Antioxidant and Antiatherogenic Responses of Black Solo Garlic in Alloxan-Induced White Rats

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ABSTRACT. The association between the consumption of black solo garlic (SG) and the increase in antioxidant enzymes and the ability to prevent atherogenicity has not been studied much. This study aims to examine the antioxidant and antiatherogenic capacities of black solo garlic in alloxan induced rats. This study used a true experimental design with a pre-
and post-test approach with a control group design. The sample of thirty Wistar rats was randomly divided into 6 groups: a healthy control, a negative control, treatment at doses of 6.5, 13.5, and 26 g/KgBW, and positive controls receiving vitamin C at a dose of 1 mg/kgBW. The outcomes demonstrated that following the induction, MDA, CRP, LDL, and IL-6 levels increased, while SOD and GSH-Px enzyme levels decreased. After treatment with black solo garlic for 14 days, there was an increase in the enzymes SOD and GSH-Px, as well as a significant decrease in levels of MDA, IL-6, CRP, and LDL in experimental animals. The SG dose of 13.5 g/kg BW is the most effective dose in this study. The flavonoid compounds in SG have a function as antioxidants in preventing atherogenic events.

Keywords: antioxidant, antiatherogenic, black solo garlic, CRP, SOD

INTRODUCTION

Previous research found that aqueous extract of black garlic containing antioxidants could improve colistin-induced acute kidney failure. However, the mechanism of action as an antioxidant and anti-
atherogenic had not been studied (Lee et al., 2019). Until recently, many studies have examined the role of multi-clove garlic and black garlic, but only a few studies described the role of black solo garlic (single bulb) for health, despite the fact many articles reported health benefits.

Black solo garlic (garlic with a single clove) is the result of fermentation of garlic at a specific time, temperature and humidity level (Kimura et al., 2017). Black garlic is a product of natural aging at certain controlled temperatures and humidity for several days without any additional treatment and is optimal for 21 days (Choi et al., 2014; Hue, 2022). Solo garlic contains more allicin than multi-clove garlic. Furthermore, black solo garlic also have more S-allylcysteine than fresh garlic (Chen et al., 2019; Qiu et al., 2020). Allicin and s-allyl cysteine have been found to have antioxidant activity.

The results of previous studies showed that black garlic can reduce malondialdehyde (MDA) and increase the body’s endogenous antioxidant enzymes (superoxide dismutase/SOD, glutathione peroxidase/GSH-Px) in rats (Wang & Sun, 2017). It has also been found that black solo garlic has an immunostimulatory effect on streptozotocin-induced Wistar rats (Saryono et al., 2021). Other studies also explain that black garlic extract can work as an anti-inflammatory which is mainly caused by reactive oxygen species (ROS) (Jeong et al., 2016). Black garlic extract also contains selenium which may increase the glutathione peroxidase enzyme (Pérez-torres et al., 2016). However, research on the use of black solo garlic as an antioxidant and anti-atherogenic has not been widely carried out. Recent literature studies show the potential of black garlic as an anti-atherosclerotic agent (Saryono & Proveravati, 2019), but laboratory studies to prove this mechanism have not been carried out. This research finding is expected to have implications for the use of black solo garlic as an antioxidant, which is useful for anti-atherogenic purposes. This study was designed to investigate the antioxidant roles measured by superoxide dismutase and glutathione peroxidase enzyme levels, and antiatherogenic roles that are expressed by malondialdehyde, interleukin-6, c-reactive protein, and low-density lipoprotein biomarkers of black solo garlic.

EXPERIMENTAL SECTION

Animal Experiments

The experimental animals, which were Wistar rats, were weighed between 150 and 250 grams. Randomly, the experimental animals were grouped
into 6 groups, each consisting of 5 experimental animals. Each experimental animal is numbered 1 to 5 on the tail by pen colour. Rats were randomly divided into six groups: (i) normal (HC), (ii) alloxan-induced rats (NC), (iii) alloxan-induced rat treated with vitamin C 1 mg/kg body weight (positive control, PC), alloxan-induced rat treated with steeping black solo garlic dose of (iv) 6.5 (low dose, SG1), (v) 13.5 (medium dose, SG2), and (vi) 26 g/kg body weight (high dose, SG3). The doses of alloxan for rat is 20 mg/200 g of rat or 100 mg/kg body weight of rat.

During the study, the experimental animals were given drinking aqua destilata and standard feed in the form of braille-II pellet (BR II) ad libitum. Prior to the study, the experimental animals were acclimatized for 7 days. Research on test animals was carried out after obtaining ethical clearance from the health research ethical commission of Mahardika High School of Health Sciences No. 043/KEPK. STIKMA/VI/2022.

Solo Garlic Fermentation

Fresh solo garlic of the same size and best quality without blemishes were selected for fermentation. Each fresh single clove of garlic was wrapped in tissue paper and placed in a modified rice cooker fermentation equipment. Each layer was given a barrier, and the temperature was maintained between 60 and 80 °C for several weeks.

The Preparation of Black Solo Garlic

Before becoming mashed with a pestle and mortar, the SG was peeled and weighed per dose. In a glass cup, fine garlic was dissolved in hot water (200 mL per dose) at 80–90 °C, agitated until well combined, and left for 15 minutes. The SG was filtered in order to obtain the brewed water, which was then allowed to cool. The rats were given the brewed SG via syringe and sonde.

Treatments

After alloxan-induction for 7 days, the animals were treated with steeping black solo garlic for 14 days on the days 15–29. The same schedule was also applied for vitamin C treatment (Figure 1). Briefly, the black solo garlic powder was measured based on the body weight of rats to obtain those three doses. The measured powder was diluted in 3.6 mL hot water and stirred for 15 minutes to obtain a steeping of black solo garlic. Steeping black solo garlic was given orally once a night based on the dose and body weight of each group.

Parameter Measurement

Prior to blood sampling, the rats are fasted for about 8 hours. Blood was taken using a hematocrit capillary pipette, which was inserted into the orbital plexus of white rats on the edge of the eye. Then the blood was accommodated in the conical tube in as much as 3 mL. Then, the blood was centrifuged for 10 minutes at 4000 rpm. The clear top, in the form of plasma, is taken and put into a test tube. Blood samples were taken on days 15 and 30 in the morning after fasting for at least 8 hours. At that time, the weight of the white rats was also measured. The independent variable was the dose of black solo garlic extract, while the dependent variable was the change in antioxidant enzymes (SOD, glutathione peroxidase), IL-6, rat CRP and LDL, and lipid peroxide products in the form of MDA.

Randox’s RanSOD kit (United Kingdom) was used to test SOD, and the RanSEL kit (United Kingdom) was used to test GSH-Px. SOD, MDA-TBARS and GSH-Px enzyme activities (Biovision kit) were measured by a spectrophotometer. The ELISA kits were used to check the cytokine IL-6 and rat CRP (BT Laboratories, Shanghai) based on the manufacturer’s protocol. Briefly, this ELISA kit uses the Sandwich-ELISA principle. The optical density (OD) was measured using the ELISA Reader (Labotrone, Germany) at a wavelength of 450 nm. LDL examination was done using the direct method.

Statistical Analysis

The characteristics of the experimental animals are presented in a table containing the mean, standard deviation and the histogram of the frequency distribution. Bivariate analysis was performed to determine differences between the control group and the treatment group. The normality test of the data used the Shapiro-Wilk test. If the results of the data normality test show that the data distribution is normal, then the homogeneity test is then carried out. Furthermore, to determine the location of the differences in each treatment, a hypothesis test was carried out using one-way ANOVA. After that, it was continued with post-hoc least significant difference (LSD) analysis. Statistical tests were carried out with a 95% level of confidence (α = 0.05).

<table>
<thead>
<tr>
<th>Day</th>
<th>acclimatization</th>
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<th>treatment (post)</th>
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</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>14 days</td>
</tr>
<tr>
<td>6</td>
<td>7 days</td>
<td>7 days</td>
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</tbody>
</table>

**Figure 1.** A graphical scheme showing timeline of alloxan and SG treatment
RESULTS AND DISCUSSION

Antioxidant Enzyme Activity (GSH-Px and SOD) and Lipid peroxidation Product (MDA and LDL levels)

The results showed that alloxan induction was proven to reduce the activity of GSH-Px and SOD. The results of the ANOVA test analysis showed that there were differences in GSH-Px and SOD activity between the alloxan-induced groups and healthy controls, but there were no differences between the induced groups (Figure 2a and Figure 2b). After administration of black solo garlic, the results of the ANOVA test also showed significant differences in GSH-Px and SOD activity at least one group between the test groups (p<0.05).

The results of Duncan’s post hoc test showed that there was no significant difference in GSH-Px activity between the SG3 and PC groups, but that it was significantly different from the SG2 group after instilling black solo garlic (p<0.05). The SG2 group had the highest average GSH-Px activity compared to the other treatment groups. Meanwhile, the results of Duncan’s post hoc test showed that there were differences in SOD activity between all groups. The SG3 group with a dose of 26 g/KgBW was the most effective in increasing the activity of the SOD enzyme.

Alloxan induction can increase MDA and LDL levels in experimental animals (Figure 3a and Figure 3b). The results of the ANOVA test showed that there was a significant difference in MDA and LDL levels before treatment with black solo garlic steeping between the healthy control group and the treatment group (p<0.05). After administration of black solo garlic, the results of the ANOVA test showed a significant difference of MDA and LDL levels at least one group between the test groups (p<0.05). The results of Duncan’s post hoc test showed no difference in MDA and LDL levels between the SG2, SG3, and positive control groups after administration of black garlic. The SG3 group with a dose of 26 g/kgBW was the most effective in reducing MDA, but SG2 was in LDL levels.
Oxidative damage by free radicals and secondary metabolites of toxic compounds can occur in body cells. Alloxan induction as a source of free radicals can cause oxidative damage. Alloxan will cause the generation of reactive oxygen species, such as superoxide anion and hydroxyl radicals. These ROS can initiate lipid peroxidation by attacking the polyunsaturated fatty acids in cell membranes. ROS can also oxidize lipids to produce a lot of malondialdehyde (MDA). High ROS production can oxidize the microvascular resulting in vascular injury and organ dysfunction. Oxidation of the endothelial walls of blood vessels causes endothelial cell activation and increases LDL (Cheng et al., 2022). Endogenous antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase will work to stop free radical chain reactions, but if ROS levels are excessive, then these enzymes will decrease.

However, the antioxidant effect of black solo garlic can reduce fat peroxidation and indirectly increase NO synthesis, thereby inhibiting the production of advanced glycosylated end products (AGEPs) (Ilmawati et al., 2017). The organosulfur component and flavonoid content of black garlic are potent antioxidants. Flavonoid can donate H⁺ from this OH group to free radical’s molecules for neutralize them. These active compounds can repair body cells by increasing the activity of antioxidant enzymes such as catalase, superoxidase dismutase, and glutathione peroxidase. Solo garlic is also known to contain various minerals, including calcium, selenium, iron, magnesium, and zinc that needed by mineral dependent antioxidant. Furthermore, the results of previous studies also showed that black garlic can increase antioxidant enzymes (Colín-González et al., 2012; Kim et al., 2019).

![Figure 3a.](image_url) MDA levels (μmol/L) before and after black solo garlic treatment

![Figure 3b.](image_url) LDL levels (mg/dL) before and after black solo garlic treatment
Inflammation Markers (Interleukin-6 and CRP)

Reactive oxygen species (ROS) such as superoxide anion, peroxyl and hydroxyl radicals will cause inflammation (Thomas et al., 2022; Yu et al., 2022), resulting in the release of pro-inflammatory mediators such as IL-6 and other inflammatory markers such as CRP. One of ROS source, Alloxan induction can cause oxidative damage resulting in inflammation. Pro-inflammatory cytokines such as IL-6 will increase after alloxan induction. CRP levels increase significantly when there is inflammation. It was proven that there was a significant increase in IL-6 and CRP levels after alloxan induction (p<0.05) (Figure 4a and Figure 4b). As an inflammation marker, IL-6 can stimulate the production of CRP. But, both of marker are not always correlated, and have different functions in the body. However, the levels of IL-6 and CRP decreased significantly after infusion of black garlic (p<0.05). The results of the ANOVA test showed significant differences in IL-6 and CRP levels in at least one group among the test groups (p<0.05).

Based on Duncan’s post hoc test, there was no difference in IL-6 levels between the SG2 and SG3 groups, but it was different from the healthy control group after being given black garlic infusion. The level of IL-6 decreased after receiving the steeping of black solo garlic. Alloxan induction causes liver cell damage resulting in inflammation. To minimize damage caused by antioxidants, endogenous antioxidant enzymes will work to prevent free radical chain reactions. Inflammatory markers begin to diminish, including pro-inflammatory mediators such as IL-2. Giving black garlic infusion can prevent free radical chain reactions, so that IL-6 levels decrease.

Figure 4a. IL-6 levels (ng/L) before and after black solo garlic treatment

Figure 4b. CRP levels (ng/mL) before and after black solo garlic treatment
The differences between pre and post-test black solo garlic treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD Mean</th>
<th>SOD SD</th>
<th>GSH-Px Mean</th>
<th>GSH-Px SD</th>
<th>MDA Mean</th>
<th>MDA SD</th>
<th>IL-6 Mean</th>
<th>IL-6 SD</th>
<th>CRP Mean</th>
<th>CRP SD</th>
<th>LDL Mean</th>
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<td>0.92</td>
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<td>0.04</td>
<td>0.004</td>
<td>0.04</td>
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<td>-0.83</td>
<td>0.34</td>
<td>-4.53</td>
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<td>23.48*</td>
<td>0.84</td>
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P value α- Anova test

0.000

*p value Anova test are significantly different (p < 0.01)

The results of Duncan's post hoc test on CRP levels after administration of black garlic showed no significant difference between the SG2, SG3, and PC groups (p<0.05). The SG2 group had the lowest CRP level compared to the other test groups. Group SG2 was the group that received the infusion of black garlic at a dose of 13.5 g/KgBW. CRP and IL-6 levels are correlated with certain health issues. Higher levels of IL-6 and CRP are frequently linked to higher risk or less favorable health outcomes. The diseases like type 2 diabetes, colorectal cancer, cardiovascular disease and successful aging are frequently found correlation between IL-6 and CRP levels (Hidayat et al., 2021; Puzianowska-Kuźnicka et al., 2016). It is crucial to remember that these studies only offer observational proof of the link; more studies are required to determine the cause-and-effect relationship between IL-6 and CRP. The result of Anova test shows that the difference of changes in levels of MDA, IL-6, CRP, LDL, SOD and GSH-Px activity showed a significant difference (Table 1). Based on Duncan's post hoc test, the SG2 group was the group with the most significant changes.

Black solo garlic contains organosulfur compounds which have potent antioxidant activity and free radical scavengers. The results of this study indicate that giving black solo garlic can reduce MDA, IL-6, CRP and oxidized LDL, but increase antioxidant enzymes such as SOD and GSH-Px. The results of this study strengthen the evidence that black solo garlic can suppress the inflammatory process due to active compound content (Arreola et al., 2015; Ilmawati et al., 2019; Lestari & Rifa'i, 2018; Nillert et al., 2017; Tsai et al., 2019; D. Wang et al., 2010; You et al., 2019). Black garlic contains lots of antioxidant compounds such as S-allyl cysteine, S-allyl mercaptocysteine, and allicin. This active compound is more soluble in distilled water compared to alcohol solvents. Black SG works by increasing the body’s antioxidant levels (Naji et al., 2017). In previous studies, the high SAC content in black SG was proven to reduce LDL in both intoxicated and normal conditions (Buu et al., 2018; Nurmwawati, 2021).

Black garlic contains S-Allil-Cysteine (a thiol group) which can function as an anti-diabetic, antioxidant and anti-inflammatory with higher bioactivity than ordinary garlic. When there is inflammation, many pro-inflammatory mediators are produced. Black garlic contain lots of flavonoids. Flavonoid donate H• to peroxynitrite so this free radical become neutral. Therefore, free radical reduction will decrease the rate of chain oxidation. Flavonoids have been shown to act as anti-inflammatories (Saryono et al., 2019). Giving black garlic can suppress pro-inflammatory mediators so that tissue damage during inflammation can be reduced. Antioxidant compounds such as S-allyl cysteine, S-allyl mercaptocysteine, and allicin show potent antioxidant activity (Saryono et al., 2022). SAC has been shown to be able to take up superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitric radicals, and peroxyl radicals produced in neuronal cells, as well as hypochlorous acid and singlet oxygen produced in microglial cells (Nillert et al., 2017).

CONCLUSIONS

Giving black solo garlic infusions can increase the activity of GSH-Px and SOD enzymes and significantly reduce IL-6, CRP, and LDL. The effective dose for increasing antioxidant enzyme activity (GSH-Px and SOD) and reducing IL-6, CRP, and LDL in this study was 13.5 g/kgBW. Increased antioxidant activity of GSH-Px and SOD enzymes after BSG administration is associated with decreased IL-6, MDA, CRP, and LDL. The high levels of antioxidants in BSG are thought to underlie the antiatherogenic mechanism.

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AUTHORSHIP CONTRIBUTION

SY, AP, and LJ conceived and designed the experiments; SY and AP performed the experiments; LJ analyzed the data; SY, and AP contributed

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chemicals/materials/analysis tools; and SY and LJ wrote the paper. The authors read and approved the final manuscript.

CONFLICT OF INTEREST
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