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EFFECT OF MELINJO SEED EXTRACT ON GSH (GLUTATHIONE) LEVELS OF HYPERURICEMIC WISTAR RATS

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ABSTRACT

Background: Hyperuricemia is an enhancement uric acid levels beyond normal. Uric acid biosynthesis by the enzyme xanthine oxidase (XO) generates free radicals that induce oxidative stress. Oxidative stress reduce GSH levels in cell. Melinjo seeds or *Gnetum gnemon L.* have contents flavonoids and stilbenoids that can act as natural XO inhibitors. **Objective:** To study the effect of melinjo seed extract on GSH levels. **Methods**: This study was a true experimental with pre and post-test controlled group design. Thirty six male wistar rats were randomly distributed into 6 groups consisting of a healthy control (KS), negative control (K1), positive control (K2), treatment 1 (P1) extract 250 mg/kgBW, treatment 2 (P2) extract 500 mg/kgBW, treatment 3 (P3) extract 2000 mg/kgBW. Hyperuricemia was caused by the application of potassium oxonate and block broth. Measurements were performed on days 21 and 35 of treatment and then analyzed using Paired-Sample T Test, followed by a One-Way Anova test and a Post Hoc LSD test. **Results**: Application of melinjo seed extract at P1, P2, and P3 significantly elevated GSH levels (P<0.05) from 1.23±0.10 to 1.55±0.16; 1.22±0.06 to 1.73±0.16; and 1.21±0.08 to 1.88±0.08. There was significant difference between the three doses with a dose of 250 mg/kgBW melinjo seed extract being more effective than 90 mg/kgBW of allopurinol. **Conclusion**: Melinjo seed extract may enhance GSH levels at the most effective dose of 250 mg/kgBW.

Keywords : *Gnetum gnemon, GSH, hyperuricemia, Melinjo seeds*

INTRODUCTION

Hyperuricemia is a condition characterized by escalation of uric acid levels in the blood.[1] High uric acid levels come from an unhealthy lifestyle such as high purine intake, alcohol, obesity, lack of exercise, drugs, impaired kidney function and increasing age.[2,3] The prevalence of hyperuricemia is rising dramatically throughout the worldwide, including in developing countries.[4] The prevalence of hyperuricemia in Indonesia is not known with certainty, but the prevalence of joint disease is 7.3% according to the diagnosis by medical staff.[5]

Hyperuricemia is a precursor to gout.[6] Several studies have also shown that chronic kidney disease (CKD), hypertension, and atherosclerotic cardiovascular disease (CVD) are also diseases with hyperuricemia as a risk factor.[7] Long-term hyperuricemia leads to the formation of renal fibrosis, glomerulosclerosis, arteriosclerosis, and thickening of arterial walls.[8]

Pharmacological therapy used in hyperuricemia includes: uricosuric drugs (probenecid, benzbromarone, and losartan) and uricostatic drugs (allopurinol, oxipurinol and febuxostat).[9] All of these drugs have many side effects such as arthralgia, nausea, skin rash, kidney failure, hypersensitivity syndrome, elevated liver transaminases, and liver toxicity.[10,11]

Uric acid biosynthesis in human is facilitated by the enzyme xanthine oxidase (XO) in the last 2 steps of in purine metabolism, the hydroxylation of hypoxanthine and xanthine.[12] This hydroxylation also generates free radicals such as superoxide anion and hydrogen peroxide that contribute to oxidative stress.[13,14] Antioxidant activity is the potency of a compound to scavenge complex metal ions or free radicals that encourage the oxidation processes.[15] Glutathione (GSH) or Tripeptide, y-L-glutamyl-L-cysteineyl-glycine, is the most effective intracellular antioxidant. The GSH:GSSG ratio describes antioxidant capacity of the cells.[16] Severe oxidative stress impairs cell function to reduce GSSG to GSH dan reduce GSH levels.[17]

Melinjo (Indonesian name; *Gnetum gnemon L.*), member of Gnetacea family and is a native plant of Southeast Asia and Melanesia.[18,19] This plant species can grow in shady places, open areas, a fairly large temperature range, heavy rainfall condition, and infertile soil.[18]

Phytochemical studies of Melinjo seeds revealed the presence of stilbenoid derivatives as major compounds, which exhibited various pharmacological

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activities.[15] Resveratrol is one of the natural stilbenoids contained in melinjo seeds.[20] Resveratrol has antioxidant properties that promote antioxidant activity and has ability to scavenge free radicals and such as GSH, SOD, and CAT.[21] In addition, flavonoid content was also found in melinjo seeds.[22] Flavonoids and stilbenoids can act as natural XO inhibitors.[23,24] Other research results showed that melinjo seeds exhibited antioxidant activity significantly ($P < 0.05$) counter to free radicals and exhibit similar activity to GSH and BHT.[25]

Based on this, a research was held utilizing melinjo seed extract to lower levels of oxidative stress. The level of oxidative stress was assessed using blood GSH as a parameter of antioxidant capacity in the body. Blood GSH spectrophotometric measurements were based on the colorimetric reaction of GSH with Ellman's reagent or 5.5'-dithiobis (2-nitrobenzoic acid) (DTNB) that yield a yellow 5′-thio-2-nitrobenzoic acid (TNB) compound and then measured by its maximum absorption at a wavelength of 412 nm.[26,27]

METHODS

This was a true experimental study with a pre- and post-trial randomized controll group design that was held from June to July 2022 in Animal Test Laboratory of the Faculty of Medicine and Biomedical Laboratory of the Faculty of Medicine, Diponegoro University, and also the Central Laboratory of RSND/FK UNDIP.

The tools used in this study include Erlenmeyer tubes for water baths, measuring cups, maceration, analytical scales, animal scales, animal cages, gastric probes, disposable syringes, centrifuges, micropipettes, test tubes, cuvettes, and spectrophotometer.

The materials used are 0.5% CMC (Sigma-Aldrich, St. Lous), melinjo seeds taken from farmers in Gunung Sari, Serang, Banten, allopurinol (Omeric, Medan), Maggie® block broth, potassium oxonate (Solarbio, Beijing), aquadest, 70% ethanol solvent, standard feed and drinking water for experimental animals. DTNB reagent (Solarbio, Beijing): 39.6 mg of DTNB dissolved in 10 ml of 0.1M phosphate buffer with a pH of 7.0, 5% TCA: 25% TCA standard solution is diluted with distilled water to obtain 5% TCA concentration, and 0.1M phosphate buffer pH 8.0 as solvent.

The samples utilized in this study were male wistar rats (*Ratuus norvegicus*), 8-12 weeks old, normal weight 140-260 grams, appearing to be in good health, active, and anatomically No abnormalities were observed.

The melinjo seed extract processs uses the maceration method. Five kg of melinjo seeds were selected with the same maturity level. Melinjo seeds were dried in an oven at 50ºC-70ºC for 24 hours and then mashed, then macerated with 70% ethanol solvent method for 3x24 hours and then filtered to obtain a liquid extract. The residue was extracted again in the same way until a clear maserate was obtained. The filtrate was evaporated using rotary evaporator vacuum for 6 hours to obtain a concentrated extract and then weighed.

Thirty-six wistar rats that fulfill the research criteria were habituated for 7 days with food and drink *ad libitum* in the laboratory then divided into 6 groups consisting of 6 samples per group based on simple random sampling.

The healthy control group (KS) was fed a standard diet, the positive control group (K1), negative control (K2), treatment 1 (P1), treatment 2 (P2), and treatment 3 (P3) were each administrated standard feed, Maggie® block broth. and potassium oxonate to induce hyperuricemia for the following 14 days.

On days 22 to 35, the KS group was given standard feed, and K1-P3 was also given Maggie® block broth and potassium oxonate. In group K2, 90 mg/kgBW allopurinol was added and P1, P2, and P3 were treated with oral melinjo seed extract at doses of of 250 mg/kgBW, 500 mg/kgBW and 2000 mg/kgBW, respectively. Wistar rat blood sampling and measurement of blood GSH levels were carried out on the 21st and 35th days.

The corresponding data were then analysed using IBM SPSS Statistics 25.0 Software. Data were analyzed using a paired T-test followed by One-Way Anova test and Post Hoc LSD.

RESULTS

This research used melinjo seeds (*Gnetum gnemon L.*) taken from farmers in Gunung Sari area, Serang city, Banten province. Before the extraction, the shells of the melinjo seeds were peeled and finely grounded and dried in order to expand the surface area of cell membranes so that the retraction of the

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active ingridient by the solvent run better. Melinjo seeds were soaked in 70% ethanol for 72 hours and then evaporated in a water bath to achieve a condensed extract consistency. This extraction yielded 63.55 grams of melinjo seed extract from 1734.55 grams of dried melinjo seeds.

A total of 36 wistar rats (*Rattus norwegicus*) used in this study were distributed into 6 groups consist of 6 wistar rats per group. Prior to treatment, the samples were adapted and fed a standard diet for 7 days. During the treatment period, which lasted for 28 days, there was 1 rat in groups K1, P1, P2, and P3 that dropped out. The final number of research samples meeting the inclusion and exclusion criteria were 32 samples.

Pre- and post-treatment plasma GSH level measurements are shown in Table 1.

Table 1. Wistar Rats Plasma GSH Levels

Group	Number of Rats	Mean ± SD Plasma GSH Level (µg/ml)	
		Before Treatment	After Treatment
KS	6	1.42 ± 0.06	1.42 ± 0.05
K1	5	1.26 ± 0.03	1.24 ± 0.05
K2	6	1.23 ± 0.07	1.81 ± 0.09
P1	5	1.23 ± 0.10	1.55 ± 0.16
P2	5	1.21 ± 0.06	1.73 ± 0.16
P3	5	1.21 ± 0.08	1.88 ± 0.08

Table 1 shows the mean plasma GSH levels before treatment is highest in the KS group. The measurement results of the plasma GSH levels after treatment in groups K2, P1, P2, and P3 relatively increased compared to the results before treatment. The average plasma GSH level is highest in the P3 group.

The hypothesis is proven by statistical analysis using IBM SPSS statistics 25.0 software. First, a normality test of the data distribution and homogeneity is done. The parameters used are Shapiro-wilk test for small samples (n<50) and Levene's analytical method. The normality test results for all groups are normally distributed ($P > 0.05$) and the homogeneity test results show homogenous population variation ($P > 0.05$). A paired t-test was used to specify the difference in mean GSH plasma levels before and after treatment in each group. The results were significantly different if $P < 0.05$.

In Figure 1, there were no significant differences ($P > 0.05$) in the KS and K1 groups; while in the K2, P1, P2, and P3 groups, there were significant differences ($P < 0.05$). Thus, there is a significant increase in plasma GSH levels between before and after treatment. As the data were homogeneous and normally distributed, the difference test was continued using the One-way ANOVA test to determine differences between groups.

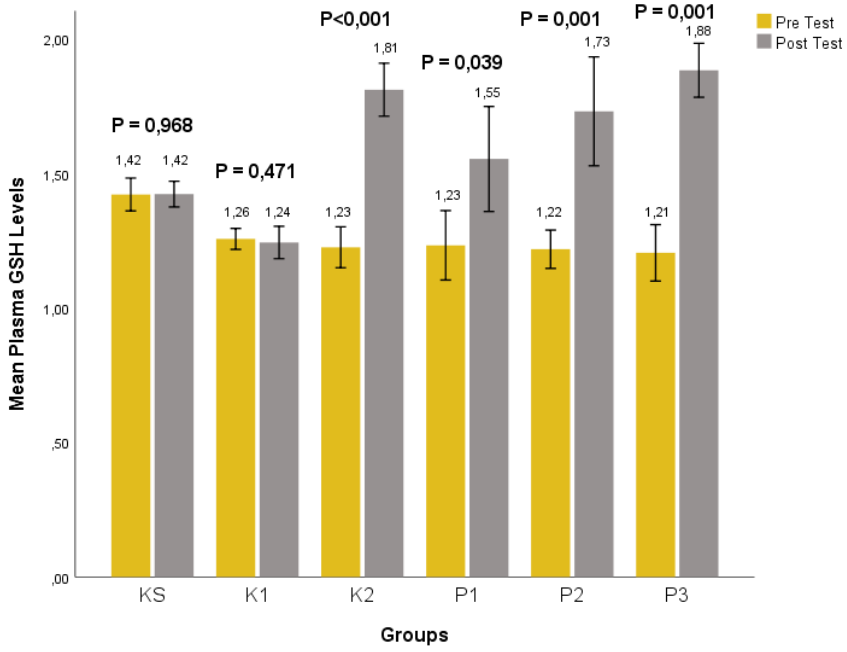

Figure 1. Bar Diagram of Paired T-Test Results Mean Differences in Plasma GSH Levels Before and After Treatment

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One-way ANOVA test results showed a significant difference ($P < 0.05$) in plasma GSH levels before and after treatment. This shows that there is an effect after the treatment was given. A Post Hoc LSD test was done to establish the level of statistical significance for each group.

The results of the post hoc LSD test before treatment shown in Table 2 showed that the KS group against K1, K2, P1, P2, and P3 was significant, it can be concluded that administration of potassium oxonate and Maggie® block broth may induce hyperuricemia so that plasma GSH levels fall.

The post-treatment post hoc LSD test resluts shown in Table 3 showed that the KS group against K1, K2, P2, and P3 was significant, while the P1 group was not significant. From this, we conclude that there was a significant difference in plasma GSH levels between the untreated KS group with hyperuricemia-induced group (K1), group receiving allopurinol 90 mg/kgBW (K2), and the group that was received 500 mg/kgBW melinjo seed extract (P2) and 2000 mg/kgBW (P3).

The comparison of groups K1 to K2, P1, P2, and P3 was significant, concluding that there was a significant difference in plasma GSH levels between the hyperuricemia-induced group with the group given allopurinol 90 mg/kgBW (K2), and melinjo seed extract (P1, P2, and P3).

A significant difference was also seen between the K2 and P1 groups, demonstrating that there was a significant difference in the increase in plasma GSH levels between the administration of allopurinol 90 mg/kgBW and 250 mg/kgBW melinjo seed extract.

The comparison of groups P1 to P2 and P3, and P2 to P3 is also significant, indicating that the effects of of 250 mg/kgBW, 500 mg/kgBW and 2000 mg/kgBW of melinjo seed extract graded doses were significantly different.

Table 2. Comparison of Plasma GSH Levels Before Treatment

Group	***P* Value Before Treatment**				
	K1	**K2**	**P1**	**P2**	**P3**
KS	0.001*	<0.001*	<0.001*	<0.001*	<0.001*
K1	-	0.476	0.598	0.401	0.260
K2		-	0.869	0.869	0.636
P1			-	0.752	0.542
P2				-	0.768
P3					-

Table 3. Comparison of Plasma GSH Levels After Treatment

Group	***P* Value After Treatment**				
	K1	**K2**	**P1**	**P2**	**P3**
KS	0.001*	<0.001*	0.053	<0.001*	<0.001*
K1	-	<0.001*	<0.001*	<0.001*	<0.001*
K2		-	<0.001*	0.224	0.273
P1			-	0.014*	<0.001*
P2				-	0.032*
P3					-

DISCUSSION

The significant differences in GSH levels before treatment between groups in the KS group to K1, K2, P1, P2, and P3 could be interpreted that the application of potassium oxonate and Maggie® block broth could reduce plasma GSH levels of wistar rats.

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Maggie® block broth contains guanylate, monosodium glutamate (MSG), and disodium inosinate.[28] MSG can cause oxidative stress by generating hydrogen peroxide and oxygen radicals. Severe oxidative stress weakens the cell's ability to reduce GSSG to GSH resulting in a decrease in GSH levels.[17] This is consistent with a research by Gad El-Hak HN, et al. They studied the effects of MSG administration on pregnant rats therewith their fetuses. The outputs showed that application of MSG at a dose of 1 g/5ml/kgBW reduced GSH and SOD levels significantly.[29]

Potassium oxonate is a selective competitive uricase inhibitor that causing hyperuricemia by inhibit the performance of hepatic uricase.[30] Potassium oxonate also stimulates oxidants such as O2- and H2O2, as well as MDA.[31] As a result, oxidative stress increases, which then causes a decrease in GSH.[32] Wang K, *et al.* in his research showed that giving 250 mg/kgBW potassium oxonate significantly reduced SOD, GSH and GSH-Px levels.[31]

The comparison of plasma GSH levels before and after treatment in the K2, P1, P2, and P3 groups also had significant results, which means that the administration of allopurinol and melinjo seed extract increased plasma GSH levels.

Allopurinol is an inhibitor of XO and other enzymes involved in purine and pyrimidine metabolism.[33] The role of inhibited XO will inhibit the production of superoxide resulting in an increase in GSH levels.[34] This is consistent with an investigation by El-Mahdy NA, *et al.* who investigated the role of XO inhibitors in a colitis model. The results showed that GSH levels were significantly elevated in the allopurinol-treated group. [35]

Gnetum gnemon L. seeds contain tannins, flavonoids, and various stilbenoids.[22,36] Flavonoids and stilbenoids can act as natural XO inhibitors.[13,23] Flavonoids have the ability to inhibit XO which produces hydrogen peroxide and superoxide anion.[37] Oxidant inhibition reduces levels of oxidative stress, so that cells can reduce GSSG to GSH again.[17] In addition, resveratrol is also known to induce an increase in intracellular GSH levels.[38]

Research conducted by Espinoza JL, *et al.* aimed to evaluate the cellular and molecular immune responses by administering melinjo seed extract orally for 28 days. The outcome indicate that application of melinjo seed extract correlated with a significant escalation in total antioxidant capacity of plasma samples. At second weeks plasma antioxidant capacity increased almost 2-fold and reached a 2.5-fold increase at the fourth week.[39]

Post-treatment plasma GSH levels at K2, P1, P2, P3 in the K1 group were significant, so there was a significant difference in plasma GSH levels between the hyperuricemia group without treatment and the hyperuricemia group given allopurinol (K2), melinjo seed extract (P1, P2, and P3).

The outputs of this study are consistent with previous studies by Radwan RR and Karam HB, where oral resveratrol lessen MDA levels and significantly elevated CAT and GSH levels activity compared to the irradiated group.[40]

Another study conducted by Adhikary M, *et al.* used wheat grass with flavonoid content. The outcome of this research showed that the groups treated 200 mg/kgBW and 400 mg/kgBW wheat grass caused a significant increases in GPX and GSH compared to the diabetic control group.[41]

Plasma GSH levels after treatment between groups in the KS group on P2 and P3 were significant. It can be concluded GSH improved at all the doses of melinjo seed extract but higher doses of resveratrol are more beneficial. This is consistent with previous investigation by Börzsei D, *et al.* This indicated that resveratrol administration significantly increased cardiac GSH levels of aging female wistar rats. Resveratrol increases cellular antioxidant capacity by decreasing ROS and simultaneously increasing GSH, so resveratrol therapy will intensify the antioxidant mechanism by increasing the GSH system.[42]

Significant differences were also found in the K2 group against P1 where it can be concluded that administration of melinjo seed extract at a dose of 250 mg/kgBW was more effective than allopurinol 90 mg/kgBW and more effective than doses of 500 mg/kgBW or 2000 mg/kgBW.

The outputs of this research are consistent with previous studies by Tang X, *et al.* conducted screening and evaluation XO inhibitory compounds in *Gnetum parvifolium*. The results showed that there were 4 ligands identified as XO inhibitors, namely piceatannol, rhaponiticin, resveratrol, and isorhapontigenin. The 4 inhibitors showed the XO inhibition superior to allopurinol and had good free

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radical scavenging abilities which resveratrol showing the highest inhibitory activity.[43] An in vitro study was performed by JA Fauzi *et al*. also indicated that the higher the concentration of melinjo seed extract, the lower the percentage of XO inhibition produced.[44]

In this study, the difference in GSH levels after treatment between groups P1, P2, and P3 was significant, that means there were differences in the effect of graded doses on the application of melinjo seed extract at 250 mg/kgBW, 500 mg/kgBW, and 2000 mg/kgBW. The outcomes of this research are consistent with the work done by Pannu N and Bhatnagar A where the application of resveratrol at a dose of 25 mg/kgBW and 50 mg/kgBW or in combination with piperine at a dose of 2.5 mg/kgBW and 5 mg/kgBW had significant therapeutic effect by increasing higher GSH levels compared to the autoimmune-induced group.[45]

There are some limitations in this study. RNS and ROS levels were not measured to demonstrate the effect of administration potassium oxonate and Maggie® block broth and melinjo seed extract on oxidative stress, and quantitative phytochemical tests were not measured so the flavonoids and stilbenoids levels in the used melinjo seed extract samples could not be measured. There was also limited amount of wistar rats blood to be measured so as to anticipate rats dying after taking blood on the 14th day of treatment.

CONCLUSION

Melinjo seed extract can increase plasma GSH levels of hyperuricemic wistar rats and there are differences in the effect of graded doses between the study treatment groups, in which 250 mg/kgBW of melinjo seed extract is more effective than 90 mg/kgBW of allopurinol.

ETHICAL APPROVAL

This research received ethical clearance from the Commission on Ethics for Medical and Health Research (KEPK) Faculty of Medicine, Diponegoro University with No.49/EC/H/FK-UNDIP/VI/2022.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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