 rats. Those were normal control group (carrier), positive control group (Colchicine 0.23 mg/kg BW rats orally), negative control or pain control group (potassium oxonic 250 mg/kg i.p BW) and the treatment group (freeze dried extract of the leaves of *Gloriosa Superba* L single dose of 121.24 mg/kg BW colchicine rats were equivalent to 0.23 mg/kg BW rats orally). Except the normal group, all experimental animals received potassium oxonic 250 mg/kg in saline intraperitoneally. Test preparation and positive controls was administered one hour after induction of hyperuricemia with potassium oxonic 250 mg/kg BW. Induction of hyperuricemia was performed for one hour [23]. Normal control, positive control, negative control and test preparation were administered once daily for three days, and blood sampling was after 2 hours of performed potassium oxonic induction. Blood sample was taken from each rat by cutting the tail tip, 1 hour after administration of the compound, at the preintervention, 0^{st}, 1^{th}, 2^{th} and 3^{th} days of the study. Determination of uric acid levels in the blood is performed by using UAsure® uric acid meter to determine uric acid levels in the blood at any given time.

Processing and Data Analysis

Obtained data were statistically analyzed using two-way ANOVA followed by the Least Significant Difference (LSD) to find out which groups showed significant differences between groups (Origin Software, IBMSPSS Amos 20).

Toxicity Study

Colchicine In *Gloriosa Superba* L Leaf extract

Animal: Male Wistar rats (2 -3 months old, 150-300 g) were maintained on a 12-h light/dark cycle. They were fed with a standard laboratory diet and allowed to consume food and water ad libitum for an acclimatization period of 7 days prior to the experiment. Tested animals were divided into 5 groups, each group consists of 4 males and 4 females. Before being treated, all rats were fasted for 18 hours (drinking is still allowed) and weighed.

Dose determination: *Gloriosa Superba* L leaf extract that has been standardized (colchicine levels in freeze drying powder *Gloriosa* ***Superba* L leaf extract** = 0.1897% (w/w)). Colchicine dose for rats was 0.23 mg/kg orally [19] and was used as the lowest given dose. In order good results we used doses in a row that would follow a geometric progression, namely [17]: $Y_N = Y_1 \times R^{N-1}$, where: Y_N = Dose to n; N = the group to...; R = geometric factor ≠ 0 or 1 multiple dose

Using a geometric factor (R) = 3, 1^{st} dose: 0.23 mg/kg, 2^{nd} dose: 0.69 mg/kg, 3^{rd} dose: 2.07 mg/kg and 4^{th} dose: 6.21 mg/kg were obtained. Each standardized extract was dissolved into distilled water and administered orally to Wistar rats. Extract was given only once in a maximum volume of 1 ml for 250 g of rat.

Pure Colchicine

Animal: Male Wistar rats (2 -3 months old, 150–300 g) were maintained on a 12-h light/dark cycle. They were fed with a standard laboratory diet and allowed to consume food and water ad libitum for an acclimatization period of 7 days prior to the experiment. Test animals were divided into 6 groups. Before being treated, all rats were fasted for 18 hours (drinking still be given) and weighed.

Dose determination: colchicine dose in human is 0.5 mg. The conversion factor from human to rat is 0.018 for 200 g rat, 70 kg human [17]. Therefor colchicines dose in rats = 0.009 or 0.045 mg/200 g BW mg/kg. This dose is defined as the lowest given dose. In order to obtained good results we used doses in a row that would follow a geometric progression, namely [17]: $Y_N = Y_1 \times R^{N-1}$, where: Y_N = Dose to n; N = the group to... ; R = geometric factor ≠ 0 or 1 multiple dose.

Using a geometric factor (R) = 10, 1^{st} dose: 0,045 mg/kg, 2^{nd} dose: 0.45 mg/kg, 3^{rd} dose: 4.5 mg/kg, 4^{th} dose: 45 mg/kg and 5^{th} dose: 450 mg/kg. Each preparation dose was dissolved into distilled water and administered orally to Wistar rats. The preparation is given only once to a maximum volume of 1 ml for 250 g of rat.

Determination of LD_{50}

The determination of LD_{50} values used multi level dose consisting of four dose variation. The extract and test preparation were orally administered in a single dose using sonde, then tested animals were observed for 30 minutes, 60 minutes and 120 minutes to find toxic symptoms appeared during observation. Observations on rats was performed at 24 hours after administration of the test solution by counting the number of dead rats from each group. Then the LD_{50} value was calculated by using the formula of Weil.

RESULTS AND DISCUSSION

Colchicine levels used in this study was based on the standardization of colchicine carried out by other researchers [24] using validated. TLC-Densitometry method standardization, and chloroform-diethylamine (9:1) as mobile phase observe on was performed under wavelength of 355 nm. Limit of detection 0.0019 µg, limit of quantitation 0.0063 µg, percent of recovery obtained by standard addition 84.0 ± 2.5% (w/w) and obtained colchicine level 0.1987 ± 0.0107% (b/b). Before the experiment began, uric acid levels of tested animals were measured early (day 0). Uric acid levels were obtained on average for 3.5 ± 0.5 mg/dl negative control, 3.9 ± 0.94mg/dl positive control, hyperuricemia treatment group 5.1 ± 1.0 mg/dl and test preparation group 3.6 ± 0.7 mg/dl. Normal uric acid levels in the serum of tested animals ranged from 1.20 to 7.5 mg/dl [25]. Thus tested animals in each group had normal uric acid levels and can be used in research. The graph (Figure 1) showed a decrease in uric acid levels of the positive control group and the test preparation group compared with negative control group which is indicated by the differences between negative control and the positive control group, and the test preparation group.

Statistical Analysis

All data obtained from each group were analyzed statistically by two-way ANOVA (Table 2) followed by Levene test, a test of between_subjects effects and LSD as post-hoc test at a price of $\alpha < 0.05$, to determine which groups showed significant differences between groups.

Table 1: Average Uric Acid Levels in Serum after Treatment in mg/dl (n=5)

Group	Dose	Uric Acid Levels Average*			
		0	I	II	III
Normal control	Vehicle	3.5 ± 0.5	3.6 ± 0.7	4.0 ± 1.6	3.2 ± 0.2
Positive control	Colchicine 0,23 mg/kg BW	3.9 ± 0.9	5.2 ± 1.9	5.5 ± 2.2	6.2 ± 3.3
Negative control	Oxonic acid potassium 250 mg/kg BW	5.1 ± 1.0	14.8 ± 6.2	8.2 ± 1.7	9.2 ± 5.0
Treatment of freeze dried extract	121,24 mg/kg BW	3.6 ± 0.75	4.9 ± 0.8	4.9 ± 2.1	4.2 ± 0.8

*Average uric acid levels per day from each test group were presented in graphical form (uric acid levels versus days).

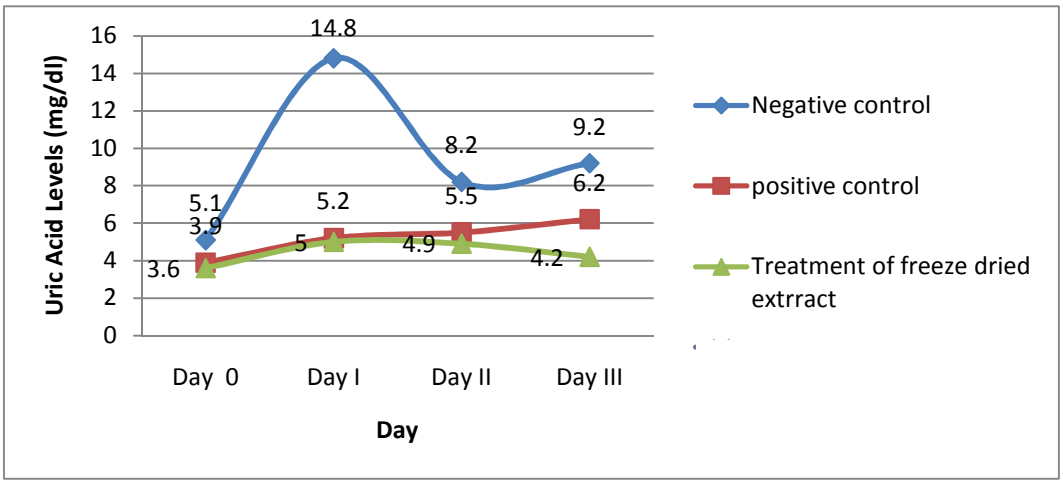

Fig. 1: Graph uric acid levels in the average serum in group positive control, negative control and treatment groups granting the extract solution freeze dried

Table 2: The results of the data analysis as a post-hoc LSD test

	Normal control	Positive Control	Negative control	Treatment of freeze dried extract
Normal Control		-1,648S= 0,057	-5,758*S= 0,000	-0,863S= 0,311
Positive Control	1,648S= 0,057		-4,110*S=0,000	-0,785S= 0,330
Negative Control	5,758*S= 0,000	4,110*S=0,000		4,895*S= 0,000
Treatment of freeze dried extract	0,863S= 0,313	-0,785S= 0,330	-4,895*S= 0,000	

*Description: Significant value <0.05 indicates a significant difference between group

The results of LSD analysis showed no significant differences between the negative control and the positive control group, testing group which was given a solution of freeze dried extract and the normal group with any significant price each group was 0,000. This indicated that the administration of potassium oxonic can increase uric acid levels on rats and showed that positive control and testing group which was given freeze-dried extract can decrease uric acid level in the blood that had been induced by potassium oxonic. Therefor, decrease of *Gloriosa Superba* L leaf extract has significant influence on the decline uric acid levels in the blood of male rats.

Test preparation group showed no significant differences with the negative control, and no significant effect on the positive control, and normal controls, so that it can be said that the *Gloriosa Superba* L leaf extract can decrease uric acid levels in hyperuricemic rats. The use of *Gloriosa Superba L* leaves in lowering uric acid has also been reported by other researchers [26] of the Faculty of Pharmacy UHAMKA Jakarta by comparing three kinds of fractions of *Gloriosasuperba* L leaves extract without a standardization and the fraction of ethanol at a dose of 5 mg/200 g BW was the best in the lowering uric acid levels.

The difference value of different levels of uric acid, can be caused by the differences in the type of extract, dose preparation, filming techniques and measurement methods of of uric acid level in blood plasma. Other researchers [26] using ethanol fraction breech leaves flowers at a dose of 5 mg/kg body weight and blood plasma collection is done through eye orbital sinus of rats, further measurement of uric acid levels used *the enzymatic photometric method* using FS TBHBA uric acid reagent (*2.4,6-Tribromo-3-hydroxybenzoic acid*). Different level of uric acid showed that both experience the effects of decreasing in uric acid levels which is likely due to uric acid metabolism of rats which eliminate uric acid out of the body through urine.

Table 3: Calculations of LD_{50} data for Colchicine in *Gloriosa Superba* L leaf extract

Group	Dose	The numberof animals per group	Animals dead	Living animals
1	Kontrol	8	0	8
2	0,23 mg/kg bw	8	0	8
3	0,69 mg/kg bw	8	0	8
4	2,07 mg/kg bw	8	0	8
5	6,21 mg/kg bw	8	0	8
6	11,5 mg/kg bw	8	0	8
7	29,44 mg/kg bw	8	1female	7

Based on the criteria of Loomis (1978), all four dose variations that has been tested is included in categories: (1). 0.23 mg/kg is equivalent to 30.31 mg/250g BW of *freeze drying* or 121.24 mg/kg (extremely toxic category), (2). 0.69 mg/kg is equivalent to 90.93 mg/250g BW of *freeze drying* or 363.73 mg/kg (extremely toxic category) (3). 2.07 mg/kg is equivalent to *freeze drying* 272.799 mg/250g BW or 1091.196 mg/kg (toxic category of medium); (4). 6.21 mg/kg is equivalent to 818.4 mg/250g Bw of *freeze drying* or 3273.6 mg/kg (toxic category of medium). Since using all doses, death is not present in the experimental animals, the dose was increased to 11.5 mg/kg and 29.44 mg/kg body weight of rats to ensure that extracts fall into mild toxic category or practically non-toxic. 11.5 mg/kg is equivalent to 1515.55 mg/250g BW of *freeze drying* or 6062.2 mg/kg (mild toxic category), and 29.44 mg/kg is equivalent to 3879.81 mg/250g BW *freeze drying* or 15519.24 mg/kg (practically non-toxic category). False LD_{50} was determined at 15519.24 mg/kg BW. Toxicity tests on *Gloriosa Superba* L extract, do not bring up the toxic effects or death to animals, because of the four-storey dose variation, none of the rats died after treatment, so it can not be determined LD_{50} values based on Table Weil. In general, the smaller the LD_{50} value, the more toxic the compound. Vice versa, the larger the LD_{50} value, the lower the toxicity. The results obtained (in mg/kg) can be classified according to the potential acute toxicity of the test compound into several classes, from extraordinary or less toxic 1 mg/kg up to a relatively less harmful 5000-15000 mg/kg [27]. Under the agreement, if the maximum dose does not cause death to experimental animals, the LD_{50} is expressed as "pseudo" by taking the maximum dose. Thus, in this study we found LD_{50} false, ie 15519.24 mg/kg BW. These results could not be included in the criteria Loomis, because LD_{50} value is not real. Oral administration of *Gloriosa Superba* L leaf extract containing colchicine 29.44 mg/kg equivalent of 15519.24 mg/kg freeze drying or approximately 206.08 x the maximum dose in humans has reached the maximum dose that can be given is technically still in tested animals, not cause death to the tested animals. Based on the criteria loomis (1978), the results of the toxicological significance that have the potential acute toxicity leaf extract of freeze drying *Gloriosa Superba* L included in the category of practical non-toxic (> 15000 mg/kg BW).

Table 5: Observation the number of tested animals dead after 24 hours Colchicines administration

Group	Dose(mg/Kg)	Log Dose	Total	Mortality	% Mortality
1	Normal	-	8	0	0
2	0,045	-1,347	8	0	0
3	0,45	-0,37	8	0	0
4	4,5	0,653	8	1	12,5
5	45	1,653	8	8	100
6	450	2,653	8	8	100

Description: Dose 4 died on the second day (1 Females), Dose 5 & 6 dead after 24 hours (each of 4 males and 4 females), Calculate the LD50 can use (1) Weil method, using tables Weil; (2).using line equationy = a + bx, wherey = % mortality and x = log dose, and (3). Using probit [17]. LD50 of pure colchicines is calculated using equation y = a + bx, and obtained LD_{50} was 7.92 mg/kg. Unlike the LD_{50} reported by other researchers [28] on pure colchicine at 5 mg/kg BW.

CONCLUSSION

Based on antihyperuricemia test data, single dose of freeze dried extract of leaf *Gloriosa Superba* L of colchicine equivalent to 0.23 mg/kg colchicines has been proven could lower uric acid level in the blood of male white rats Wistar strain-induced potassium oxonic. And the results of LD_{50} for *Gloriosa Superba* L leaf extract is included in the category of practically non-toxic (greater 1500 mg/kg body weight), were obtained by colchicine LD_{50} of 7.92 mg/kg.

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