Hematological Parameters in Subchronic Toxicity Test of Black Garlic Ethanol Extract in Rats

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Abstract
The community has used black garlic since ancient times for hypercholesterolemia. Until now, people still consume both raw and cooked black garlic. Black garlic is included in processed garlic products. People on the Asian continent have used black garlic for the past 10 years. This study aims to determine the sub-chronic toxic effect of black garlic ethanol extract on male and female white rats using hematological parameters consisting of hematocrit, hemoglobin, erythrocytes, leukocytes, platelets, MCV, MCH, and MCHC. This research method was a completely randomized design with the administration of ethanolic extract of black garlic to rats in 5 treatment groups, including a negative control group, a dose group of 1000 mg/kg BW, a dose group of 2000 mg/kg BW, a negative control satellite group and a satellite group with a dose of 2000 mg/kg BW. Data were analyzed statistically using one-way ANOVA with a 95% confidence level and SPSS version 20. The study results of black garlic ethanol extract showed no significant effect or were at normal levels on hematological parameters. Therefore, it can be concluded that the ethanolic extract of black garlic is safe for human use as a treatment for hypercholesterolemia.

Keywords: Black Garlic; Hematology; Subchronic Toxicity Test

INTRODUCTION
The community has used black garlic for hypercholesterolemia. Until now, people still consume both raw and cooked black garlic. Black garlic is one of the processed garlic products. People on the Asian It has a sweet, chewy taste and a distinctive aroma. Black garlic contains carbohydrates, amino acids, total polyphenols, and flavonoids. In addition, there are changes in several bioactive compounds such as S-allyl cysteine, vitamins, phenolic acids and flavonoids in black garlic during the heating process. S-allyl cysteine, one of the main components of sulfur-containing amino acid compounds, is five to six times higher than fresh garlic. The decrease in alliin content in black garlic is that alliin is converted to S-allyl cysteine. S-allylmercapto-cysteine, arginine and other compounds are not defined when the heating process. According to the 2016 Minister of Health Regulation, garlic is included as a native plant of Indonesia that can be used as

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Herbal medicine. It has conditions proven to be safe, efficacious and of good quality. The use of black garlic as a processed garlic product is a native Indonesian herbal medicine included in the herbal medicine category called jamu. Several studies of black garlic in vivo have also been carried out over the last 20 years, including Prof. Dr. Jin Ichi Sasaki in Japan, revealing that black garlic has anti-tumor activity. Black garlic initially resulted in many Japanese people producing black garlic using simple tools such as rice cookers and other heating devices so that black onions can be consumed. In addition to black garlic as an anti-tumor, many other activities were investigated by several researchers, such as research by Wang et al. in 2012 related to the content of black garlic, namely S-allyl-cysteine, which was found to be able to reduce 50% of the size of fibrosarcoma in mice. Other research also investigated that black garlic has other biological activities such as antioxidant activity, anticancer on human leukemic cells, antiobesity where black garlic is given to obese mice, anti-inflammatories, hypoallergenic. Research conducted by Nuristika in 2018 concluded that black garlic did not cause death in mice, so the LD₅₀ was unknown. Another study revealed that the administration of Dayak onion ethanol extract did not affect the hematological profile in white rats. The development of an herbal medicinal product that has been tested in vivo, apart from toxicity testing, parameters can also be studied to obtain a toxic effect. A natural ingredient can also be investigated for its toxicity, including changes in body weight, clinical symptoms, hematological parameters, clinical biochemistry, macro pathology, histopathology, target organs, mortality, and other general or specific effects. Testing of a natural ingredient was carried out on a biological system such as oral subchronic toxicity testing on rats for 28 days or 90 days with five dose groups, namely the negative control group, a dose of 1000 and 2000 mg/kg BW, as well as the negative control satellite group and satellite group of 2000 mg/kg BW. At the end of the period of administering the test preparations, all living experimental animals were autopsied and subjected to mactopathological, hematological, clinical biochemical and histopathological observations. The test was carried out to determine the cumulative effect and reversibility effect after repeated exposure to the test preparation for a certain period. This study aims to identify if people with a history of hypercholesterolemia consuming black garlic continuously or in the long term causes toxic effects, as seen from the description of the blood hematology parameters.

METHOD

The research design for the oral subchronic toxicity test was a completely randomized design (CRD), in which there were treatment and control groups with homogeneous environmental factors. The research was conducted at the Phytochemical Laboratory of Santo Borromeus College of Health and the Pharmacology and Toxicology Laboratory of Padjadjaran University. This research was started from February to May 2019. The sample used was garlic (Allium sativum, L) determined in advance at the Taxonomy Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University. 1 kg of garlic was processed into black garlic by the maceration method for 5x24 hours with 70% ethanol solvent. The garlic was processed with a rice cooker at 70°C heating for 21 days. The preparation of tested animals used were 25 male rats and
25 female Wistar rats with a bodyweight of approximately 100-200 grams according to the Cage Space Guidelines for Animals Used in Biomedical Research (2008), adapted for 1 week. Before carrying out subchronic toxicity testing, it is necessary to have a research code of ethics (ethical clearance) from the Research Ethics Commission of the Faculty of Medicine, the University of Padjadjaran, with number 528/UN6.KEP/EC/2019. The black garlic ethanol extract was administered for 28 days (measurement of rats' body weight) and 29 days for the negative control group, and 43 days for the satellite test examination. Hematological examination on mice took 1 mL of blood into a K2EDTA vacutainer tube containing 0.5 mL of ethylene diamine tetraacetate (EDTA). The blood was then examined using a hematological analyzer (XP-100). The data obtained included hemoglobin, erythrocytes, leukocytes, platelets, MCV, MCH, and MCHC. The graph was created to determine the increase in the body weight of rats. Statistical analysis of blood hematological parameter data used One Way ANOVA and SPSS version 20. The research materials included garlic obtained from Lembang-West Java. All other chemicals and reagents were sourced commercially, such as ethanol from Merck (Germany) and Pulvis Gummi Arabicum from J. Trading Co. Ltd. (Thailand). Research tools included thermostatic water bath (China), Rotary Evaporator RE 100-S Dlab (China) and Hematological Analyzer XP-100 (China).

RESULTS AND DISCUSSION

Ethanol extract of black garlic
The maceration results were revealed after being conducted for 5 days. This method was used to extract the active substance that has pharmacological activity. Hypercholesterolemia obtained a yield of 49.584%, as shown in table 1.

| Black Garlic | Extract of black garlic | Yield value of black garlic |
|-------------|-------------------------|-----------------------------|
| 1.000 gram  | 495.84 gram             | 49.584%                     |

Oral subchronic toxicity test
The graph of the weight development of male rats is shown below. In administering test preparations at a dose of 1000 mg/kg BW and 2000 mg/kg BW, the bodyweight development of rats fluctuated every day, as shown in Figure 1.

![Figure 1. Bodyweight development of male rats](image-url)
There were fluctuations in the body weight of rats in the negative control group and doses of 1000 and 2000 mg/kg BW until the 28th day. An increase in body weight of rats occurred in the negative control satellite group, and the satellite dose of 2000 mg/kg BW significantly until the 42nd day. The graph of the bodyweight development of female rats is shown below. In administering test preparations at a dose of 1000 mg/kg BW and 2000 mg/kg BW, the bodyweight development of rats fluctuated every day, shown in Figure 2.

There were fluctuations in the body weight of female rats in the negative control group and doses of 1000 and 2000 mg/kg BW until the 28th day. An increase in body weight of rats occurred in the negative control satellite group, and the satellite dose of 2000 mg/kg BW significantly until the 42nd day. In addition to weighing the body weight of rats, the average increase in body weight was also observed to determine the magnitude of administering black garlic ethanol extract with certain doses, as shown in Table 2.

Table 2. Average Body Weight of Male and Female Rats

| Groups                  | Male Rats | Female Rats |
|-------------------------|-----------|-------------|
|                         | n         | Average     | n         | Average     |
| Negative Control        | 5         | 191.6±14.18 | 5         | 175.6±12.29 |
| 1000 mg/kg BW           | 5         | 187.6±11.09 | 5         | 166.7±12.97 |
| 2000 mg/kg BW           | 5         | 184.7±12.95 | 5         | 151.8±13.15 |
| Satellite 2000 mg/kg BW | 5         | 194.3±17.34 | 5         | 174.4±9.09  |
| Satellite Negative Control | 5   | 191.07±16.87| 5         | 169.21±10.45|

Note: n = Number of rats in each treatment group
The statistical data analysis was carried out regardless of the difference in the increase in body weight of rats among groups, as shown in Table 3.

**Table 3.** Result of Statistical Analysis of Body Weight of Male and Female Rats

| Groups                  | Statistical Analysis Results (p>0.05) |
|-------------------------|--------------------------------------|
|                         | Male Rats                           | Female Rats |
| Day 1-28                | ANOVA                                | ANOVA       |
| Negative Control        | 0.034                                | 0.345       |
| 1000 mg/kg BB           | 0.144                                | 0.434       |
| 2000 mg/kg BB           | 0.312                                | 0.146       |
| Day 29-42               | **Kruskal Wallis**                   | **Kruskal Wallis** |
| Satellite Negative Control | 0.140                      | 0.337       |
| Satellite 2000 mg/kg BB |                                       |             |

The research data were tested for normality and homogeneity using ANOVA and non-parametric analysis methods for $K$ independent samples (Kruskal Wallis). If the significance is more than 0.05 ($p>0.05$), there is no significant difference, and no further test is needed. If the significance is less than 0.05 ($p<0.05$), a significant difference can be further identified by conducting the Tukey test. The administration of black garlic ethanol extract in the negative control group, a dose of 1000 and a dose of 2000 mg/kg BW of male and female rats for 28 days, did not significantly increase the bodyweight of the tested rats (28 days) and the satellite group (42 days).

**Hematological Parameter Test**

The hematological examination was carried out to determine abnormalities in the quantity and quality of blood cells. It also examined changes in plasma that played a role in the blood clotting process and identified if there was inflammation or infection, as indicated in table 4 and table 5 below.
### Table 4. Value of Hematological Parameters Analysis of Male Rat

| Parameter | Groups                                      | Normal Level Male | Grade Value | ANOVA (p > 0.05) |
|-----------|---------------------------------------------|-------------------|-------------|------------------|
| Hemoglobin (g/dL) | Negative Control | 13.7-17.6         | 12.5        | 0.259            |
|           | 1000 mg/kg BW                                |                   | 13.3        |                  |
|           | 2000 mg/kg BW                                |                   | 14.9        |                  |
|           | Satellite 2000 mg/kg BW                      |                   | 14.1        |                  |
|           | Satellite Negative Control                    |                   | 16.8        |                  |
| Hematocrit (%) | Negative Control                             | 39.6-52.5         | 33.46       | 0.239            |
|           | 1000 mg/kg BW                                |                   | 40.01       |                  |
|           | 2000 mg/kg BW                                |                   | 41.10       |                  |
|           | Satellite 2000 mg/kg BW                      |                   | 40.81       |                  |
|           | Satellite Negative Control                    |                   | 49.87       |                  |
| Leukocytes (10³/µL) | Negative Control                             | 1.96-8.25         | 10.31       | 0.565            |
|           | 1000 mg/kg BW                                |                   | 1.96        |                  |
|           | 2000 mg/kg BW                                |                   | 9.98        |                  |
|           | Satellite 2000 mg/kg BW                      |                   | 10.04       |                  |
|           | Satellite Negative Control                    |                   | 9.29        |                  |
| Erythrocytes (10⁶/µL) | Negative Control                             | 7.27-9.65         | 5.86        | 0.280            |
|           | 1000 mg/kg BW                                |                   | 7.83        |                  |
|           | 2000 mg/kg BW                                |                   | 7.96        |                  |
|           | Satellite 2000 mg/kg BW                      |                   | 7.71        |                  |
|           | Satellite Negative Control                    |                   | 8.51        |                  |
| Platelets (10³/µL) | Negative Control                             | 638-1177          | 784         | 0.487            |
|           | 1000 mg/kg BW                                |                   | 638         |                  |
|           | 2000 mg/kg BW                                |                   | 487         |                  |
|           | Satellite 2000 mg/kg BW                      |                   | 645         |                  |
|           | Satellite Negative Control                    |                   | 676         |                  |
| MCV (fL) | Negative Control                             | 48.9-57.9         | 57          | 0.550            |
|           | 1000 mg/kg BW                                |                   | 53          |                  |
|           | 2000 mg/kg BW                                |                   | 52          |                  |
|           | Satellite 2000 mg/kg BW                      |                   | 53          |                  |
|           | Satellite Negative Control                    |                   | 59          |                  |
| MCH (pg)  | Negative Control                             | 17.1-20.4         | 21.4        | 0.679            |
|           | 1000 mg/kg BW                                |                   | 17.8        |                  |
|           | 2000 mg/kg BW                                |                   | 18.7        |                  |
|           | Satellite 2000 mg/kg BW                      |                   | 18.3        |                  |
|           | Satellite Negative Control                    |                   | 19.7        |                  |
| MCHC (g/dL) | Negative Control                             | 32.9-37.5         | 37.5        | 0.755            |
|           | 1000 mg/kg BW                                |                   | 32.9        |                  |
|           | 2000 mg/kg BW                                |                   | 36.2        |                  |
|           | Satellite 2000 mg/kg BW                      |                   | 34.6        |                  |
|           | Satellite Negative Control                    |                   | 33.7        |                  |
### Table 5. Value of Hematological Parameters Analysis of Female Rat

| Parameter       | Groups                              | Normal Level | Grade Value | ANOVA (p>0.05) |
|-----------------|-------------------------------------|--------------|-------------|----------------|
| Hemoglobin (g/dL) | Negative Control 1000 mg/kg BW | 13.7-16.8   | 12.4        | 0.520          |
|                 | 2000 mg/kg BW                  |             | 13.2        |                |
|                 | Satellite2000 mg/kg BW          |             | 14.3        |                |
|                 | Satellite Negative Control      |             | 13.0        |                |
|                 |                                    |             | 14.4        |                |
| Hematocrit (%)  | Negative Control 1000 mg/kg BW   | 37.9-49.9   | 34.42       | 0.122          |
|                 | 2000 mg/kg BW                  |             | 38.74       |                |
|                 | Satellite2000 mg/kg BW          |             | 38.57       |                |
|                 | Satellite Negative Control      |             | 38.86       |                |
|                 |                                    |             | 44.11       |                |
| Leukocytes (10⁶/µL) | Negative Control 1000 mg/kg BW | 1.13-7.49   | 7.24        | 0.437          |
|                 | 2000 mg/kg BW                  |             | 5.82        |                |
|                 | Satellite2000 mg/kg BW          |             | 6.17        |                |
|                 | Satellite Negative Control      |             | 6.07        |                |
|                 |                                    |             | 5.74        |                |
| Erythrocytes (10⁶/µL) | Negative Control 1000 mg/kg BW | 7.07-9.03   | 5.87        | 0.552          |
|                 | 2000 mg/kg BW                  |             | 6.02        |                |
|                 | Satellite2000 mg/kg BW          |             | 7.23        |                |
|                 | Satellite Negative Control      |             | 6.35        |                |
|                 |                                    |             | 7.27        |                |
| Platelets (10⁹/µL) | Negative Control 1000 mg/kg BW  | 680-1.200   | 44.2        | 0.077          |
|                 | 2000 mg/kg BW                  |             | 743         |                |
|                 | Satellite2000 mg/kg BW          |             | 394         |                |
|                 | Satellite Negative Control      |             | 688         |                |
|                 |                                    |             | 537         |                |
| MCV (fL)        | Negative Control 1000 mg/kg BW   | 49.9-58.3   | 59          | 0.111          |
|                 | 2000 mg/kg BW                  |             | 56          |                |
|                 | Satellite2000 mg/kg BW          |             | 53          |                |
|                 | Satellite Negative Control      |             | 61          |                |
|                 |                                    |             | 61          |                |
| MCH (pg)        | Negative Control 1000 mg/kg BW   | 17.8-20.9   | 21.1        | 0.658          |
|                 | 2000 mg/kg BW                  |             | 20.2        |                |
|                 | Satellite2000 mg/kg BW          |             | 19.8        |                |
|                 | Satellite Negative Control      |             | 20.4        |                |
|                 |                                    |             | 19.8        |                |
| MCHC (g/dL)     | Negative Control 1000 mg/kg BW   | 33.2-37.9   | 35.9        | 0.010          |
|                 | 2000 mg/kg BW                  |             | 36.0        |                |
|                 | Satellite2000 mg/kg BW          |             | 37.1        |                |
|                 | Satellite Negative Control      |             | 33.4        |                |
|                 |                                    |             | 32.7        |                |
Male rats in the 2000 mg/kg BW and satellite 2000 mg/kg BW groups experienced an increase in the production of leukocytes to fight infection or inflammation caused by immune system disorders\textsuperscript{16,17}. In female rats in the 2000 mg/kg BW satellite dose group, the MCV value increased due to a lack of folate/vitamin B12 nutrition and the possibility of liver infection, which could be examined through further histopathological examination\textsuperscript{18,19}.

The suspension of ethanolic extract of black garlic in the negative control group, doses of 1000 mg/kg BW, 2000 mg/kg BW, the negative control satellite group and the satellite group of 2000 mg/kg BW in male and female white rats for 28 days to 42 days did not significantly affect blood levels, such as hemoglobin, hematocrit, leukocytes, erythrocytes, platelets, MCV, MCH, and MCHC. A study on black garlic extract increased hematological parameters in high doses as observed in aged black garlic increasing blood cell counts and blood lipids in albino Wistar rats\textsuperscript{20}.

**CONCLUSION**

The study showed that black garlic ethanol extract had no significant effect or was at normal levels on hematological parameters. Therefore, it can be concluded that the ethanolic extract of black garlic is safe for human use as a treatment for hypercholesterolemia.

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